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EVALUACIÓN DE LA CAPACIDAD ANTIOXIDANTE GLOBAL DE LOS COMPONENTES DE LA DIETA ESPAÑOLA MEDIANTE SU DIGESTIÓN *IN VITRO:* EFECTO DEL PROCESADO TÉRMICO Y DE LA MICROBIOTA INTESTINAL.

TESIS DOCTORAL

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“Hazlo, o no lo hagas, pero no lo intentes”

Yoda, El Imperio Contraataca.

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ABREVIATURAS

MR: Reacción de Maillard

MRP: Productos de la reacción de Maillard

HMF: Hidroximetilfurfural

TEAC: Trolox Equivalent Antioxidant Capacity

SCFA: Short Chain Fatty Acid

HPLC: Cromatografía líquida de alta resolución

RESUMEN

La capacidad antioxidante de los alimentos está estrechamente relacionada con la mayoría de las enfermedades no transmisibles (síndrome metabólico, cáncer, enfermedad de Parkinson, enfermedades cardiovasculares, diabetes, etc.). Por ello es necesario conocer que capacidad antioxidante nos está aportando la dieta. Sin embargo, para ello es necesaria una metodología adecuada. Es por ello que en esta tesis doctoral se desarrolla: por un lado, un procedimiento fisiológico de extracción de compuestos antioxidantes procedentes de la dieta a partir de un sistema *in vitro* de digestión gastrointestinal junto a un proceso de fermentación, también *in vitro.* Este proceso fisiológico permite evaluar la efectividad tanto de la digestión como de la actividad de la microbiota intestinal en la extracción y transformación de compuestos con capacidad antioxidante. Por otro lado, se desarrollan varios métodos de determinación de capacidad antioxidante *in vitro,* que simulen de una forma más apropiada las condiciones de temperatura, pH y fuerza iónica que se encuentran en el organismo humano. Mediante el empleo de esta metodología se estudia la capacidad antioxidante de los principales alimentos de la dieta española y el efecto que sobre ella tiene el tratamiento térmico, ya sea industrial o casero (tipo de cocinado). Además, gracias al modelo de digestión-fermentación *in vitro,* también se estudia el efecto que distintos tipos de alimentos, así como el tratamiento térmico, tienen sobre la microbiota intestinal y su funcionalidad. Estas investigaciones han permitido observar el efecto que diversos alimentos tienen. Así mismo, también se ha desarrollado un proceso que permite obtener compuestos bioactivos de un producto de desecho (posos de café) y por otro lado mejorar la composición de un alimento nutricionalmente no recomendable (salchichón) para hacerlo más antioxidante y que mejore la funcionalidad de la microbiota intestinal.

ABSTRACT

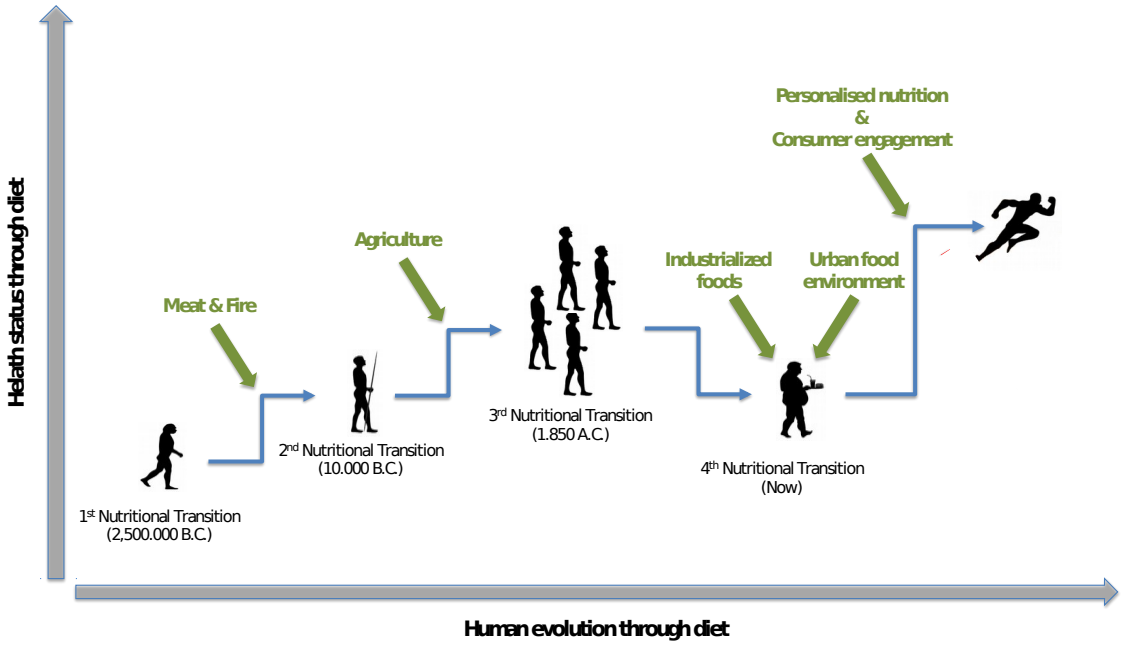
Antioxidant capacity is strongly linked to most chronic diseases (metabolic syndrome, cancer, cardiovascular diseases, Parkinson's disease, diabetes, etc.). Therefore, it is mandatory to know the antioxidant capacity provided by diet. However, to achive this goal, it is needed a proper methodology. Accordingly, in this doctoral thesis, it has been developed: on one hand, a physiological extraction process composed of an *in vitro* gastrointestinal digestion followed by an *in vitro* fermentation. This physiological process will allow us to evaluate gastrointestinal digestion of foods as well as gut microbiota role in food degradation and biotransformation of bioactive compounds. On the other hand, it has also been developed three different *in viro* methods to determine antioxidant capacity physiologically closer conditions such as pH, temperature, and ionic strength. By using all this methodology, it has been studied the antioxidant capacity of the main foodstuffs of the Spanish diet and the effect of thermal processing (industrial or home-performed) on the antioxidant power. Moreover, thanks to the *in vitro* digestion-fermentation process, it has been studied the effect of different foodstuffs as well as the effects of different cooking techiniques on gut microbiota and its functionality. This research not only has allowed us to observe the effects of different foodstuffs but also it has allowed to obtain bioactive compounds from a food waste (spent coffee grounds) and also to improve the composition of a nutritionally poor food (salami) by increasing its antioxidant capacity and improving gut microbiota functionality.

INTRODUCCIÓN

1. Alimentación

A través de la historia se ha ido produciendo un cambio gradual (**Figura 1**) hacia la comida de alta calidad, alta densidad energética y fácilmente absorbible gracias al avance de la tecnología (Katz, 2003). La evolución humana está conectada con cambios en la dieta. Así, a lo largo de la historia humana se han atravesado diferentes transiciones nutricionales: el descubrimiento del fuego y su uso para cocinar, el desarrollo de la agricultura y la industrialización y producción de alimentos. La primera transición nutricional ocurrió hace 2.5 millones de años cuando los seres humanos pasaron de ser vegetarianos a omnívoros gracias al uso del fuego. La segunda transición tuvo lugar hace entre 10000-5000 años cuando la agricultura fue desarrollada por primera vez contribuyendo a una elevada producción de alimentos ricos en carbohidratos además de permitir una mayor disponibilidad de los mismos a lo largo del año. La tercera transición ocurrió en el siglo XIX cuando gracias al vapor y la electricidad la industria empezó su auge. De esta manera, se incrementó el consumo de azucares refinados y grasas idustrilizadas mientras que disminuyo el consumo de alimentos poco procesados y fibra. Esta profunda simplificación de nuestra dieta desde que empezó la industrialización ha tenido lugar en un periodo de tiempo muy corto, evolutivamente hablando, de forma que el genoma humano no ha podido adaptarse (Konner & Eaton, 2010) y ha sido probablemente el causante de la perdida de diversidad en la microbiota intestinal que ha ido heredándose generación tras generación (Sonnenburg et al., 2010). Se ha sugerido que esta mala adaptación a la dieta moderna sea la razón subyacente tras el origen de las “enfermedades de la civilización” como el cáncer, la obesidad, las enfermedades cardiovasculares, la diabetes o incluso el declive cognitivo (Rabassa et al., 2015), lo cual constituiría la cuarta transición nutricional, que necesita ser redireccionada hacia una vertiente más saludable. Esta cuarta transición nutricional está realacionada con la nutrición personalizada teniendo por objetivo adecuar la ingesta de nutrientes a cada persona de acuerdo con su carga genética y relación microbiota intestinal-hospedador.

Figura 1. Transiciones nutricionales a lo largo de la historia.



Cerca de 2000 millones de personas en el mundo padecen sobrepeso (OMS, 2017) lo cual contribuye a un aumento en la incidencia de enfermedades no transmisibles que matan a unos 38 millones de personas al año (OMS, 2017). Las más comunes son enfermedades cardiovasculares, cáncer, enfermedades respiratorias crónicas y diabetes. Además, casi la cuarta parte de las personas con ocupación laboral sufren algún trastorno crónico, la mayoría de ellos relacionados con la obesidad lo cual además lleva a una pérdida sustancial de la productividad (UNEP, 2016). La prevalencia de este tipo de enfermedades en niños es también alarmante. El sobrepeso en niños y adolescentes ha aumentado drásticamente desde el 4% en 1975 hasta el 18% en 2016 (OMS, 2017). Estos niños tienen mayor riesgo de padecer problemas físicos, enfermedades cardiovasculares, diabetes y desórdenes músculo-esqueléticos. Además su bienestar social y emocional así como su autoestima se ven comprometidos (Kaur, Hyder, & Poston, 2003). El sobrepeso y la obesidad infantil han sido caracterizados por la OMS como uno de los problemas de salud pública más serios del siglo XXI. Adicionalmente, otras patologías relacionadas con la alimentación están en auge; la

enfermedad celiaca ocurre en alrededor del 1% de las personas en la mayoría de las poblaciones y se cree que el aumento no se debe simplemente a un aumento de las personas diagnosticadas sino a un aumento real de la incidencia (Lebwohl, Sanders, & Green, 2018). Por otro lado, las alergias alimentarias han aumentado rápidamente su prevalencia durante los últimos 30 años y están consideradas también como una amenaza grave contra la calidad de vida de los niños y sus familias (Du Toit, Foong, & Lack, 2017).

Las razones detrás de estos cambios en la alimentación son muy diversas y están relacionados con cambios en el abastecimiento y la demanda de alimentos, dependiendo el uno del otro. Las políticas y el proceso de globalización a través del crecimiento de la industria alimentaria a escala internacional, incluyendo la aparición de supermercados y el marketing en masa; el cambio en las condiciones laborales, salarios, horarios, etc., que conducen a cambios en los hábitos alimentarios. Todas estas razones están implicadas en los cambios en los patrones dietéticos que están estrechamente relacionados con las condiciones de salud (Ruel, Garrett, Yosef, & Olivier, 2017). Todos estos factores están relacionados con el proceso de "urbanización" ya que el cambio hacia un mayor uso de grasas y azúcares refinados en lugar del empleo de carbohidratos complejos ocurría más rápidamente en ciudades que en poblaciones rurales (Popkin, 1999). Las poblaciones urbanas tienden a consumir más calorías aunque la proporción de éstas que procede de cereales o carbohidratos es menor que en medios rurales (Kelles & Adair, 2009).

La dieta en las ciudades está también influenciada por los ingresos ya que determina que tipo de alimentos se compran (Hawkes et al., 2015). En las áreas urbanas, la mayor parte de los alimentos son comprados así que los ingresos de las familias van a jugar un papel importante en su capacidad para adquirir alimentos lo que afectará a su dieta y salud. Sin embargo, muchos de los cambios negativos están ocurriendo tanto en familias con elevado poder adquisitivo como en familias con bajo poder adquisitivo. Además, mientras que los alimentos con mayor densidad de nutrientes suelen ser más caros (frutas, verduras y alimentos de origen animal), los de mayor

densidad calórica son más baratos lo que puede suponer una barrera para cierta población. Así, ambas opciones, saludable y no saludable, están disponibles para los ricos pero para los pobres la oferta o posibilidades están mucho más orientadas hacia lo no saludable (Imamura et al., 2015). Además, la pobreza está aumentando en muchos países lo que hace que cada vez un mayor número de personas se vea obligada a realizar elecciones no saludables en lo respectivo a la dieta (Ruel, Haddad, & Garrett, 2001).

No solo los ingresos son importantes, igualmente el acceso físico a los distintos tipos de alimentos marcará que comemos. De esta manera, en lo que se refiere a alimentación, el sector minorista, pequeños comercios, supermercados e hipermercados, está creciendo rápidamente, aunque generalmente lo hace primero en ciudades y luego en pequeñas poblaciones (Bermudez, Gao, & Valdes, 2008). Como resultado, las poblaciones urbanas tienen acceso a distintos tipos comercios. Los comercios minoristas modernos son importantes para los cambios en la alimentación ya que tienden a especializarse en alimentos procesados. Globalmente, cerca del 60% de los alimentos procesados se distribuye a través de supermercados y éstos dominan en países de rentas alta-media (UNEP, 2016). Además, la elección de un alimento u otro también está influenciada por sus propiedades hedónicas y la comodidad (Hawkes et al., 2015). Así, el marketing influencia de manera significativa el deseo de comprar o no ciertos alimentos. Las empresas que venden snacks, comida rápida y bebidas azucaradas con algún tipo de valor añadido invierte fuertemente para que sus productos estén disponibles para cuanta más gente mejor, incluyendo lugares cercanos a escuelas y otros lugares donde la gente se reúne.

1.1. La cuarta transición nutricional y la microbiota intestinal

Teniendo en cuenta toda esta información, está claro que es necesario un cambio profundo en la actual dieta humana. Este profundo cambio sería la clave de esa cuarta transición nutricional, la cual podría corregir las actuales modas gracias a la nutrición personalizada y al conocimiento en sistemas biológicos. La nutrición se ha ido preocupando cada vez más del metabolismo humano y en la respuesta metabólica individual de cada persona al consumo de macro y micronutrientes, lo que se conoce como "nutrigenómica". Sin embargo, aunque la nutrición personalizada es frecuentemente considerada en el contexto de interación dieta-genes, varios estudios han mostrado que las recomendaciones de cambios de estilo de vida basadas en riesgo genético no incrementa significativamente el cumplimiento. Cuando se consideran los problemas éticos asociados con la provisión de información genética a terceros, puede hacerse difícil llevar a cabo estrategias de nutrigenómica en el marco de nutrición personalizada (Daniel, Byrne, & Raats, 2016).

Por otro lado, la fisiología de las personas depende no solo de los genes humanos sino también de los genes de los microorganismos que forman parte de la microbiota intestinal. El intestino alberga una comunidad microbiana que posee un número de células mucho mayor que el de células eucariotas que forman nuestro cuerpo. El colon es el principal sitio en el que la microbiota intestinal ejerce su actividad metabólica lo cual mejora la obtención de energía de los nutrientes e influencia la síntesis, biodisponibilidad y función de nutrientes (Tremaroli & Bäckhed, 2012). Esta actividad produce distintos compuestos beneficiosos que actúan en la regulación de la salud del individuo. Entre ellos destacan, por ejemplo, ácidos grasos de cadena corta (SCFAs), metabolitos fenólicos, compuestos neuroactivos, etc. Esto hace que podamos considerarnos "supraorganismos", compuestos por nuestras propias células y las microbianas dependiendo ambas unas de otras para la supervivencia (Hooper & Gordon, 2001). La habilidad para adaptarse a cambios ambientales, concretamente dietéticos, estará determinada por la actividad metabólica coordinada de la microbiota y el individuo, siendo la microbiota la parte más maleable y adaptable

del ecosistema humano. En este sentido, la evolución de la dieta humana ha estado acompañada por un descenso en la diversidad y un aumento en el metabolismo de carbohidratos y xenobióticos por la microbiota intestinal (Gomez et al., 2016). Así, la modulación de la microbiota tendrá que ser un componente principal de la cuarta transición nutricional y un elemento clave en futuras SPN (Smart Personalized Nutrition).

El conocimiento actual establece claramente que la microbiota intestinal tiene un fortísimo impacto en la salud humana y además que puede ser modificada por la dieta. Las comunidades bacterianas intestinales son diversas y variables de una persona a otra pero tienen una estabilidad temporal sustancial dentro de un individuo y comparten un elevado grado de capacidades funcionales a través de individuos sanos (Faith et al., 2013). El medio ambiente, el estilo de vida y la dieta son factores críticos ya que determinan la configuración de la microbiota intestinal (Song et al., 2013; Zhernakova et al., 2016) mientras que la información genética parece jugar un papel menor (Zhang et al., 2010). Los efectos dietéticos sobre la microbiota intestinal pueden ocurrir tanto a largo o corto plazo. Así, una ingesta aguda de energía y nutrientes puede inducir crecimientos bacterianos en cortos periodos de tiempo (David et al., 2013) aunque los patrones dietéticos a largo plazo son los que parecen ser responsables de la riqueza genética y de especies de la microbiota intestinal (Cotillard et al., 2013; Chatelier et al., 2013). El perfil de macronutrientes puede modular la microbiota: grasa saturada y proteína animal disminuye la diversidad microbiana y enriquece en *Bacteroidetes* y *Actinobacteria*, mientras que dietas basadas en vegetales con alto contenido en carbohidratos incrementa la diversidad microbiana, vinculada con *Firmicutes* y *Proteobacteria* (Wu et al., 2011). Thaiss et al., (2016) mostraron que la administración de flavonoides (un grupo de polifenoles) en ratones disminuía la ganancia de peso después de la dieta al modificar la funcionalidad de la microbiota obesogénica. Además, dietas altas en fibra se asocian con una saludable y bien estructurada microbiota caracterizada por una liberación lenta de energía de los nutrientes, que protege de la inflamación y enfermedades no infecciosas del colon (De Filippo et al.,

2010). En ratones, la privación de fibra ha mostrado recientemente que induce a la degradación de la mucosa colónica que actúa de barrera por la microbiota intestinal (Desai et al., 2016), mientras que la suplementación con fibra junto con el probiótico *Akkermansia miciniphila* mejora la salud metabólica. (Dao et al., 2016).

Además, no sólo la composición de la comida juega un papel importante en la modulación de la microbiota intestinal sino que también los métodos de cocinado podrían influir debido a cambios químicos durante el procesado. Hay unos pocos estudios respecto a este tema pero Shen, Chen, & Tuohy, (2010) encontraron que la ternera frita incrementó más los niveles de *Clostridium spp.* y *Bacteroides spp.* que la ternera cocida, mientras que disminuyó los niveles de SCFAs. En este sentido, Marungruang, Fåk, & Tareke, (2016) encontraron que la dieta rica en lípidos con tratamientos térmicos altera la composición y funcionalidad de la microbiota intestinal en ratones e incrementa la adiposidad y la inflamación de bajo grado, en comparación con la misma dieta no sometida a procesado térmico. Ya que durante el procesado térmico, se transforman las proteínas, carbohidratos y lípidos debido a la Reacción de Maillard, oxidación lipídica y otras (J.A. Rufián-Henares & Pastoriza, 2016). Estas reacciones producen nuevos compuestos, como las llamadas melanoidinas, las cuales pueden ejercer tanto actividad antimicrobiana como prebiótica (José A. Rufián-Henares & de la Cueva, 2009). De esta manera, uno de los grandes retos de la nutrición es conocer las interaciones existentes dentro de matrices complejas de alimentos que incluyen un amplio abanico de compuestos biológicamente activos. Esto hace que nos preguntemos si hay ingredientes específicos que ejerzan un efecto selectivo más fuerte que otros a la hora de mejorar la diversidad y funcionalidad de las comunidades microbianas y también como diferentes tratamientos térmicos o culinarios pueden afectar o modular a la microbiota intestinal.

De esta manera, la importancia de las intervenciones dietéticas para prevenir el desarrollo de las enfermedades que se han mencionado es actualmente ampliamente reconocida. La microbiota intestinal parece que juega un papel importante tanto en la enfermedad celíaca como en las alergias

alimentarias (Wacklin et al., 2014; Aitoro et al., 2017). En el caso de la enfermedad celíaca, una dieta libre de gluten puede no ser todo lo que se necesita para su tratamiento. Un estudio finlandés (Wacklin et al, 2014) encontró menos diversidad bacteriana y un desequilibrio en ciertos tipos de microorganismos en pacientes celiacos con síntomas persistentes. Hallazgos similares se encontraron en personas con alergias alimentarias (Aitoro et al., 2017), demostrando que ambas enfermedades pueden mejorar restaurando la diversidad de la microbiota intestinal con el uso de prebióticos, incrementando la producción de SCFAs como butirato, el cual mejora la modulación del sistema inmune.

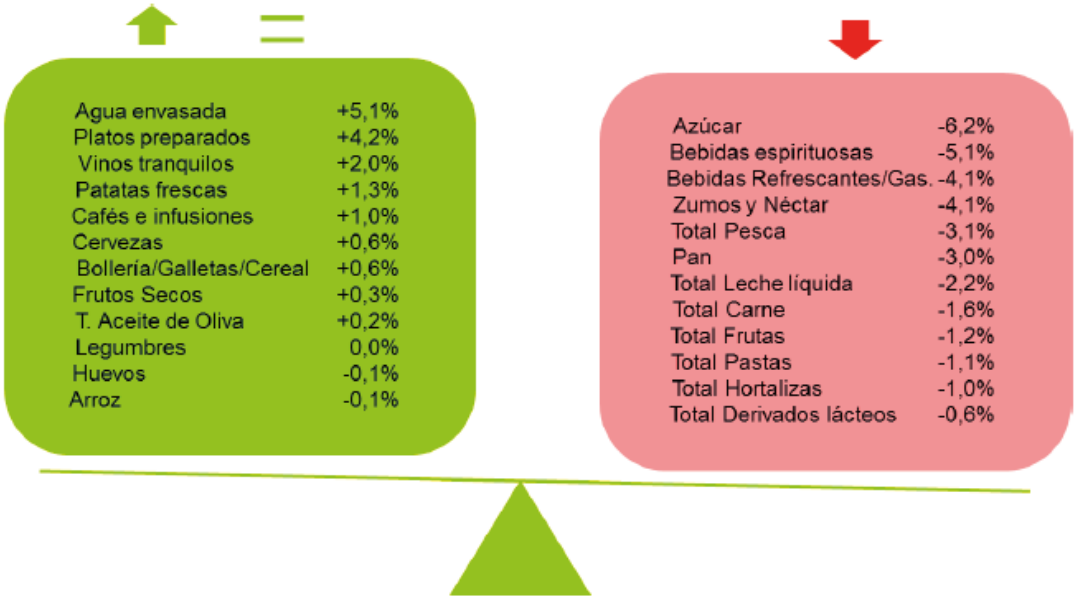
2. Alimentación en España.

En España, por categorías, destaca el consumo de productos frescos en los hogares que supone el 41% del total y el 44.1 % del presupuesto destinado a la alimentación (MAPAMA, 2018). Por tipos de productos, destaca el crecimiento del consumo de alimentos como platos preparados (+4.2 %), patatas (+1.2 %), bollería, pastelería, cereales y productos navideños (+0.6%) y frutos secos (+0.3%) (MAPAMA, 2018). Las legumbres, por otro lado, han mantenido su consumo estable (**Figura 2**). Sin embargo, hay otros alimentos como aceite, leche, derivados lácteos, carne, pescado, frutas frescas y hortalizas frescas, frutas y hortalizas transformadas, pan, pasta y huevos en los que el consumo ha disminuido (MAPAMA, 2018). Además, respecto a las bebidas, se ha producido un aumento en el consumo de vinos tranquilos con DOP, los vinos espumosos y gasificados con DOP, los vinos con IGP, el agua envasada, las bebidas refrescantes y las cervezas, y un descenso en el caso de los vinos con DOP/IGP, los zumos y néctares y las bebidas espirituosas (MAPAMA, 2018).

El gasto realizado en los hogares españoles en 2017 ascendió a 67095 millones de euros lo que supone un incremento del 0.1% con respecto al año anterior. Sin embargo, se ha registrado un

descenso en el consumo del 0.7 % con lo que el incremento anterior es debido al aumento de precio (0.8 %)(MAPAMA, 2018). El mayor gasto se realiza en supermercados y autoservicios (43.9 %) seguido de tiendas tradicionales (19.8 %) aunque estas últimas han sufrido un descenso del gasto empleado en ellas. Por el contrario, ha aumentado el gasto en supermercados, tiendas descuento, comercio electrónico y en "otros canales" (MAPAMA, 2018).

Figura 2. Evolución de las compras de los principales productos sobre el total de alimentación (% en volumen)



Por otro lado, los productos frescos constituyen una parte importante de la alimentación en los hogares, representando un 41% del volumen total consumido y un 44% del gasto. Sin embargo, en el último año se ha producido una disminución del volumen de un 1.3% y una disminución del gasto del 0.8%.

- **Aceite.**

El consumo doméstico total de aceite disminuyó un 0.5% aunque la facturación aumentó debido al aumento de precio del aceite. El 67.2% del aceite consumido es aceite de oliva (39.6% aceite de oliva y 19.2% AOVE), el 25.3% es aceite de girasol, el 6% es aceite de semillas, el 1.3% aceite de orujo y el 0.1% restante se divide entre el resto de aceites. El consumo per cápita de aceite total sin embargo aumento un 1.1 % respecto al año anterior.

Se ha producido un aumento de la compra de aceite de oliva compra de un 4.2% con respecto al año anterior. El consumo por persona y año también ha aumentado con respecto al año anterior un 5.9%. La compra de AOVE aumentó un 5.2% el último año. Además, el consumo por persona y año es de 2.43 litros, un 6.9% más respecto al año anterior. Por otro lado, el aceite de oliva virgen sufrió un descenso debido al aumento de precio.

El aceite de girasol es el segundo más consumido con un 25.3% del consumo total de aceite. Además, durante el último año su consumo aumentó un 1.3%, puede que debido a la bajada de precio.

El aceite de semillas, el cual representa un 6% del consumo de aceite, ha sufrido un descenso en su consumo ya que ha sufrido un aumento de precios que ha hecho que no sea tan asequible como antes (MAPAMA, 2018).

- **Leche líquida**

El volumen de leche líquida consumida se ha reducido un 2.2%. El consumo per cápita también ha sufrido una bajada del 0.6%. Desde 2010 existe una tendencia negativa en su consumo.

Por otro lado, el consumo de derivados lácteos también ha disminuido. De los derivados lácteos, los que ocupan el mayor porcentaje del consumo son las leches fermentadas, seguidas del queso, postres lácteos, batidos y, por último, helados y tartas.

- **Carne**

  El volumen de carne consumido a nivel doméstico ha disminuido un 1.6%. Por otro lado, el consumo per cápita se mantiene estable. La mayor parte de la carne para consumo doméstico, 74%, es carne fresca. Le sigue con un 23.5% la carne transformada mientras que la carne congelada solo representa un 2.5%.

- **Pescado**

  El volumen de pescado consumido a nivel doméstico cayó un 3.1%. El consumo doméstico de pescado está en una tendencia de descenso que se mantiene durante los últimos 7-8 años. Salvo las conservas, el resto de presentaciones han visto disminuido su consumo. Sin embargo, el pescado fresco sigue siendo el más consumido seguido de las conservas y en tercer lugar los maricos.

- **Frutas frescas**

  Mientras que el volumen de frutas frescas adquirido para consumo doméstico disminuyó el pasado año, el consumo per cápita se mantiene estable. Las frutas más consumidas son naranjas, plátanos y manzanas con un 20%, 12% y 11% del volumen del mercado respectivamente.

- **Hortalizas frescas**

  El volumen de hortalizas frescas adquiridos para el consumo doméstico ha sufrido una ligera caída del 0.4%. Los productos con más peso en el volumen de compra son las patatas con un 27%, los tomates con un 14%, cebollas con 9% y pimientos con 6%.

  El consumo per cápita por otro lado ha aumentado un 3% en el caso de las patatas y un 0.6% en el caso de las hortalizas en general.

- **Pan**

  El volumen de pan que se ha comprado para uso doméstico se ha reducido un 3% durante el último año. De igual manera, el consumo per cápita ha disminuido un 1.4% respecto al año anterior. El pan blanco es el que mas volumen representa con 74.7% mientras que el pan blanco industrial se sitúa en segunda posición. En último lugar se encuentra el pan integral con un 7.4% de cuota.

- **Bollería, pastelería, galletas, cereales, productos navideños.**

  El consumo per cápita de este tipo de productos se ha incrementado durante el último año un 2.3%. La bollería/pastelería son los que más contribuyen al crecimiento suponiendo un 43% de la cuota de mercado situándose en segundo lugar las galletas con un 38.7% de cuota. Las familias con hijos cualquier edad son el grupo que más contribuye a su consumo.

- **Legumbres**

  El consumo de legumbres se mantiene estable.

- **Pasta**

  El consumo de pasta ha disminuido un 1.1% respecto al año anterior.

- **Frutos secos**

  Mientras que el volumen de frutos secos dedicado al consumo doméstico es similar al año anterior, el consumo per cápita ha aumentado un 1.9%.

- **Platos preparados**

  El consumo per cápita de platos preparados aumentó un 6% respecto al año anterior así como el volumen de compra dedicado al consumo doméstico (4.2%).

- **Cafés e infusiones**

  El volumen dedicado a consumo doméstico aumenta un 1% respecto al año anterior así como el consumo per cápita que aumenta un 2.6%.

- **Huevos**

  Mientras que el volumen dedicado a consumo doméstico se mantiene estable, el consumo per cápita ha aumentado un 1.5%.

- **Arroz**

  El volumen de arroz que se compra para consumo doméstico se mantiene estable pero el consumo per cápita aumenta un 1.5%.

- **Azúcar**

  Tanto el volumen de azúcar dedicado a consumo doméstico con el consumo per cápita ha disminuido (6.2 y 4.7%, respectivamente).

- **Especias y condimentos**

  El volumen de compra de especias y condimentos ha aumentado un 7.2% mientras que el consumo per cápita lo ha hecho en un 8.9%.

- **Sal**

La sal ha visto disminuido tanto su volumen de compra, un 3%, como su consumo per cápita, un 1.5%.

- **Vino**

El volumen de vino dedicado a consumo doméstico aumento en un 0.8% mientras que el consumo per cápita hizo lo propio en un 2.4%. La mayor cuota de mercado la ocupan los vinos tranquilos con DOP (37%) seguidos con un 7.3% de cuota por los vinos espumosos con DOP y los vinos gasificados con DOP.

- **Agua**

Tanto el volumen como el consumo per cápita de agua ha aumentado un 5.1% y un 6.8%, respectivamente.

- **Bebidas refrescantes**

Las bebidas refrescantes han visto disminuido su consumo, tanto el volumen de compra para uso doméstico como el consumo per cápita (3.7 y 2.2%, respectivamente).

- **Cerveza**

El consumo de cerveza ha aumentado volumen dedicado para uso doméstico en un 0.6% y el consumo per cápita un 2.2%.

- **Zumos y néctares**

El volumen de zumos y néctares dedicados al consumo doméstico ha disminuido un 4.1% mientras que el consumo per cápita lo hace un 2.6%.

- **Bebidas espirituosas**

  El volumen de uso doméstico ha disminuido un 5.1% mientras que el consumo per cápita también lo ha hecho en un 3.5%.

3. Tipos de tratamientos culinarios

Cocer un alimento significa exponerlo a la acción de un foco de calor, o de unas radiaciones, con el propósito de elevar su temperatura. Como consecuencia de ese calentamiento, el alimento, experimenta cambios que pueden ser físicos, químicos e incluso biológicos, que implican modificaciones relacionadas con su calidad, tanto organoléptica como nutricional. Tales modificaciones pueden resultar favorables o desfavorables, según el tipo de cocción y las condiciones bajo las que se realice. Por ello, la cocción puede ser considerada como aquella operación capaz de transformar de modo físico y/o químico el aspecto, la textura y el valor nutritivo de un alimento mediante la acción del calor, con el fin de mejorar las características organolépticas (Bello Gutierrez, 1998).

Se pueden distinguir varios tipos de cocciones en función de factores diversos: sistema calorífico utilizado, materiales empleados, modalidades de trabajos, etc. (Bello Gutierrez, 1998).

3.1. Cocciones en medio no líquido.

Se denominan cocciones con calor seco, porque el alimento se calienta a través de su parte superficial, puesta en contacto con una atmosfera de aire caliente. Sus objetivos son mejorar la palatabilidad del alimento, haciéndolo más tierno, digerible y con unas propiedades sensoriales específicas. Este calor puede llegar al alimento:

a) Mediante fuego directo

i. Plancha

Cocción a temperatura elevada del alimento situado sobre una placa caliente, que transfiere por conducción el calor recibido desde un foco calorífico de ascuas, electricidad o gas.

ii. Parrilla

Cocción a temperaturas elevada sobre parrilla que recibe el calor por radiación, a una distancia adecuada, desde un foco calorífico que normalmente son brasas.

b) Mediante fuego indirecto

i. Horno

Cocción en un recinto cerrado, donde le calor se transfiere, en parte por radiación y en parte por corrientes de convención.

3.2. Cocciones en medio graso

Se tratan de métodos culinarios que utilizan un medio graso como transferencia de calor.

a) Salteado

Consiste en la cocción total o parcial de un alimento en poca cantidad de cuerpo graso previamente calentado. Es un método de cocción rápido que se caracteriza por la poca cantidad de grasa que se utiliza.

b) Fritura

En la fritura el alimento debe ser sumergido en un cuerpo graso, que ha sido previamente calentado a una temperatura más o menos elevada, en función de la naturaleza y del grosor de la pieza tratada. Con ello, se consigue obtener preparaciones culinarias doradas y crujientes. Es uno de los más antiguos procesos de cocción aplicado en los países

del área mediterránea donde abundaba el aceite de oliva. Se distinguen dos tipos de fritura, en la práctica que son, la fritura superficial y la fritura profunda.

- En la **fritura superficial** el alimento está sumergido sólo en parte en el cuerpo graso de manera que es esa parte la que realmente se fríe. El resto se cuece por el vapor de agua generado en su interior.

- En la **fritura profunda** el alimento queda totalmente sumergido en el aceite de manera que se fríe de manera uniforme por toda su superficie. Las temperaturas aplicadas en la fritura suelen oscilar entre 160ºC-200ºC, suelen ser las necesarias para conseguir una textura crujiente. En ocasiones se llevan a cabo una serie de labores preparativas del alimento consistentes en recubrirlo con harina, huevo, leche, etc. Se realiza con la finalidad de proteger al alimento de la penetración de un calor excesivo y perdida demasiado elevada de humedad. Cabe distinguir:

- **Enharinado**: el alimento se recubre de harina. Más realizado en pescados.

- **Rebozado**: operación que consiste en proteger el alimento con una ligera capa de ciertas características de acuerdo con los ingredientes empleados (harina de trigo, proteína de soja, gluten de trigo, albúmina de huevo, leche, sal común, etc.) y determinado grosor, con la finalidad de mejorar textura, sabor, color, presencia, etc., cuando se someten a procesos de fritura.

- **Empanado**: el alimento se recubre con pan rallado.

3.3. Cocciones en medio acuoso

Existen técnicas de cocción en la que se emplea un fluido acuoso como medio de transferencia de calor para el tratamiento térmico del alimento. Para ellos, el alimento toma contacto con agua, caldo corto, jarabe o, incluso, vapor de agua normal o sobrecalentado, dentro de sus posibilidades de realización, se puede partir de un medio acuoso líquido, más o menos frio, como un líquido en ebullición o de vapor de agua.

La diversidad del medio acuoso elegido tendrá, de modo indudable, su reflejo y sus consecuencias prácticas en los mecanismos de transferencias de energía y de masas. En la práctica pueden distinguirse cuatro tipos principales de cocciones en medio acuoso:

a) **Escaldado.** Se considera más bien una operación previa que se suele realizar para inactivar sistemas enzimáticos. Consiste en una cocción incompleta del alimento en agua hirviendo durante un periodo de tiempo corto. Si este tiempo es más prolongado, recibe el nombre de sancochado.
b) **Cocer o hervir:** Consiste en la cocción del alimento por inmersión del mismo en agua u otro caldo que puede estar a distintos grados de temperatura inicialmente: fría, caliente o en ebullición. En cualquier caso, los tiempos de cocción vendrán determinados por la calidad y volumen de algunos alimentos.
c) **Escalfado:** Consiste en la cocción de un alimento en un medio líquido que se encuentra justo por debajo de su punto de ebullición. El medio líquido empleado puede ser de naturaleza muy diversa: agua, jarabe, leche. Se trata de un método de cocción lento.
d) **Cocción al vapor:** Cocción mediante vapor de agua, que puede ser realizada bajo dos modalidades:
    i. A presión normal
    ii. A presión elevada

En la cocción al vapor los objetivos son varios: facilitar la digestión, proporcionar una textura más agradable y comestible, minimizar las pérdidas de nutrientes. La textura del alimento varía de acuerdo con la naturaleza del mismo, el tipo de vapor y el grado de calor aplicado. Posee varias ventajas como: mejora la retención de nutrientes, reduce el riesgo de sobrecocción, la elevada presión hace más rápida la llegada al punto final, hace algunos alimentos más fáciles de digerir y ligeros por lo que puede ser más recomendable en clínica.

4. Influencia del tratamiento culinario en valor nutritivo del alimento

Como consecuencia del tratamiento culinario, se van a producir una serie de cambios físicos y químicos en el alimento que en algunos casos serán positivos y en otros negativos. Estos cambios que tienen lugar en el alimento durante la cocción van a depender sobre todo del binomio tiempo-temperatura aplicado, del medio de cocción y de la naturaleza del alimento (Bello Gutierrez, 1998). En este caso nos vamos a centrar en los efectos que los tratamientos térmicos puedan generar sobre el valor nutritivo y contenido en micronutrientes de los alimentos.

En alimentos proteicos, los tratamientos culinarios que buscan un choque térmico (como asado, plancha o parrilla) provocan una rápida coagulación de las proteínas externas que aparte de ayudar a mantener la jugosidad pueden contribuir a favorecer la retención de nutrientes en el interior del alimento disminuyendo las perdidas. Un tratamiento demasiado prolongado podría llevar a destrucción de las estructuras por pirolisis de proteínas (Bello Gutierrez, 1998).

Durante el calentamiento de los alimentos se produce el **pardeamiento no enzimático o Reacción de Maillard,** la cual está ampliamente descrita en el **apartado 5.** Esta tiene lugar por la reacción del grupo carbonilo de un azúcar reductor o de un lípido oxidado con el grupo amino libre de un aminoácido, péptido o proteína o incluso vitamina. Esta reacción se ve favorecida por el

tiempo y la temperatura aunque es capaz de desarrollarse a temperatura ambiente pero lentamente. En cualquier caso, además de influir en las características organolépticas va a modificar el valor nutritivo de los alimentos. Por un lado puede provocar pérdidas de aminoácidos esenciales. Sin embargo, también se ha visto que compuestos generados durante la reacción de Maillard poseen determinadas actividades beneficiosas para la salud como antioxidante o prebiótica (Tagliazucchi & Bellesia, 2015; Pastoriza & Rufián-Henares, 2014; Morales, Somoza, & Fogliano, 2012). De esta manera, el tratamiento térmico de alimentos podría mejorar algunas de sus cualidades beneficiosas como la antioxidante o prebiótica a través de esta vía (Kusznierewicz, Śmiechowska, Bartoszek, & Namieśnik, 2008; Michalska, Amigo-Benavent, Zielinski, & del Castillo, 2008).

En tratamientos térmicos en los que se aplica temperaturas más elevadas, como asado, plancha o fritura, se va a producir una deshidratación del alimento que va a dar lugar a una concentración de nutrientes en su interior (Bello Gutierrez, 1998).

Por otro lado, la cocción en medio acuoso va a provocar una difusión de nutrientes al medio de cocción enriqueciéndolo. Sin embargo, si no se consume el liquido empleado el valor nutritivo final del alimento puede verse disminuido. De esta manera, se ha visto que se puede producir una pérdida de vitaminas hidrosolubles asi como de compuestos fenólicos reduciendo por tanto la **actividad antioxidante** del alimento en cuestión (Bello Gutierrez, 1998); Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & de la Serrana, 2015). También se ha visto en espinacas un descenso de carotenoides después del hervido. De igual manera se observó un descenso de los polifenoles totales en espinacas, col, coliflor y brócoli después del hervido probablemente debido a su solubilización en el medio de cocción. Sin embargo, también se ha visto que el tratamiento térmico puede aumentar su disponibilidad ya que los compuestos fenólicos suelen encontrarse unidos a estructuras vegetales de pectínicas o celulósicas. Este tratamiento favorecería la desunión de estos compuestos haciéndolos más biodisponibles para su absorción

(Bunea et al., 2008). Se ha comprobado que mediante el cocinado al vapor las pérdidas de compuestos hidrosolubles se evita (Soares, Carrascosa, & Raposo, 2017).

Por otro lado, la cocción en medio graso al emplear temperaturas más elevadas podría provocar la pérdida de nutrientes termolábiles. Sin embargo, cuando el medio graso empleado es aceite de oliva virgen extra se produce un enriquecimiento en compuestos fenólicos del alimento debido a la penetración del aceite en el alimento. De esta manera se mejora su perfil fenólico (Ramírez-Anaya et al., 2015).

Además, se ha visto que la cocción, ya sea en medio acuoso o graso, puede favorecer y aumentar la actividad antioxidante de los vegetales (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2007). Los autores de este estudio propusieron como respuesta que el tratamiento térmico favorece el ablandamiento y degradación de estructuras vegetales favoreciendo así la liberación de compuestos haciendo que fueran más bioaccesibles ya que de otra forma quedarían retenidos en el interior del vegetal puesto que nuestros sistemas enzimáticos no pueden degradarlos. Además, aunque de manera general, en lo que se refiere a compuestos individuales como luteína, ácido cafeico, ácido clorogenico, ácido cumárico se observa una disminución con respecto al material crudo, la actividad antioxidante aumente. Según estos autores es posible que el procesado y el medio favorezcan la formación de nuevos compuestos con elevada capacidad antioxidante. Estos compuestos generados durante el tratamiento térmico podría proceder de la reacción de Maillard como otros autores han puesto de manifiesto anteriormente (Kusznierewicz et al., 2008; Michalska et al., 2008; Pastoriza & Rufián-Henares, 2014).

Como conclusión, el valor nutricional del alimento se verá afectado por la temperatura, el tiempo y el medio de cocción. Aunque la temperatura pueda degradar ciertos compuestos, otros se generan como los generados durante la **reacción de Maillard** que contribuyen a la **actividad antioxidante**. Además, la cocción favorece la biodisponibilidad de los nutrientes al hacerlos más

accesibles. El medio de cocción es también importante puesto que la cocción acuosa puedo provocar perdidas de compuestos hidrosolubles mientras que la cocción al vapor no. La cocción en medio graso con aceite de oliva virgen extra puede mejorar el perfil fenólico y antioxidante del alimento.

5. Pardeamiento químico

El pardeamiento químico (también conocido como pardeamiento no enzimático) es un conjunto de reacciones muy complejas producidas en los alimentos tratados térmicamente y que da lugar a la formación de productos pardos (Cheftel & Cheftel, 1980). Se produce durante el procesado y almacenamiento de diversos alimentos, se acelera con el calor y se acusa especialmente durante las operaciones de cocción, pasteurización, esterilización y deshidratación. Estas reacciones provocan modificaciones en el olor, color y sabor de los alimentos. Los problemas derivados de este tipo de transformaciones en algunos alimentos, obliga a la industria alimentaria a establecer normas de procesado y control que reduzcan este tipo de alteraciones.

El pardeamiento químico puede producir efectos indeseables durante la preparación y almacenamiento de alimentos líquidos (leche, zumos de frutas y jarabes) y deshidratados (leche, huevos, carne) pero también puede producir efectos favorables para ciertos alimentos (Cheftel and Cheftel, 1980).

El pardeamiento químico agrupa las reacciones de degradación del ácido ascórbico, caramelización (transformación de carbohidratos) y reacción de Maillard (interacción proteína-carbohidrato).

5.1. Degradación del ácido ascórbico

El ácido L-ascórbico o vitamina C es una sustancia muy soluble en agua que posee propiedades ácidas y fuertemente reductoras, debido a su estructura de enodiol conjugado con el grupo carbonilo de una lactona, por lo que es una molécula muy sensible a diversas formas de degradación (Finholt, Alsos, & Higuchi, 1965).

La degradación del ácido ascórbico se realiza sin la presencia de grupos amino a pH ligeramente ácido, con a_w media/alta y temperatura moderada (Badui Dergal, 2006). Sigue tanto una vía oxidativa (Velí'sek, Davídek, Kubelka, Zelinková, & Pokorny, 1976) como no oxidativa (Kurata & Sakurai, 1967) produciendo ambas diversos compuestos como furfural, 3-hidroxi-2-pirona, ácido 2-furancarboxílico, ácido acético y 2-acetilfurano. Algunas de estas sustancias contribuyen al aroma de ciertos alimentos como es el caso de las patatas, café y el pan (Velí'sek et al., 1976).

5.2. Caramelización

La caramelización se presenta cuando los azúcares son calentados por encima de su temperatura de fusión. Se produce en ausencia de oxígeno y grupos amino, a a_w bajas y a pH tanto ácidos como básicos. En ella, los monosacáridos forman enoles como paso inicial de la reacción (Badui Dergal, 2006). Las pentosas generan 2-furaldehido como principal producto de degradación, mientras que las hexosas producen 5-hidroximetil-2-furaldehido (HMF) y otros compuestos como 2-hidroxiacetilfurano e isomaltol. La fragmentación de estos productos primarios da lugar a la formación de compuestos como ácido fórmico, acetal, diacetilo, ácido acético, etc. Algunos de estos productos poseen intenso olor y pueden conferir fuertes aromas deseables o indeseables (Cheftel & Cheftel, 1980).

5.3. Reacción de Maillard

La reacción de Maillard ocurre principalmente en aquellos alimentos que contienen azúcares reductores o lípidos oxidados y grupos amino de un aminoácido, péptido o proteína, que sufren un procesado térmico (calentamiento o conservación a temperatura ambiente) y se produce por lo general a a_w intermedias.

5.3.1. Química de la reacción de Maillard

La reacción de Maillard es un conjunto de reacciones químicas en cadena que dan lugar a la formación de pigmentos pardos con modificaciones del olor, color y sabor de diversos alimentos. Se desarrolla a a_w intermedias y diversos pH, necesitando un aporte de calor moderado (Badui Dergal, 2006). De acuedo con Baxter (1995), se origina entre el grupo amino de un aminoácido, péptido o proteína y el grupo carbonilo de un azúcar reductor o un lípido oxidado, dando lugar a los denominados productos de la reacción de Maillard (PRMs).

Esta reacción es especialmente importante para la industria alimentaria, ya que se da con frecuencia durante el almacenamiento de los alimentos y en procesos como el horneado, tostado, fritura, etc. confiriéndoles nuevos colores, olores, sabores y texturas agradables para el consumidor (Rizzi, 1997), aunque también pueden originarse sustancias aromáticas y compuestos pardos indeseados (Baltes, 1982). Además, la reacción de Maillard puede disminuir el valor nutritivo de los alimentos, principalmente el afectar la calidad de las proteínas, debido a la destrucción de aminoácidos o disminución de su biodisponibilidad y la de otros nutrientes (Finot & Magnenat, 1981).

La reacción de Maillard puede dividirse en tres etapas:

1. **Etapa temprana.** En esta etapa aún no aparece el pardeamiento. Comienza con la condensación entre el grupo carbonilo libre de un azúcar reductor o un lípido oxidado y el grupo amino de un aminoácido, péptido o proteína que, tras deshidratación, da lugar a una base de Schiff inestable que se transforma en glicosamina-N-sustituida. Esta reacción es reversible ya que en un medio fuertemente ácido se regenera el azúcar y el aminoácido. Las glicosilaminas son más estables cuando proceden de aminas aromáticas que de aminoácidos (Pigman & Johnson, 1953; Finot & Magnenat, 1981).

2. **Etapa intermedia.** En esta etapa se produce la inmediata reorganización irreversible de la glicosilamina-N-sustituida. Cuando se parte de una aldosilamina-N-sustituida, mediante la **transposición de Amadori,** se genera 1-amino-1-desoxi-2-cetosa, mientras que cuando se parte de una cetosilamina-N-sustituida, mediante la **transposición de Heyns** se genera 2-amino-2-desoxialdosa. Los compuestos de Amadori (Mauron, 1981) y de Heyns (McPherson, Shilton, & Walton, 1988) han sido encontrados en diversos alimentos y en el organismo humano.

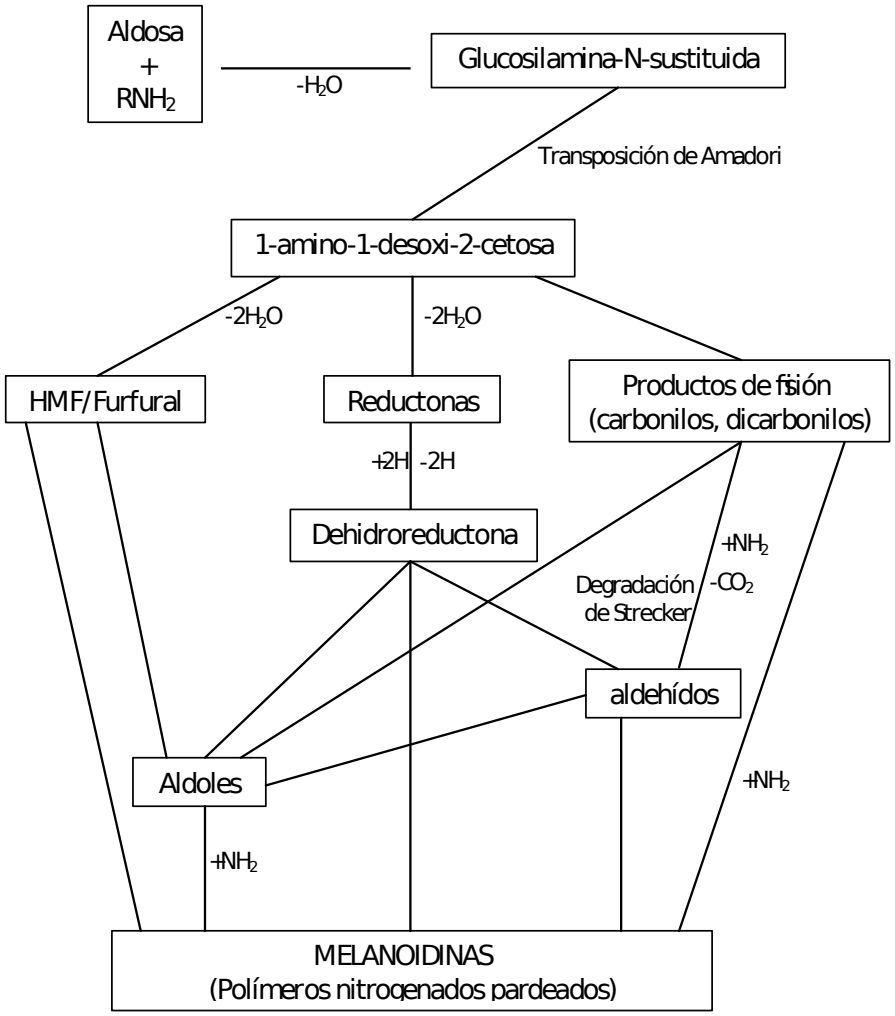
Durante esta fase, se produce la deshidratación de los azúcares por isomerización enólica dando lugar a furfural y derivados. También se presentan mecanismos de fragmentación de los azúcares enólicos y se favorece la formación de compuestos de bajo peso molecular como aldehídos, cetonas, ácidos, alcoholes, etc.

3. **Etapa final**. Se produce la polimerización y formación de sustancias coloreadas como las melanoidinas y los compuestos avanzados de la reacción de Maillard. Las melanoidinas son polímeros pardos producidos mediante la condensación de compuestos aminados procedentes de las etapas intermedias de la reacción de Maillard como son pirroles N-sustituidos, 2-formilpirroles N-sustituidos y 2-furaldehido (Tressl, Wondrak, Garbe, Krüger, & Rewicki, 1998).

Las melanoidinas varían ampliamente en peso molecular y poseen rasgos distintivos en la región visible del espectro (Clark & Tannenbaum, 1970).

La **Figura 3** muestra esquemáticamente como se desarrolla la Reacción de Maillard.

Figura 3. Esquema de la reacción de Maillard



5.3.2. Variables y prevención de la reacción de Maillard

El desarrollo de la reacción de Maillard depende de una serie de factores como son la estructura química de los reactantes y su concentración, la actividad de agua, el pH, la temperatura y el tiempo de calentamiento, la presión hidrostática, etc. (O'brien & Morrissey, 1997; Wijewickreme, Kitts, & Durance, 1997).

1. Sustratos

Principalmente, los sustratos de esta reacción son grupos carbonilos de azúcares reductores (o de lípidos oxidados) y los grupos amino de aminoácidos libres, péptidos o proteínas. Es una reacción isomolecular, pero se produce una pérdida de azúcares al producirse paralelamente la caramelización.

En función del azúcar implicado en la reacción se aumentará o disminuirá la intensidad de la reacción. En general, los carbohidratos de bajo peso molecular son más reactivos que los de alto peso molecular por tener un menor impedimento estérico (J.A. Rufián-Henares & Pastoriza, 2016).

Compuestos con grupos carbonilo (Montgomery & Day, 1965; Velí'sek et al., 1976) como los formados por degradación de Strecker (formaldehido, acetal, propional) o los producidos durante la degradación de los compuestos de Amadori (furfural e hidroximetilfurfural) (Obretenov, Argirov, & Rashkov, 1983) pueden reaccionar con los grupos amino e iniciar otra vez la reacción sin necesidad de azúcares (Clegg, 1964).

Por parte del grupo amino, todas las proteínas son susceptibles de reaccionar con los azúcares salvo las insolubles. Sin embargo, algunos no son susceptibles al encontrarse ocultos en la cadena proteica, por lo que el grupo amino terminal es el más reactivo seguidos de los aminoácidos básicos. Los aminoácidos básicos libres no reaccionan con los azúcares a una velocidad marcadamente superior al resto de aminoácidos, sino que es la configuración estereoquímica la que

determina su comportamiento. Si logramos transformar el grupo amino en amido mediante el uso de transglutaminasas, lograremos impedir la reacción (Friedman, Wilson, & Ziderman, 1990; Friedman, 1996).

2. pH

El desarrollo de la reacción de Maillard está fuertemente condicionado por el pH inicial de los reactantes así como por la capacidad tampón del sistema (Nursten, 1981). En general, un incremento del pH favorece su desarrollo y la aparición de color (Ames, 1990) mientras que a pH ácido la velocidad es mínima y su desarrollo escaso (Lea & Hannan, 1949) alcanzando su máximo cuando el pH se eleva a 10 (Ashoor & Zent, 1984). El avance de la reacción supone una disminución del pH debido a la formación de ácidos grasos de cadena corta y a la desaparición de aminoácidos básicos (J.A. Rufián-Henares & Pastoriza, 2016). La reactividad del grupo amino es mayor cuando el aminoácido está en forma aniónica y este valor depende del carácter ácido-base del aminoácido. En aminoácidos ácidos (ácido aspártico y glutámico) la forma aniónica se produce a pH 3, pero en aminoácidos básicos (lisina y arginina) se necesita un pH de 10 (Song & Chichester, 1967; Powell & Spark, 1971). A pH mayor de 10, el descenso de protones necesario para la catálisis de la reestructuración de Amadori y Heyns, puede ser la causa del descenso de esta reacción (Carson, 1955; Isbell & Frush, 1958).

3. Actividad de agua

Uno de los factores que más influencia tiene en el desarrollo de la reacción de Maillard es el agua presente en los alimentos, expresado como actividad de agua (a_w) (Labuza & Saltmarch, 1982; Ames, 1990). La velocidad de la reacción aumenta de manera exponencial con el contenido de humedad del sistema (Labuza, Tannenbaum, & Karel, 1970) hasta un máximo de a_w entre 0.3 y 0.7 (Eichner & Karel, 1972) y ya a partir de 0.8 la velocidad de reacción disminuye debido a la dilución del soluto en la fase acuosa (Kane & Labuza, 1989).

Esta cinética se explica de la siguiente forma: con un contenido en agua muy bajo está frenada la difusión de especies químicas entre sí. La adición o aumento del agua facilitan la difusión de los reactantes (Schwartz & Lea, 1952; Wolfrom & Rooney, 1953; Labuza et al., 1970) y aumenta la velocidad de pardeamiento. Sin embargo, con cantidades superiores de agua, las concentraciones de sustancias reactivas en solución disminuyen y se reduce, de acuerdo con la ley de acción de masas, la velocidad de la reacción (Eichner & Karel, 1972). En sistemas con una humedad pequeña o nula la reacción de Maillard podría proseguir por el agua que aparece en las deshidrataciones de las primeras etapas de la reacción.

El estado fisicoquímico en sistemas de alimentos puede también afectar a la reacción de Maillard. La isoterma de sorción dependerá de si el sistema es cristalino o amorfo y de otros factores tales como el porcentaje en grasa (O'brien & Morrissey, 1997). A bajas a_w un sistema de alimentos amorfo absorbe más agua en el espacio que hay entre las moléculas, mientras que en un sistema cristalino, la absorción del agua sólo puede darse en la superficie del entramado del cristal. En alimentos desecados como la leche en polvo, un cambio del estado amorfo al cristalino ocurre a valores de a_w = 0.6 a 0.7 (O'brien & Morrissey, 1997). La fase amorfa absorbe agua hasta que las moléculas adquieren suficiente movilidad y espacio para formar el entramado del cristal. Como la cristalización se ha iniciado, el agua es expulsada y puede quedar atrapada en áreas localizadas del alimento (H. Erbersdobler, 1971). Esta agua está entonces disponible para la interacción con otros componentes y afectará al grado de la reacción de Maillard, a no ser que se evapore (H. Erbersdobler, 1971).

Por lo general, es durante la deshidratación cuando los riesgos de pardeamiento son mayores y en especial en la fase del proceso en que el contenido en agua es inferior al 20% y la temperatura es elevada. Tratamientos a temperaturas más bajas presentan el inconveniente de alargar el proceso de deshidratación y el alimento permanece más tiempo con un contenido crítico de agua. Así por ejemplo en la elaboración de pan la baja actividad de agua favorece la formación

de color durante el horneado, pero a medida que avanza la cocción, aumenta la temperatura y disminuye la actividad de agua por lo que se acelera el pardeamiento y acumulación de compuestos coloreados en la corteza (Purlis, 2010).

4. Temperatura

La reacción de Maillard se produce tanto a temperatura ambiente (durante el almacenamiento) como a altas temperaturas (esterilización). A mayor temperatura el desarrollo de la reacción de Maillard es más intenso, aunque el factor clave no solo es la temperatura utilizada sino el tiempo empleado. Así se puede obtener el mismo grado de pardeamiento si el producto se calienta a una temperatura elevada durante un periodo de tiempo corto como si el tiempo empleado es mayor y la temperatura que se alcanza es menor. Por eso es importante tener en cuenta que la mejor variable para predecir el pardeamiento es la carga de calor: cantidad de calorías aplicadas al producto (Rufián-Henares & Pastoriza, 2016).

Debido a las fuertes energías de activación de algunas de las reacciones de pardeamiento, no deben someterse los alimentos a tratamientos térmicos demasiado enérgicos y además hay que conseguir que el almacenamiento sea a una temperatura moderada para minimizar el avance de la reacción de Maillard.

5. Presencia de metales

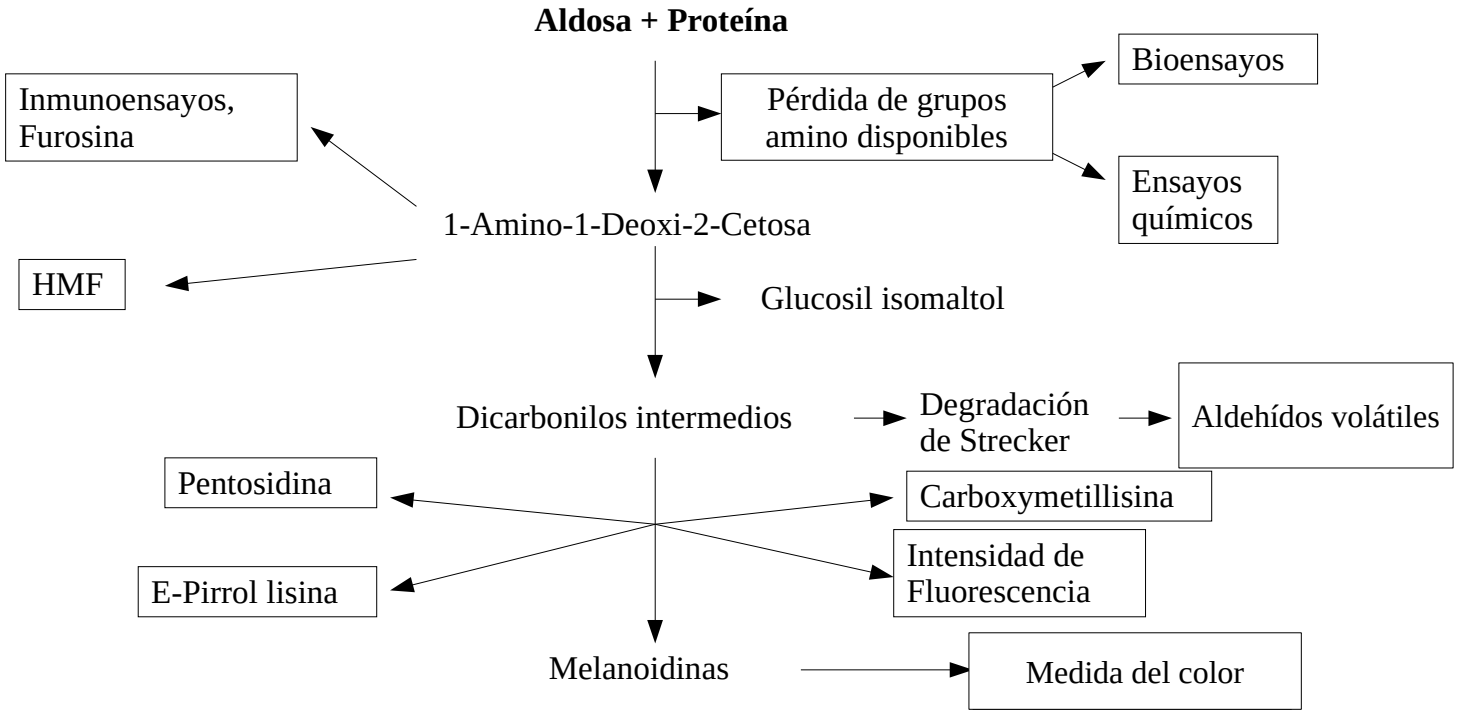
La presencia de cationes metálicos afecta a la reacción de Maillard ya que mediante reacciones de oxidación pueden favorecer la formación de compuestos dicarbonílicos que participan en la reacción (Wolf, 1996) o bien de complejos capaces de catalizarla (Kato, Watanabe, & Sato, 1981; O’brien & Morrissey, 1997)y por tanto incrementar el desarrollo del pardeamiento. Las sales de cobre e hierro aceleran la reacción mientras que el manganeso la inhibe (Bohart & Carson, 1955). Hay que hacer notar que los primeros estudios atribuyen efectos a los cationes metálicos debidos al descenso del pH que se produce en su incorporación al sistema (Powell & Spark, 1971).

5.3.3. Indicadores químicos y biológicos de la reacción de Maillard

La extensión de la reacción de Maillard en alimentos puede seguirse con numerosos métodos que incluyen desde los clásicos ensayos químicos a los ensayos biológicos. Los objetivos principales son definir en el alimento, el estado nutricional, características organolépticas y su posible toxicidad después del procesado y/o almacenamiento, para así optimizar los procesos de elaboración y conservación y conseguir productos con una buena calidad final y un alto valor nutritivo.

Según el método utilizado se obtendrá información de las diferentes etapas de la reacción (**Figura 4**).

Figura 4. Indicadores de la Reacción de Maillard

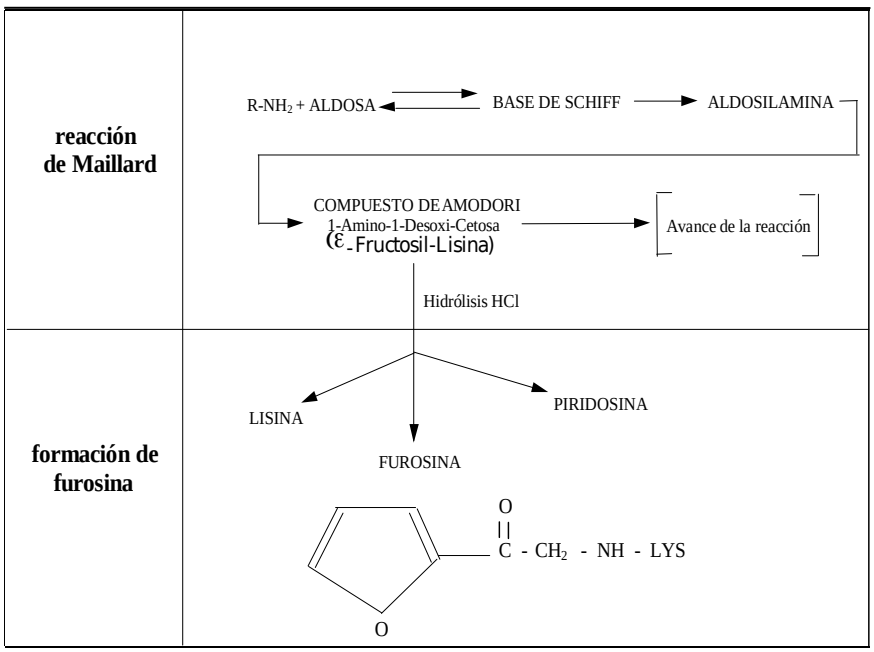


- **Furosina**

Este compuesto fue uno de los primeros productos que se identificaron para la reacción de Maillard. Fue cuantificada en alimentos como un indicador de los compuestos de Amadori (Erbersdobler, Dehn, Nangpal, & Reuter, 1987). Algo que se debe tener en cuenta es que la cantidad de furosina varía dependiendo de las condiciones de hidrólisis (Guerra-Hernandez, Corzo, & Garcia-Villanova, 1999).

En 1966 se detectó un compuesto que eludía en la zona de los aminoácidos básicos después de la arginina tras analizar un hidrolizado de leche en polvo desnatada secada con rodillos mediante cromatografía de intercambio iónico. Además, daba positiva la reacción de la ninhidrina. La concentración de este compuesto aumentaba en función del tratamiento térmico aplicado a la leche. Finot, Bricout, Viani, & Mauron, (1968) y Heyns, Heukeshoven, & Brose, (1968) la sintetizaron e identificaron llamándole furosina (ε-N-furoil-metil-L-lisina). La **Figura 5** muestra la formación de furosina a partir de una aldosa de forma simplificada.

Figura 5. Formación de furosina a partir de la ε-fructosillisina.



Los primeros métodos que surgieron para su determinación consistían en cromatografía de intercambio iónico de aminoácidos (Erbersdobler et al., 1987; Messia, Panfili, Marconi, Cubadda, & Acquistucci, 2001). Sin embargo, eran métodos caros y con una sobreestimación del daño en la lisina (Henle, Walter, Krause, & Klostermeyer, 1991; Henle, Walter, & Klostermeyer, 1991). Otro método utilizado es la cromatografía de gases, previa derivación (Ruttkat & Erbersdobler, 1994) y la electroforesis capilar (Tirelli, 1998; Delgado-Andrade, Rufián-Henares, & Morales, 2005). No obstante, la cromatografía liquida de alta resolución (HPLC) ha sido la técnica más utilizada para la determinación de furosina. Resmini, Pellegrino, & Battelli, (1990) propusieron la separación de furosina en fase reversa con par iónico (columna C8) y elución con gradiente. Posteriormente Delgado, Corzo, Santa-María, Jimeno, & Olano, (1992) pusieron a punto un método en el que separaron furosina utilizando fase reversa con par iónico (columna C18) y elución isocrática. Ambos métodos son sensibles, reproducibles y rápidos.

- **5-Hidroximetilfurfural (HMF)**

Mediante la determinación del 5-Hidroximetilfurfural (HMF), podemos seguir las etapas intermedias de la reacción de Maillard. Para su cuantificación se puede utilizar el método colorimétrico con ácido tiobarbitúrico propuesto por Keeney & Bassette, (1959) que permite obtener los valores de HMF libre, y el HMF libre más el HMF potencial, derivado de otros intermediarios de pardeamiento (HMF total).

La cromatografía líquida de alta resolución (HPLC) es la técnica que más se utiliza en la actualidad, ya que permite estimar el contenido de HMF de forma más exacta al separarlo de posibles interferencias (Ferrer, Alegría, Fairé, Abellan, & Romero, 1999). Las muestras líquidas son sometidas a una clarificación previa con reactivo de Carrez (Garcia-Villanova, Guerra-Hernandez, Martinez-Gomez, & Montilla, 1993) o ácido tricloroacético (TCA), separándose el sobrenadante obtenido con columnas de fase reversa y condiciones isocráticas (Porretta, 1992; Garcia-Villanova

et al., 1993). Aunque la técnica parece adecuada, en alimentos con proteínas lácteas se han encontrado compuestos que coeluyen con el HMF (van Boekel, 2006), por lo que es necesario controlar los componentes de las fases móviles (Morales, Romero, & Jiménez Pérez, 1992) o purificar las muestras con solventes orgánicos (Rufian-Henares, Garcia-Villanova, & Guerra-Hernandez, 2001).

- **Furfural**

El furfural es un compuesto furánico que se produce en las reacciones de degradación del ácido ascórbico, caramelización y en las etapas intermedias de la reacción de Maillard. Este compuesto es formado durante tratamientos térmicos, deshidratación o almacenamiento a temperaturas inadecuadas.

El furfural no se detecta en los zumos frescos, sin embargo, este se incrementa en condiciones de calentamiento o almacenamiento a temperaturas elevadas. Se ha encontrado una buena correlación entre el contenido de furfural y aromas no deseables en estos productos. Por tanto, el furfural es un buen indicador de calidad (Lee & Nagy, 1988; Espinoza Mansilla, Salinas, Nevado, & J, 1992). En la actualidad, la determinación de furfural se realiza casi exclusivamente por HPLC ya que esta técnica permite la cuantificación de forma individual.

6. Actividad antioxidante.

6.1. Radicales libres y especies reactivas de oxigeno

Por radical libre se entiende cualquier molécula o especie química con un electrón libre desapareado en el orbital más externo de su estructura atómica. Esto le confiere cierta inestabilidad haciendo que se trate de moléculas altamente reactivas (Gutiérrez & Justo, 2002).

Las especies reactivas del oxígeno (ROS) es un término general que se aplica no sólo a los radicales libres derivados del oxígeno sino también a moléculas no radicales que se forman como consecuencia de la reducción molecular del oxígeno y que son también muy reactivas como el peróxido de hidrógeno (H_2O_2) o el ácido hipocloroso (Halliwell, 2006). También existen especies oxidantes derivadas del nitrógeno (**Tabla 1**).

Tabla 1. Especies reactivas derivadas del oxígeno.

ESPECIES REACTIVAS DERIVADAS DEL OXÍGENO			
Radical libre		***Pro-radical***	
Anión superóxido	($^{\cdot}O_2$)	Peróxido de hidrógeno	(H_2O_2)
Radical hidroxilo	($^{\cdot}OH$)	Ácido hipocloroso	(HOCl)
Radical peroxílo	($ROO^{\cdot}$)	Ácido hipobromoso	(HOBr)
Radical alcoxilo	($RO^{\cdot}$)	Ozono	(O_3)
Hidroperoxilo	($HOO^{\cdot}$)	Oxígeno singlete	($^1O_2$)
ESPECIES REACTIVAS DERIVADAS DEL NITRÓGENO			
Radical libre		***Pro-radical***	
Óxido nítrico	($NO^{\cdot}$)	Ácido nitroso	(HNO_2)
Dióxido de nitrógeno	($NOO^{\cdot}$)	Catión nitrosilo	(NO^+)
		Anión nitrosilo	(NO)
		Peroxinitrito	($ONOO^-$)
		Ácido peroxinitroso	(ONOOH)
		Catión nitrilo	(NO_2^+)

Estos agentes oxidantes pueden ser tanto de origen endógeno (mitocondria, peroxisomas, lipooxigenasas, NADPH oxidasa, citocromo P450 y células fagocitarias) como exógeno (radiaciones ultravioleta, radiaciones ionizantes, medicamentos, citoquinas inflamatorias y toxinas) (Finkel & Holbrook, 2000). A bajas concentraciones describen efectos beneficiosos, debido a que intervienen en diversas rutas de señalización celular, en la defensa frente a agentes infecciosos y en la inducción de la respuesta mitogénica (Seifried, Anderson, Fisher, & Milner, 2007). Sin embargo, a elevadas concentraciones (hábitos como el tabaquismo, alcoholismo, exposición descontrolada a radiaciones solares, contaminación ambiental, situaciones de estrés, ejercicio físico extenuante o dietas muy energéticas y grasas) pueden provocar efectos perjudiciales derivados del estrés oxidativo, hecho que puede originar daños celulares en biomoléculas tales como lípidos, proteínas y ADN (Aruoma, 2003).

6.2. Estrés oxidativo

La generación de especies reactivas de oxígeno y otros radicales libres durante el metabolismo celular aeróbico es un proceso normal y fisiológico que generalmente se encuentra compensado por los sistemas de defensa de antioxidantes endógenos, los cuales ayudan a mantener el equilibrio redox (Davies, 1995; Valko et al., 2007). No obstante, ciertas condiciones ambientales, estilo de vida o situaciones patológicas pueden derivar en un exceso de radicales libres que conduzca a una situación de estrés o daño oxidativo. El desequilibrio entre especies oxidantes y antioxidantes se conoce como estrés oxidativo. Dicho estrés se debe, por un lado, a la sobreproducción de radicales libres y por otro, al déficit de antioxidantes enzimáticos y no enzimáticos. El estrés oxidativo está asociado al proceso normal de envejecimiento y a numerosas patologías como carcinogénesis, enfermedad cardiovascular, diabetes, neurodegeneración (Alzheimer y Parkinson) o procesos inflamatorios (Valko et al., 2007).

Un estado de estrés oxidativo va a provocar alteraciones en biomoléculas como lípidos, proteínas, carbohidratos y ácidos nucléicos, lo que puede provocar alteraciones en el metabolismo y regulación celular (peroxidación lipídica, inactivación y desnaturalización proteica, lesiones en el citoesqueleto celular, pérdida de fluidez de membrana y procesos de mutagénesis) que pueden producir acumulación de agregados intracelulares, disfunción mitocondrial, citotoxicidad y apoptosis (Hicks, Torres-Ramos, & Sierra-Vargas, 2006). De igual forma, la oxidación también afecta a los alimentos, siendo la causa principal del deterioro químico, originando enraciamiento y reducción de la calidad nutricional, color, sabor, textura e inocuidad (Laguerre, Lecomte, & Villeneuve, 2007; Roginsky & Lissi, 2005).

6.3. Sistemas de defensa antioxidante

La exposición a radicales libres de una variedad de fuentes ha hecho que los organismos desarrollen una serie de mecanismos de defensa que incluyen (Londoño, 2012):

1. Remoción catalítica de radicales libres por enzimas.
2. Unión de proteínas a metales prooxidantes como hierro y cobre.
3. Protección contra daño, como las proteínas de choque térmico.
4. Estabilización de radicales libres con donadores de protones o electrones como glutatión, vitamina E o vitamina C.

Estos sistemas de defensa antioxidante pueden clasificarse en función de su origen en sistemas antioxidantes endógenos, enzimáticos y no enzimáticos, y sistemas antioxidantes exógenos, que se adquieren a través de la dieta (Ugartondo, 2009):

1. **Sistemas de defensa antioxidantes endógenos:**

    a. **Sistemas enzimáticos:** Los antioxidantes enzimáticos constituyen la primera línea de defensa antioxidante y previenen el daño oxidativo interaccionando directamente con las ROS. Destacan las enzimas superóxido dismutasa, catalasa y glutatión peroxidasa (Ugartondo, 2009).

    b. **Sistemas no enzimáticos:** Constituyen la segunda línea de defensa y está constituido básicamente por antioxidantes de bajo peso molecular que forman un numeroso conjunto de compuestos capaces de prevenir el daño oxidativo por interacción directa o indirecta con las ROS. Destacan el tripéptido glutatión, la bilirrubina, el ácido úrico y la histidina (Ugartondo, 2009).

2. **Sistemas de defensa antioxidantes exógenos:** Formados por los denominados antioxidantes dietarios. La capacidad de los sistemas de defensa antioxidantes endógenos es limitada, por lo que nuestro organismo necesita cierta ayuda externa. Los nutrientes básicos que ingerimos a través de la dieta (proteínas, lípidos, vitaminas y minerales) ayudan a los mecanismos de defensa endógenos contra las oxidaciones no deseadas, ya sea actuando como antioxidantes por sí solos o actuando de cofactores de los sistemas antioxidantes endógenos. A través de la dieta también obtenemos una de las principales fuentes de antioxidantes exógenos, las denominadas sustancias fitoquímicas, entre las que destacan las sustancias fenólicas (Ugartondo, 2009).

Existen varias estrategias de defensa celular frente a los procesos mediados por las especies reactivas de oxígeno, según se desarrolla la reacción en cadena de la oxidación. La primera línea de defensa está formada por los antioxidantes preventivos, a continuación intervienen los secuestradores de radicales libres y finalmente actúan los reparadores del daño oxidativo.

Los **antioxidantes preventivos** impiden la formación de radicales libres a través de mecanismos de tipo enzimático (capaces de metabolizar las especies reactivas a estructuras más

estables) o de tipo no enzimático como agentes quelantes (capaces de secuestrar metales que participan en la formación de radicales libres). Los **antioxidantes eliminadores de radicales libres** estabilizan radicales al inhibir la cadena de inicio o romper la de propagación; dentro de este grupo se encuentran tanto antioxidantes de origen endógeno como exógeno obtenidos a partir de la dieta. Los **antioxidantes reparadores** actúan cuando las biomoléculas ya han sufrido el daño oxidativo, reconstituyendo la membrana y subsanando el daño producido; son los sistemas enzimáticos de reparación o de síntesis *de novo*.

A diferencia de los enzimas antioxidantes, los compuestos antioxidantes se modifican al reaccionar con los radicales libres y necesitan ser sustituidas por agotarse. Algunos antioxidantes de origen endógeno (glutation, ácido úrico, coenzima Q y proteínas plasmáticas) pueden ser reemplazos por síntesis (Laguerre et al., 2007), mientras que si son de origen exógeno, necesitan ser reabastecidos a través de la dieta. Así, el consumo de alimentos con compuestos bioactivos como vitaminas, carotenoides o compuestos fenólicos, juega un papel muy importante en la prevención de enfermedades, al tratarse de una alternativa de fácil modificación e implementación (Diplock et al., 1998; Patil, Jayaprakasha, Chidambara Murthy, & Vikram, 2009).

6.4. Antioxidantes presentes en los alimentos

Los efectos nocivos del estrés oxidativo sobre la salud humana pueden ser reducidos a través de la ingesta de antioxidantes, presentes en diversos alimentos de origen vegetal como frutas, verduras, hierbas y tés (Cao, Sofic, & Prior, 1996; Prior, 2003; Gülçin, 2012).

Algunos de los principales antioxidantes que encontramos en alimentos son la vitamina C, los tocoferoles, los carotenoides, los polifenoles y los compuestos de Maillard. Además diversos minerales participan como componentes o cofactores de enzimas antioxidantes (Diplock et al., 1998; Pérez-Jiménez et al., 2008), participando en la defensa antioxidante (Masella, Di Benedetto, Varì, Filesi, & Giovannini, 2005).

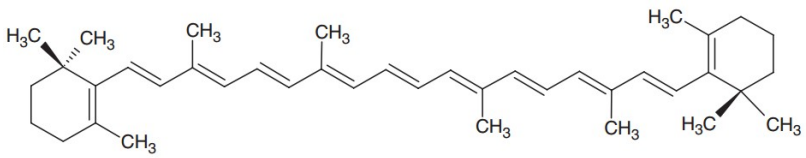
De esta forma, el efecto de los vegetales y las frutas como promotores de la salud no sólo está relacionado con el perfil nutricional general de este grupo de alimentos (alto contenido en fibra, bajo en grasa y sal, baja densidad energética y ricos en vitaminas y minerales). Además lo está con una amplia gama de fitoquímicos y componentes antioxidantes (como flavonoides y otros compuestos fenólicos) (Szeto, Tomlinson, & Benzie, 2002; Aruoma, 2003; Balasundram, Sundram, & Samman, 2006), cuyo mecanismo de actuación consiste en evitar o reducir el daño oxidativo (Shi, Noguchi, & Niky, 2001).

Por otro lado, la adición de algunas plantas a los productos alimentarios pueden prevenir su deterioro oxidativo, inhibiendo el proceso de peroxidación lipídica y mejorando la calidad y valor nutricional de dichos alimentos (Kähkönen et al., 1999; Mariutti, Barreto, Bragagnolo, & Mercadante, 2008).

- **Carotenoides**

Son una familia pigmentos sintetizados por algunas plantas y microorganismos, responsables del característico intenso color amarillo, anaranjado o rojo de un gran número de vegetales. De acuerdo a su estructura química se dividen en dos grupos, los carotenos (α, β y γ-carotenos, así como el licopeno) y las xantofilas (zeaxantina, luteína, violaxantina, etc). Algunos de ellos son precursores de la vitamina A, como el β-caroteno, el cual es el carotenoide de mayor importancia por sus potentes propiedades antioxidantes (Krinsky, 1989).

Figura 6. Estructura del β-caroteno.



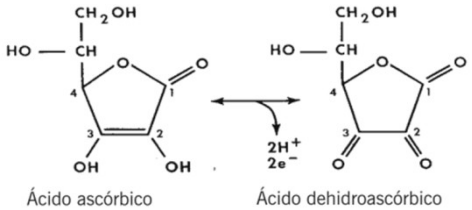
Las propiedades antioxidantes de los carotenoides residen en su estructura (**figura 6**), formada por largas cadenas de dobles enlaces conjugados (Namitha & Negi, 2010). Esta disposición les permite actuar como captadores de radicales libres, siendo eficaces antioxidantes contra el oxígeno singlete, los radicales peróxilo e hidroxilo, el anión superóxido, el ácido hipocloroso y otras especies reactivas (Handelman, 1991), contribuyendo de esta manera al sistema de defensa antioxidante lipofílico del organismo (Young & Lowe, 2001). También pueden presentar efectos sinérgicos con otros antioxidantes como la vitamina E (Zanfini, Corbini, Rosa, & Dreassi, 2010).

Debido a su estructura, los carotenoides están sujetos a muchos cambios químicos inducidos por las distintas condiciones de procesado alimentario (Meléndez-Martínez, Vicario, & Heredia, 2006) que afectan su contenido, perfil y biodisponibilidad, por lo que factores como tratamientos térmicos elevados, exposición a la luz o presencia de oxidantes favorecen su degradación (Wong, 1995).

- **Vitamina C**

Es sintetizada por la mayoría de los vegetales y está presente en muchas frutas, verduras y hortalizas (Schlueter & Johnston, 2011). Generalmente es conocida como ácido L-ascórbico, compuesto hidrofílico de seis carbonos que contiene un grupo enediol (**figura 7**) esencial para su actividad antioxidante. Este grupo hace al ácido ascórbico, un potente agente reductor que reacciona fácilmente con radicales, los neutraliza y se transforma reversiblemente en el radical dehidroascórbico.

Figura 7. Estructura de la vitamina C.

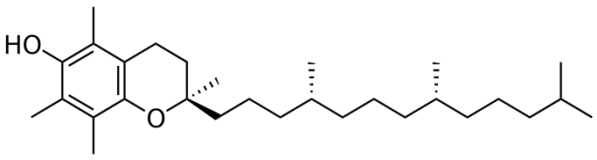


La vitamina C en su forma oxidada (ácido dehidroascórbico), es transportada al interior de la mitocondria, donde es reducida. La cadena respiratoria puede donar electrones para la reducción del ácido dehidroascórbico y regenerarla a su estado reducido (ascorbato) por medio de la dihidroascorbato reductasa y glutatión reducido. El glutatión oxidado es regenerado por la glutatión reductasa, a expensas de NADPH. El ácido ascórbico puede salir de la mitocondria por transporte activo; de esta manera, las moléculas de ácido ascórbico se convierten en captadores de especies reactivas de oxígeno, protegen el genoma mitocondrial y evitan la depolarización de la membrana mitocondrial (Mandl, Szarka, & Bánhegyi, 2009). Por otro lado actúa sinérgicamente con el tocoferol para regenerar los radicales tocoferilo. Además, puede prevenir o reducir la peroxidación lipídica inducida por H_2O_2 y la formación de OH-deoxiguanosina, producida como consecuencia de la oxidación del ADN (Yen, Duh, & Tsai, 2002).

- **Vitamina E**

El término genérico vitamina E engloba varios tocoferoles y tocotrienoles (α, β, γ, δ) que son sintetizadas por organismos fotosintéticos y se encuentran en todos los tejidos verdes y en las semillas (Meydani, Han, & Wu, 2005). Es considerado el principal antioxidante secuestrador de radicales lipofílicos *in vivo* (Pryor, 2000). La acción antioxidante reside en su capacidad para neutralizar los radicales superóxido, hidroxilo y lipoperoxilo a formas menos reactivas, además de ser capaz de romper la reacción en cadena de peroxidación lipídica protegiendo las membranas celulares (Burton, Ingold, Cheeseman, & Slater, 1990).

Figura 8. Estructura del α-tocoferol.



El α-tocoferol (**figura 8**) es la forma más abundante en la naturaleza y además la que posee una mayor actividad biológica (Brigelius-Flohé & Traber, 1999; Rodrigo, Guichard, & Charles, 2007). La actividad de este compuesto se debe al carácter reductor del grupo hidroxilo de su anillo cromanol (Maes et al., 1996). Al actuar como antioxidante se transforma en radical tocoferilo, más estable porque el electrón desapareado del átomo de oxígeno puede ser deslocalizado dentro de la estructura del anillo aromático. Otros antioxidantes como el ascorbato y el GSH actúan de manera sinérgica con la vitamina E, regenerándola y reduciendo el carácter prooxidante del radical tocoferilo, por lo que son considerados los reductores fisiológicos de este radical (Burton et al., 1990).

El nivel de tocoferoles en un determinado producto alimentario depende de las operaciones y las condiciones en que se ha elaborado (Tiwari & Cummins, 2009). Durante el almacenamiento se reduce el contenido de vitamina E en los alimentos debido a la oxidación, exposición a altas temperaturas y humedad, a la luz solar y a la acción de microorganismos (Riaz, Asif, & Ali, 2009).

- **Productos de la reacción de Maillard**

La reacción de Maillard, o pardeamineto no enzimático, engloba un conjunto de reacciones químicas en cadena que dan lugar a la formación de diferentes compuestos intermedios y finalmente a la formación de polímeros pardos llamados melanoidinas. Los compuestos generados también se conocen como productos de la reacción de Maillard (Baxter, 1995). Los productos hidrosolubles del pardeamiento no enzimático generados durante el tratamiento térmico ejercen un efecto como captadores de radicales (Plaza, Amigo-Benavent, del Castillo, Ibáñez, & Herrero, 2010). Esta actividad antioxidante es proporcional al grado de pardeamiento (Yilmaz & Toledo, 2005; Tubaro, 2009) y tiene una correlación estrecha con los productos derivados de las etapas intermedias y tardías y con el tipo de azúcar involucrado en la reacción (Chen & Kitts, 2011). La actividad

antioxidante de los productos de la reacción de Maillard se basa en varios mecanismos de actuación como la inactivación de los radicales libres lipídicos, la inhibición de la formación de peróxidos, la habilidad de captar tanto los iones metálicos como los catalizadores de las reacciones de primer orden y la acción como trampas de oxígeno (Yen, Wang, Chang, & Duh, 2005).

Los compuestos de bajo peso molecular, poseen una mayor actividad antioxidante que los de alto peso molecular (del Castillo, Ames, & Gordon, 2002; Delgado-Andrade & Morales, 2005. Es decir, a pesar de la pérdida parcial de compuestos naturales con actividad antioxidante que se puede producir durante el procesado de los alimentos, las propiedades antioxidantes podrían mantenerse e incluso aumentar debido a la formación de nuevos compuestos.

- **Minerales**

Ciertos minerales pueden tener carácter antioxidante, principalmente el hierro, cobre, zinc, manganeso y selenio debido a que actúan como cofactores de enzimas antioxidantes endógenas implicadas en reacciones que impiden la formación de radicales hidroxilo, atrapan radicales superóxido y favorecen la eliminación de peróxido de hidrógeno (Bravo et al., 2007).

El **selenio** facilita la absorción de la vitamina E, protege a las células frente al radical superóxido y aumenta la actividad de algunas selenioenzimas como la glutatión peroxidasa (Combs, 1999; Tinggi, 2008). La estructura de la catalasa, por otra parte, contiene hierro hemo (Fang, Yang, & Wu, 2002). El **zinc** protege los grupos sulfhidrilo de las proteínas contra la oxidación (Gibbs & Jordan, 1981) y previene la generación de especies reactivas del oxígeno mediante competencia por los sitios de unión con metales como el hierro y el cobre (Zago & Oteiza, 2001), además de formar parte del centro activo de la superóxido dismutasa citosólica, junto con el cobre (Prohaska, 1990; Martínez-González et al., 2002). El **manganeso** forma parte de la enzima superóxido dismutasa

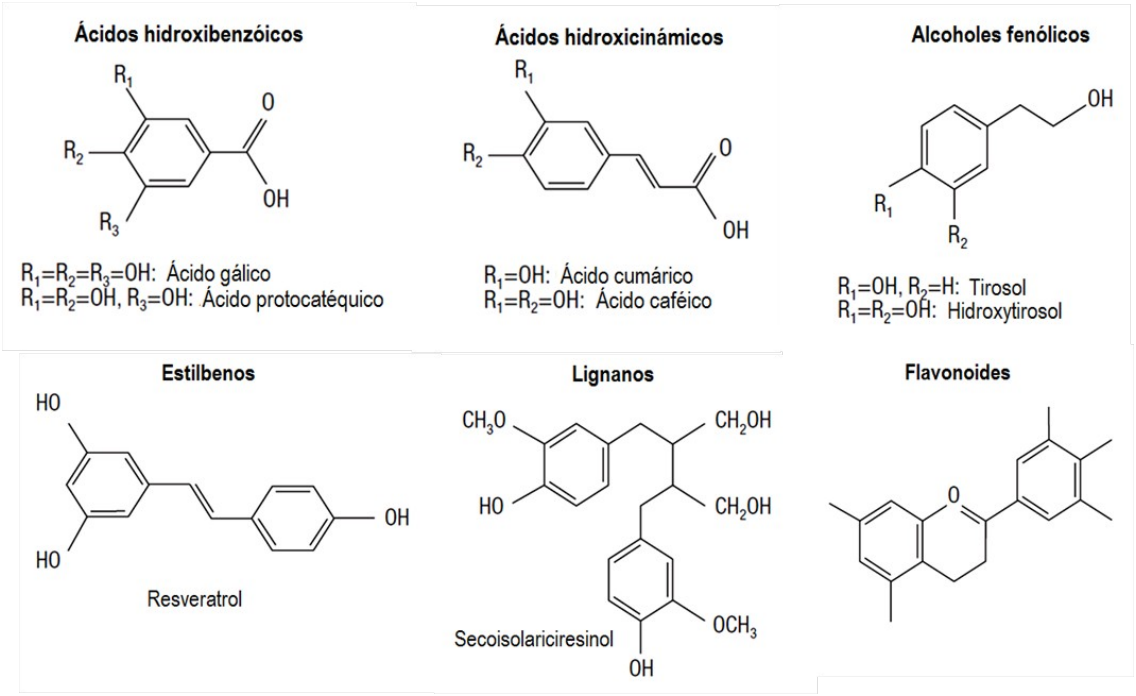
mitocondrial, protege contra la peroxidación lipídica, atrapa radicales hidroxilo y superóxido e induce la síntesis de metalotioneínas (Molina, 2012).

No obstante, cabe destacar que aunque el hierro y el cobre tiene importantes propiedades antioxidantes, también pueden actuar como fuentes pro-oxidantes de radicales libres descomponiendo el peróxido de hidrógeno en radical hidróxilo (Gaetke & Chow, 2003; Turi, Yang, Garrick, Piantadosi, & Ghio, 2004).

- **Compuestos fenólicos**

Constituyen un amplio grupo de compuestos (**Figura 9**) producto del metabolismo secundario de las plantas (Van Sumere, 1989). Son sintetizados por los vegetales durante su desarrollo como respuesta a diversas condiciones adversas (Dixon & Paiva, 1995). Estructuralmente se identifican por poseer anillos aromáticos con dobles enlaces conjugados y sustituyentes hidroxilo, que suelen aparecer en sus formas glicosiladas al combinarse con azúcares como la glucosa y ramnosa (Thomson, 1964). De acuerdo con el número de anillos fenólicos que contienen y a los elementos estructurales que se unen estos anillos, los polifenoles se clasifican principalmente en flavonoides, ácidos fenólicos, alcoholes fenólicos, estilbenos y lignanos (D'Archivio et al., 2007) (**Tabla 2**). El grupo de los flavonoides constituye la subclase de polifenoles más abundante (Quiñones, Miguel, & Aleixandre, 2012).

Figura 9. Principales tipos de compuestos fenólicos.



Los compuestos fenólicos son importantes para la calidad de los vegetales ya que van a influir en sus características organolépticas y por otro lado en su calidad nutricional (Cheynier, 2005; Lesschaeve & Noble, 2005). Además, van a favorecer la conservación de los vegetales retrasando la degradación oxidativa y mejorando así la calidad y valor nutritivo de los alimentos (Kähkönen et al., 1999).

Los efectos beneficiosos asociados al consumo de vegetales están en su mayoría relacionado con estos compuestos y su capacidad antioxidante (Balasundram et al., 2006).

Tabla 2. Clasificación estructural de los compuestos fenólicos.

GRUPO ESTRUCTURAL	*COMPUESTO FENÓLICO*
FLAVONOIDES	**FLAVONOLES** *Quercitina, kaempferol, mirecetina y rutina* **FLAVONAS** *Apigenina, luteolina y diosmetina* **FLAVANONAS** *Naringenina, hesperetina y eriodictiol* **FLAVANOLES** *Catequina, epicatequina, galocatequina, epigalocatequina y proantocianidinas (taninos condensados)* **ISOFLAVONAS** *Daidzeína y genisteína* **ANTOCIANIDINAS** *Cianidina, delfinidina, pelargonidina y malvidina*
ÁCIDOS FENÓLICOS	**ÁCIDOS HIDROXICINÁMICOS** *Cumárico, ferúlico, sinápico, caféico y clorogénico* **ÁCIDOS HIDROXIBENZÓICOS** *Gálico, elágico, siríngico y vaníllico*
ALCOHOLES FENÓLICOS	*Tirosol e hidroxytirosol*
ESTILBENOS	*Resveratrol*
LIGNANOS	*Secoisolariciresino*

Los efectos beneficiosos de los polifenoles están relacionados con la prevención de enfermedades asociadas al estrés oxidativo como el cáncer, enfermedades cardiovasculares y trastornos neurodegenerativos (Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010; Quiñones et al., 2012). La actividad antioxidante de los compuestos fenólicos depende de su estructura química, número de grupos hidroxilo, sustituciones, conjugaciones y grado de

polimerización (Rice-Evans, Miller, & Paganga, 1996). De esta forma actúan presentando un doble mecanismo de acción antioxidante:

1. Cesión del radical hidrógeno de un grupo hidroxilo aromático a un radical libre, gracias a su capacidad de deslocalización de cargas en el sistema de dobles enlaces del anillo aromático (Duthie, Gardner, & Kyle, 2003).

2. Quelación iones metálicos (principalmente hierro y cobre) inhibiendo la formación de radicales libres a través de reacciones de Fenton (Khokhar & Owusu Apenten, 2003).

Algunos polifenoles, además de su propia acción antioxidante, también pueden actuar potenciando la actividad de enzimas antioxidantes como la catalasa, glutation peroxidasa, glutation reductasa y superóxido dismutasa (Lin et al., 1998; Fernández-Pachón et al., 2009). Al mismo tiempo inhibien la actividad de enzimas involucradas en procesos oxidativos como la lipooxigenasa, ciclooxigenasa, xantina oxidasa y fosfolipasa A2, evitando la generación de especies reactivas del oxígeno (Higdon & Frei, 2003; Sun, Wang, Simonyi, & Sun, 2008).

6.5. Evaluación de la capacidad antioxidante

El creciente interés por los posibles efectos beneficiosos de los antioxidantes, tanto sobre la salud humana como en aspectos de calidad alimentaria, ha hecho que se desarrollen multitud de métodos para determinar la capacidad antioxidante de los alimentos o su efecto protector sobre las biomoléculas. Para ello, se han unificado las condiciones que debería reunir un procedimiento estandarizado de medida de capacidad antioxidante (Frankel & Meyer, 2000; Prior, Wu, & Schaich, 2005):

1. Especificar el sustrato de oxidación.
2. Usar distintas fuentes de radicales.

3. Asegurar que el sustrato y el modo de inducir la oxidación son relevantes como fuentes de daño oxidativo.
4. Tener un mecanismo conocido y un punto final definido.
5. Ser adaptable para medir antioxidantes hidrofílicos y lipofílicos.
6. Ser un método sencillo.
7. Emplear una instrumentación fácilmente disponible.
8. Tener una buena reproducibilidad.
9. Ser adaptable para análisis rutinarios a gran escala.

Sin embargo, debido a la variedad de agentes oxidantes y a los diferentes mecanismos antioxidantes, en la actualidad no existe un único método de medida que refleje de forma completa el perfil antioxidante de un alimento o compuesto, lo cual hace necesario el aplicar diferentes ensayos para generar un perfil contrastado sobre la capacidad antioxidante (Schlesier, Harwat, Böhm, & Bitsch, 2002; Aruoma, 2003; Roginsky & Lissi, 2005; Rivero-Pérez, Muñiz, & Gonzalez-Sanjosé, 2007). Además, hay que destacar que la capacidad antioxidante de un alimento está relacionada con su composición global siendo más importante la sinergia entre compuestos antioxidantes que la actividad de cada uno por separado (Ghiselli, Serafini, Natella, & Scaccini, 2000).

En general, existen clasificaciones de los métodos utilizados en la evaluación de la capacidad antioxidante total de una muestra, siendo una de ellas la que agrupa los métodos en directos e indirectos (Roginsky & Lissi, 2005; Laguerre et al., 2007).

Los **métodos directos** son aquellos que evalúan el efecto inhibitorio de una sustancia potencialmente antioxidante sobre la degradación oxidativa de un sustrato sometido a condiciones de oxidación, involucrando siempre la utilización de un sustrato oxidable. Estos métodos suelen

estar relacionados principalmente con ensayos de inhibición de la peroxidación lipídica, que implican una estrategia de medición basada en la detección de los productos de oxidación (Laguerre et al., 2007). No obstante, también se podrían incluir en esta clasificación de métodos directos otros ensayos basados en modelos cinéticos, donde los antioxidantes compiten con el sustrato oxidable por eliminar los radicales libres generados (Roginsky & Lissi, 2005) y cuyos productos de reacción se caracterizan por seguir un mecanismo de transferencia de hidrógeno (Ou et al., 2002).

Por otro lado están los **métodos indirectos**, basados en la estabilización de un radical libre artificial (por transferencia de hidrógeno o electrones) o en la reducción de un metal de transición prooxidante (por transferencia de electrones). Generalmente son los más utilizados para evaluar la capacidad antioxidante de diversos alimentos (Sánchez-Moreno, 2002; Huang, Ou, & Prior, 2005; Roginsky & Lissi, 2005; Pérez-Jiménez & Saura-Calixto, 2006; Magalhães, Segundo, Reis, & Lima, 2008; Moon & Shibamoto, 2009).

En la práctica, para evaluar la capacidad antioxidante de una muestra, siempre deben combinarse al menos dos métodos, ambos con distintos fundamentos, puesto que aunque todos son métodos adecuados, al mismo tiempo todos tienen algún inconveniente que limitaría su utilización como único método de cuantificación (Pérez-Jiménez et al., 2008).

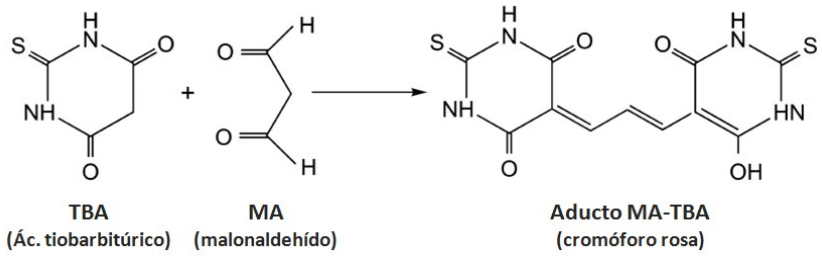
Algunos investigadores recomiendan la combinación de ensayos basados en la capacidad de reducción de metales como el hierro, con algún otro método que determine la capacidad de reducción de radicales libres (Pérez-Jiménez & Saura-Calixto, 2006), por lo que muchos estudios en plantas y alimentos han utilizado esta conjunción (Moon & Shibamoto, 2009). Otros investigadores argumentan que existen métodos como el ensayo de fenoles totales por el método Folin-Ciocalteu, que a pesar de no destinarse directamente para el análisis de capacidad antioxidante, podría ser útil para tales estudios, especialmente en combinación con otros métodos (Roginsky & Lissi, 2005), incluso algunos autores proponen su utilización como un método más para la evaluación de la

capacidad antioxidante, el cual estaría basado en reacciones de transferencia de electrones (Huang et al., 2005).

- **Método del ácido tiobarbitúrico (TBA)**

El TBA es un compuesto químico que reacciona con los compuestos carbonílicos formados en la peroxidación lipídica (**Figura 10**), formando unos aductos de color rosa que absorben a una longitud de onda de 535 nm (Waravdekar & Saslaw, 1959). El malonaldehído es un compuesto carbonílico secundario de la oxidación lipídica cuya concentración puede determinarse específicamente tras su reacción con TBA y separación mediante cromatografía líquida de alta resolución o cromatografía gaseosa. El total de compuestos carbonílicos que reaccionan con el TBA se denominarán sustancias reactivas de TBA (TBARS). Antioxidantes como el BHT, vitamina A y vitamina C se utilizan como controles.

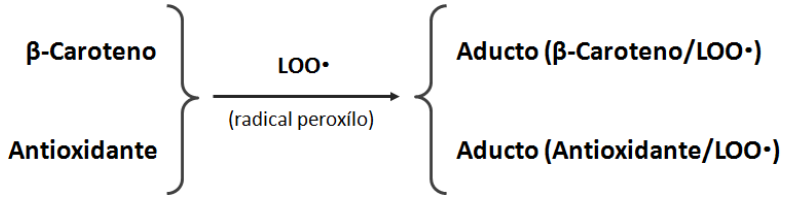
Figura 10. Formación del aducto coloreado (MA-TBA) desde TBA y MA.



- **Blanqueamiento del β-caroteno**

El beta-caroteno es un antioxidante que reacciona con el radical peroxilo, formado en la peroxidación lipídica, para dar un aducto estable (Kennedy & Liebler, 1991). En presencia de antioxidantes, éstos competirán con el β-caroteno por el radical (**Figura 11**). Por tanto, los efectos antioxidantes serán fácilmente medibles mediante el blaqueamiento de la muestra a una absorbancia de 470 nm (absorbancia máxima del β-caroteno).

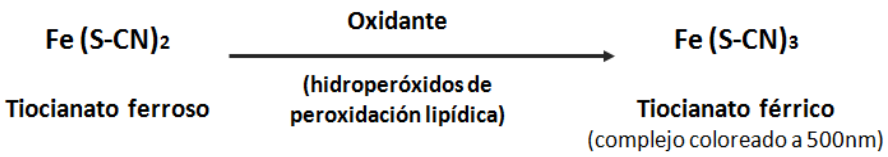
Figura 11. Formación de aductos de β-carpteno y antioxidante a partir de radicales peroxílo.



- **Método del ferrotiocianato (FTC)**

Es un método basado en la oxidación del hierro ferroso (Fe^{2+}) a férrico (Fe^{3+}). Esta reacción (**Figura 12**) se puede cuantificar espectrofotométricamente monotorizando la formación, a partir de tiocianato-Fe^{2+}, de un complejo de tiocianato-Fe^{3+} con una coloración rojiza que tiene una absorción máxima a 500nm (Kikuzaki & Nakatani, 1993). La actividad antioxidante de una sustancia puede evaluarse como el efecto inhibidor sobre la formación de hidroperóxidos o por su capacidad para donar un electrón al compuesto férrico. Es un ensayo simple y reproducible, pero su espectro de absorción no es muy adecuado y existen pocos estudios sobre antioxidantes naturales que utilicen este método.

Figura 12. Formación del complejo de tiocianato-Fe^{3+} a partir de tiocianato-Fe^{2+}.

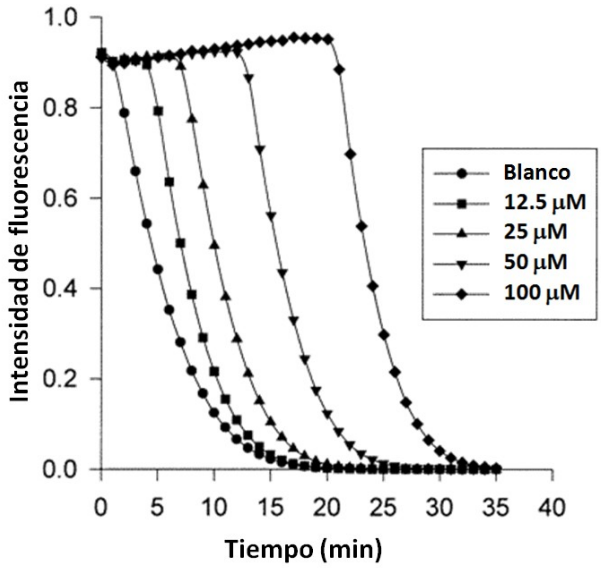


- **Método ORAC (Oxygen radical absorbance capacity assay)**

Es un método cinético que mide la reacción entre una especie reactiva (radicales peroxilo) y un sustrato oxidable (fluoresceína) en presencia de antioxidantes. El método se basa en la utilización de fluoresceína, compuesto sintético con una elevada fluorescencia que desaparece al oxidarse (Ou et al., 2002). Como agente generador de radicales peroxilo se utiliza el AAPH (2,2´-azobis (2-metilpropionamidina)) que inicia la reacción al formar los radicales que oxidan a la fluoresceína. La fluorescencia se monitoriza mediante espectrofluorimetría, observando una disminución de su intensidad. En presencia de antioxidantes el descenso de la fluorescencia es menor.

La cuantificación se lleva a cabo mediante la técnica del área bajo la curva cinética (**Figura 13**) de descenso de fluorescencia, que integra los porcentajes de inhibición del radical libre con el tiempo completo de reacción. Los resultados se expresan en TEAC (capacidad antioxidante equivalente de Trolox), utilizando como patrón el Trolox. Algunos de los inconvenientes que presenta este método radican en la necesidad de un equipo de medida costoso, que precisa de unos tiempos de cuantificación largos y presenta una elevada sensibilidad al pH del medio. Además, ciertos aminoácidos y ácidos urónicos no antioxidantes presentes en alimentos vegetales pueden mostrar un efecto interferente en este ensayo (Pérez-Jiménez & Saura-Calixto, 2006).

Figura 13. Curvas cinéticas del efecto del Trolox frente a la oxidación de la fluoresceína.

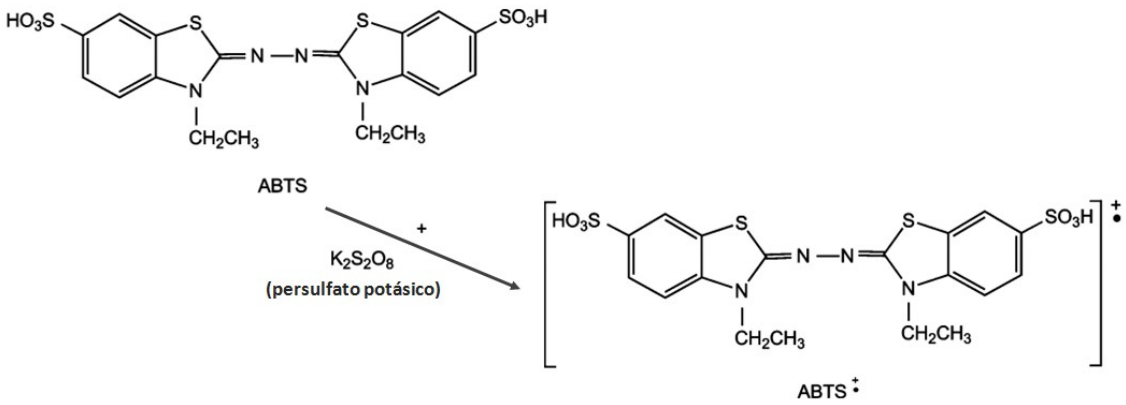


- **Método del ABTS (ácido 2,2'-azinobis (3-etilbenzotiazolín)-6-sulfónico)**

El radical $ABTS^{+\cdot}$ se produce por la oxidación del ABTS (**Figura 14**). Esta oxidación puede generarse de forma enzimática, química (dióxido de manganeso, persulfato potásico, radicales peroxilo) o electroquímica (Re et al., 1999). El radical catiónico obtenido es un compuesto de color verde-azulado estable con una absorción máxima a 734 nm.

Es un método indirecto basado en la reducción del radical $ABTS^{+\cdot}$ a ABTS por la adición de una muestra que contiene antioxidantes. La reacción es monitorizada mediante espectofotómetro, determinando la decoloración del ABTS a 734 nm, utilizando como patón de referencia el Trolox (ácido 6-hidroxi-2,5,7,8-tetrametil-croman-2-carboxílico) (análogo sintético hidrosoluble de la vitamian E) y expresando los resultados como TEAC (Re et al., 1999).

Figura 14. Formación del radical ABTS $^{+\cdot}$.

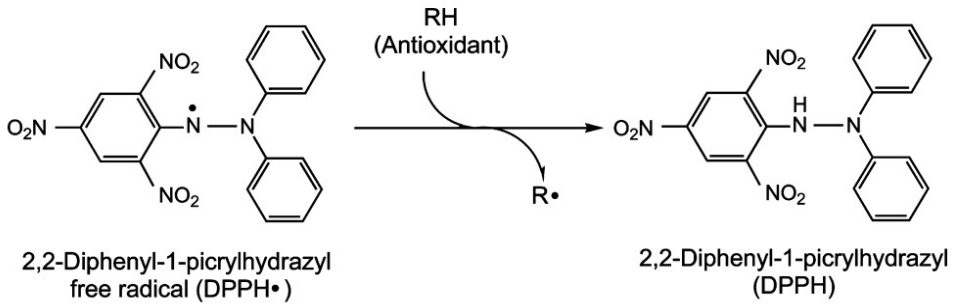


La ventaja de este ensayo es que puede realizarse tanto en muestras hidrosolubles como liposolubles, eligiendo el disolvente apropiado en cada caso y que proporciona rápidamente resultados reproducibles empleando un equipo relativamente habitual en laboratorio. Asimismo, como la longitud de onda a la que se realizan las medidas de absorbancia no es común en los alimentos, hace que este método sea particularmente interesante para el estudio de extractos vegetales, al eliminar la posibilidad de interferencias de color. Por otro lado, entre las limitaciones que presenta esta técnica destaca la poca selectividad del radical ABTS$^{+\cdot}$ en reacciones con donadores de átomos de hidrógeno, pudiendo reaccionar con el grupo hidroxilo de cualquier compuesto aromático independientemente de su potencial antioxidante real. Es un ensayo dependiente del tiempo, siendo relativamente lento, tanto en la generación del radical ABTS$^{+\cdot}$ como en su reacción con los antioxidantes de la muestra. Al igual que ocurre en todos los métodos indirectos, no es representativo de la capacidad de inhibir el proceso oxidativo.

- **Método del DPPH (2,2-difenil-1-picrilhidracil)**

Es un ensayo muy popular para el estudio de antioxidantes naturales debido a su simplicidad y alta sensibilidad (Brand-Williams, Cuvelier, & Berset, 1995). Este método indirecto se basa en la teoría de que todo donador de hidrógeno es un antioxidante. El DPPH· (uno de los pocos radicales de nitrógeno estables que son comerciales) acepta un hidrógeno del antioxidante para formar DPPH (**Figura 15**), de forma que el efecto antioxidante es proporcional a la desaparición de DPPH.

Figura 15. Captación del radical libre del DPPH y formación de DPPH.

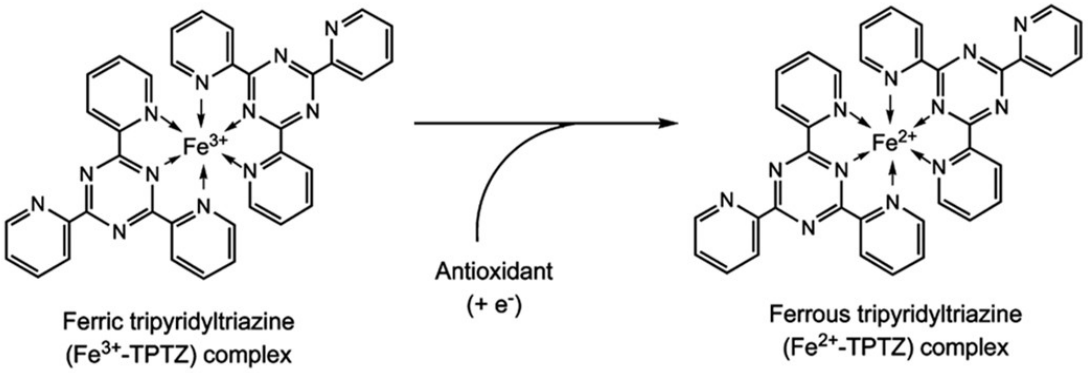


Existen varios métodos para su monitorización, pero el más común es mediante espectrofotometría UV, por su facilidad y precisión. Este radical presenta un máximo de absorción a 517 nm, volviéndose amarillo cuando se forma DPPH, de forma que el efecto antioxidante puede ser fácilmente evaluado siguiendo la disminución de la absorbancia, utilizando para ello un patrón de referencia (Trolox) y expresando los resultados como TEAC. No obstante, algunos antioxidantes que presentan un espectro de absorción similar al del radical $DPPH^{\cdot}$, como es el caso de los carotenoides, pueden causar interferencias (Prior et al., 2005).

- **Método FRAP (Reducción del hierro férrico a ferroso)**

Es un método indirecto basado en la reducción, por un antioxidante y en condiciones ácidas, del hierro férrico (Fe^{3+}, prooxidante) a hierro ferroso (Fe^{2+}). Esta reducción (**Figura 16**) se puede cuantificar gracias a la acción del TPTZ (cloruro de 2,3,5-trifeniltetrazolio), compuesto químico capaz de quelar el hierro.

Figura 16. Formación del complejo (Fe^{2+}-TPTZ) desde el complejo (Fe^{3+}-TPTZ) por la acción del antioxidante.

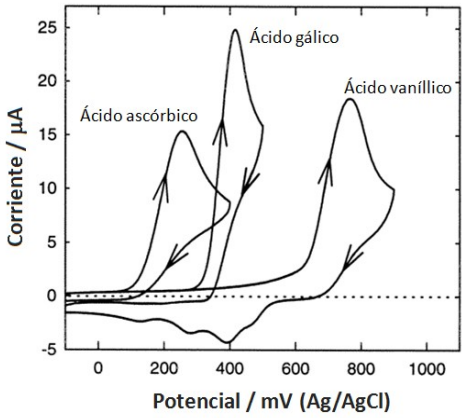


El complejo Fe^{3+}-TPTZ tiene una intensa coloración azul con un máximo de absorción a 595 nm mientras que el complejo Fe^{2+}-TPTZ tiene coloración amarillenta (Benzie & Strain, 1996). Por tanto, el efecto antioxidante (capacidad de reducción) se evaluará monitorizando la formación de este complejo con un espectrofotómetro. Este ensayo proporciona resultados reproducibles de forma rápida, pero presenta la desventaja de tener que realizarse en una matriz acuosa, debiendo por tanto usar como antioxidante de referencia uno que sea hidrosoluble, como el ácido ascórbico o el Trolox.

- **Método electroquímico de voltametría cíclica (VC)**

Es un método electroquímico utilizado como una herramienta promisoria para evaluar la capacidad antioxidante (o reductora) de una muestra mediante voltamogramas cíclicos (**Figura 17**). En este ensayo indirecto la muestra es introducida en una celda con un sistema formado por un electrodo de trabajo (carbono vítreo), un electrodo de referencia (Ag/AgCl) y un electrodo auxiliar (platino). El poder reductor está compuesto por el pico de potencial y la corriente anódica (Kilmartin, 2001).

Figura 17. Voltamogramas cíclicos de disoluciones 0.5 mM de ácido ascórbico, gálico y vaníllico.



Un bajo valor de pico potencial significa una mayor capacidad de la muestra para donar electrones al electrodo. La intensidad de la corriente anódica incrementa debido a la oxidación de compuestos antioxidantes en la superficie del electrodo. Los estudios de voltametría no permiten la determinación de antioxidantes individuales pero dan información sobre la cantidad de grupos funcionales responsables de los picos en el voltamograma (Cosio, Buratti, Mannino, & Benedetti, 2006; Piljac-Žegarac, Valek, Stipčević, & Martinez, 2010). Entre las desventajas del método está que no todos los antioxidantes donan electrones a los electrodos de carbono generalmente utilizados. Los compuestos tipo tiol no son detectados de esta forma y requieren electrodos de Au/Hg.

- **Métodos de determinación de polifenoles totales**

  ✔ **Ensayo del reactivo Folin-Ciocalteu**

Es un método utilizado para determinar el contenido de fenoles totales presentes en un alimento, empleando como estándar el ácido gálico. Su determinación se basa en la reducción de los fenoles por una mezcla de ácidos fosfotúngstico y fosfomolíbdico (reactivo Folin-Ciocalteu), lo que genera óxidos de tungsteno y molibdeno de coloración azul con un máximo de absorción en torno a los 750 nm, que es proporcional al contenido de compuestos fenólicos (Singleton & Rossi, 1965). Al tratarse de una reacción redox, puede considerarse como un método indirecto de medida de capacidad antioxidante (Prior et al., 2005; Huang et al., 2005). El mecanismo se basa en una transferencia de electrones en medio alcalino, por parte de los polifenoles (así como otras especies reductoras presentes en la muestra) al reactivo Folin-Ciocalteu (Magalhães et al., 2008). Tiene la ventaja de ser un método relativamente simple y reproducible, pero presenta el inconveniente de sufrir interferencias con sustancias de naturaleza no fenólica como azúcares (fructosa y sacarosa), proteínas, ciertos aminoácidos, ácidos orgánicos (ácido ascórbico) y algunas sales inorgánicas (Prior et al., 2005).

  ✔ **Ensayo del reactivo Fast Blue BB (FBBB)**

Es un método directo para la detección de compuestos fenólicos en alimentos y bebidas. Su mecanismo está basado en las interacciones directas de los fenoles con el reactivo Fast Blue BB (cloruro hemi (-zinc cloruro) de 4-benzoilamino-2.5 dimetoxibenzendiazonio), que en medio alcalino forman complejos azoicos coloreados detectables a una absorbancia de 420 nm y cuantificables mediante un patrón de ácido gálico (Medina, 2011). Es un método sencillo, que a diferencia del método Folin-Ciocalteu, presenta la ventaja de no sufrir interferencias con otros compuestos, proporcionando una estimación más precisa del contenido en fenoles totales por su

selectividad frente a los grupos hidroxilo de los polifenoles (Lester, Lewers, Medina, & Saftner, 2012).

OBJETIVOS

El **objetivo general** de la presente tesis doctoral consiste evaluar la capacidad antioxidante de los alimentos de la dieta española y cómo éstos pueden modular la composición y funcionalidad de la microbiota intestinal. Así mismo se estudia cómo el procesado térmico de los alimentos puede modificar tanto su capacidad antioxidante como el efecto que tiene sobre la microbiota intestinal. De esta forma, los **objetivos específicos** de la tesis doctoral son:

1. Desarrollar métodos de determinación de actividad antioxidante en condiciones fisiológicas.

2. Modificar el ya existente método GAR (Global Antioxidant Response) de Pastoriza et al. (2011) para incorporar una fase de fermentación que simule el efecto de la microbiota intestinal sobre el residuo de la digestión y la consiguiente liberación de compuestos con actividad antioxidante y moduladores de la microbiota intestinal.

3. Evaluar la capacidad antioxidante de los principales alimentos de la dieta española (té, café, frutas, hortalizas y alimentos cárnicos como el salchichón) tras ser sometidos a un proceso de digestión gastrointestinal y fermentación *in vitro.*

4. Estudiar la modulación de la microbiota intestinal mediante los alimentos anteriormente citados, prestando especial atención en las posibles modificaciones tecnológicas del café y del salchichón.

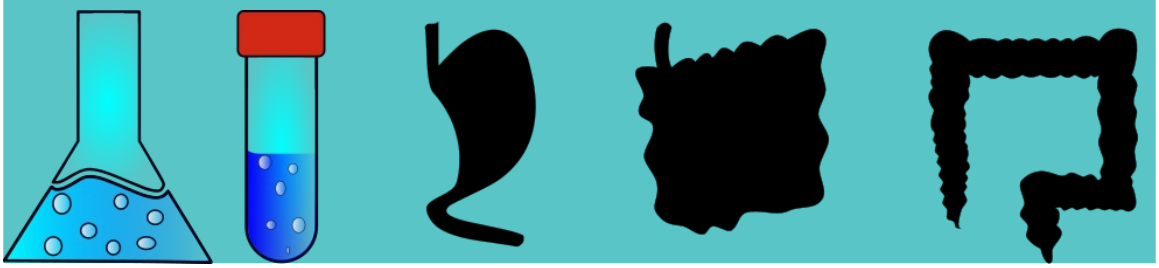
5. Estudiar el efecto del procesado térmico tanto sobre la capacidad antioxidante de los alimentos como sobre la microbiota intestinal.

OBJECTIVES

The **general objective** of this doctoral thesis is to evaluate the antioxidant capacity of the different components of the spanish diet and how they can modulate gut microbial community structure and its functionality. Moreover, it has been studied how thermal processing of foods can modify its antioxidant capacity as well as the effect that such thermal treatment could have on gut microbiota. Therefore, the **specific objectives** are:

1. To set up different methods to determine antioxidant capacity under physiological conditions.
2. To modify the already developed GAR (Global Antioxidant Response) method from Pastoriza et al. (2011) to incorporate an *in vitro* fermentation step afterwards the *in vitro* gastrointestinal step. This will allow to simulate the action of gut microbiota over digestion residue and study the release of antioxidant compounds and others that can modulate gut microbes.
3. To evaluate antioxidant capacity of the main components of the Spanish diet (tea, coffee, fruits, vegetables, and meats such as salami) through *in vitro* gastrointestinal digestion and fermentation.
4. To study the modulation of gut microbiota by the above mentioned foodstuffs, highlighting possible technological modifications of coffee and salami.
5. To study the influence of thermal treatment in both, antioxidant capacity and gut microbiota.

Capítulo I. Desarrollo de métodos *in vitro*.



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Towards an improved Global Antioxidant Response method (GAR+): physiological-resembling in vitro antioxidant capacity methods

Este trabajo describe la puesta a punto de varios métodos para determinar actividad antioxidante bajo condiciones fisiológicas, lo cual está íntimamente ligado con la temática de la presente tesis doctoral, además de ser uno de sus objetivos principales.

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Towards an improved Global Antioxidant Response method (GAR+): physiological-resembling *in vitro* antioxidant capacity methods

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ABSTRACT

Many methods have been developed to measure the antioxidant capacity of foods under non physiological-relevant conditions. In this study, three methods ($TEAC_{OH}$, $GEAC_{RED}$ and $TEAC_{AAPH}$) are developed to measure antioxidant capacity at physiological pH, using indigo carmine as a redox dye. $TEAC_{OH}$ and $TEAC_{AAPH}$ determine foodstuffs' scavenging capacity against hydroxyl (OH·) and AAPH· radicals, while the third method measures the global reducing capacity of the sample. The results obtained for commercial teas, commercial beverages containing tea as the main ingredient and different solid foods (spinach, onion, salami, etc.) were compared with well-established protocols. The new methods demonstrated good linearity-reproducibility, providing reliable data about the antioxidant capacity of foods under physiological-resembling conditions. The new methods were also useful for evaluating the antioxidant capacity of human plasma after acute intake of tea. The physiological-resembling conditions of these assays and the use of absorbance readings make them suitable for application by any laboratory.

KEYWORDS: Antioxidant capacity, ABTS, FRAP, ORAC, tea.

1. Introduction

Generalised interest in antioxidants was aroused in the 1990s, when relationships between antioxidant capacity and various health benefits were first established. Oxidation is directly related to damage to biological tissues and to substances essential for cell function. Studies have shown that the hydroxyl radical (OH·) is one of the most harmful and common radicals generated inside living cells, and that it reacts avidly with biologically relevant molecules such as DNA, membrane lipids and proteins (Lee, Koo & Min., 2004). Oxidative damage is known to be one of the causes of various chronic diseases, including cancer, liver disease, aging, Alzheimer's disease, inflammation, diabetes, atherosclerosis and Parkinson's disease (Moon & Shibamoto, 2009). Hence, the consumption of antioxidants is known to be an important means of preventing or delaying the appearance of such diseases. Accordingly, many foods and beverages, especially vegetables, are viewed with great interest because of their antioxidant capacity (Roginsky & Lissi, 2004). This characteristic is related to the presence of bioactive compounds such as flavonoids and carotenoids, which produce many beneficial effects on human health (Patil, Jayaprakasha, Chindambara-Murthy & Vikram, 2009).

The antioxidant capacity of many different foods has been measured, using various methods, to provide an overview of their influence against oxidative damage. These methods can be classified in many different forms. One is to distinguish between assays focused on analysing compounds derived from lipid peroxidation (thiobarbituric acid, conjugated diene, malonaldehyde or β-carotene bleaching assays, among others) and assays focused on the scavenging activity against free radicals such as ABTS, DPPH, FRAP, ferric thiocyanate assays, among others (Moon & Shibamoto, 2009). On the other hand, antioxidant capacity methods can also be classified according to their chemical basis: those based on hydrogen atom transfer (ORAC, TRAP or crocin bleaching assays) and those based on electron transfer like Folin-Ciocalteu, FRAP, ABTS or DPPH assays (Huang, Ou & Prior, 2005). All of these methods are widely used but most present

drawbacks. For example, some are performed in a non-aqueous reaction medium (DPPH), or under non-physiological pH (FRAP, Folin-Ciocalteu) or use non-physiological radicals (ABTS). Although the ORAC method is carried out at physiological pH and the radical used (AAPH) is widely accepted, it is based on fluorescence measurement, which can give rise to quenching problems.

In order to correctly describe the antioxidant capacity of a foodstuff, it is necessary to apply a combination of methods, based on different chemical reactions (Huang et al., 2005; Moon & Shibamoto, 2009). In this respect, it has been demonstrated that H_2O_2 and the OH· radical are both inevitable intermediates generated in the reduction of oxygen to water inside the body (Lee et al., 2004). The analysis of the overall reducing capacity of an extract is also a good indicator of its antioxidant activity (Benzie & Strain, 1996). Therefore, from the plethora of antioxidant methods available, both an antioxidant method including OH· radicals and one measuring the reducing capacity under physiological-resembling conditions should be included. The aim of this study is to derive a new method for measuring the antioxidant capacity of *in vitro* digested foods (tea infusions and tea-containing beverages). This method is performed at physiological pH (7.24), with relevant physiological (OH·) or widely accepted (APPH·) radicals and can be carried out using a simple spectrophotometer, thus avoiding problems produced by fluorescence.

These methods have been mainly evaluated against tea since it is one of the most widely consumed beverages worldwide, due to its sensory properties (Hilal & Engelhardt, 2007), to socio-cultural factors and to certain health properties that have been attributed to it (Cabrera, Artacho & Giménez, 2006). Thus, tea has been related to beneficial effects on neurodegenerative diseases, cardiovascular diseases, diabetes, obesity and, basically, to any disease which involves oxidative stress. This protection is most probably due to the wide range of bioactive compounds present in tea, including flavonoids, other polyphenols, caffeine and theanine (Vuong, 2014).

2. Materials and methods

2.1. Chemicals

6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®), methanol, 2,2'-azobis(2-methylpropionamide) (AAPH), sodium dihydrogen phosphate monohydrate, ferric chloride hexahydrate, hydrogen peroxide, carmin indigo, gallic acid, catechin hydrate, naringenin, resveratrol, genistein and phosphotungstate were purchased from Sigma-Aldrich (Germany).

2.2. Samples

The first set of samples selected were four commercial teas, packaged in sachets, of each type available (green, black, red and white teas), together with commercial beverages that are widely consumed in Spain and that have tea (either green or white) declared on the label as a major ingredient. These beverages were frozen at -80^{0}C until they were needed for the analysis. The teas were prepared in 60 mL of boiling water, leaving the sachet immersed for 7 minutes. Enough samples of each type of tea were obtained and stored to carry out all the analyses at the same temperature.

A second set of samples was analysed to extend the usefulness of the method to other solid and liquid samples. Coffee, spinach, green pepper, onion, lettuce, fermented milk, salami and chamomile were bought in local supermarkets (Granada, Spain) and prepared in the laboratory. Coffee was bought as whole coffee beans and homogenized by using a coffee grinder. All the samples (except the chamomile and coffee, which were prepared as infusions in the same way as the teas) were ground using an Ultraturrax homogeniser (model T25, IKA, Spain) at 13000 rpm, aliquoted and stored at -80^{0}C until analysis. The samples were then digested in vitro by the GAR (Global Antioxidant Response) method described by Pastoriza, Delgado-Andrade, Haro & Rufián-Henares (2011), slightly adapted, following the technique described by Minekus et al. (2014). GAR method consists on an *in vitro* gastrointestinal digestion which simulates gastric step using pepsin

and intestinal step using pancreatin and bile acids. The antioxidant capacity was calculated as previously reported with the GAR method (Pastoriza et al., 2011).

Finally, the usefulness of the methods considered was assessed in an *in vivo* human intervention. A randomised placebo-controlled trial was conducted with 30 healthy male-female volunteers (aged 18-47 years). Written informed consent was obtained from all participants. All procedures of the protocol were approved by the Bioethics Committee of the University of Granada. Participants were randomly divided into three groups with the same number of male-female participants in each group (n = 10): green tea (GT), black tea (BT) and control group (C). The test was completed in the morning after overnight fasting. Participants received either a daily single dose (150 mL) of green/black tea infusion (selected from the samples analysed in the first assay) or a placebo (hot boiled water). Blood samples were collected from each participant before tea consumption and after 2 hours. Plasma was obtained by centrifugation and stored at -80°C until further analysis.

2.3. Antioxidant capacity methods

$TEAC_{OH}$ method (Trolox equivalent antioxidant capacity against hydroxyl radicals): The principle underlying this method is to isolate and determine scavenging activity against OH· radicals, using indigo carmine as a redox indicator. For this purpose, OH· radicals were generated by the Fenton reaction using H_2O_2 and ferric ions. The assay was carried out using a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37^0C) and measuring the absorbance at 610 nm. A transparent 96-well polystyrene microplate (Biogen Científica, Spain) was used, in which each well contained 15 μL of indigo carmine indicator (1 mM), 150 μL of phosphate buffer (0.5 M; pH 7.24), 30 μL of ferric chloride hexahydrate (0.1 μM) chelated with phosphotungstate (0.1 μM) in proportion 1:1 (v/v), 15 μL of freshly prepared H_2O_2 (4M) and 90 μL of sample or standard. Each assay was carried out with two blanks: one containing indicator and buffer and the

other with indicator, buffer and the Fenton reagents. Absorbance was measured every 60 seconds in kinetic mode and the absorbance values used were those obtained at 60 minutes. The calibration curve was obtained using Trolox, ranging from 0.01 mg/mL to 10 mg/mL, and the results are expressed as μmol Trolox equivalent per mL of sample.

$TEAC_{AAPH}$ method (Trolox equivalent antioxidant capacity against AAPH radicals, absorbance method): This method was performed to reveal the scavenging activity against AAPH· radicals, using indigo carmine as a redox indicator. The reagent solution contained 1.5 g of AAPH in 10 mL of distilled water. The assay was carried out using a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37^0C) and measuring the absorbance at 610 nm. Each well of the transparent 96-well polystyrene microplate used (Biogen Científica, Spain) contained 40 μL of indigo carmine indicator (1 mM), 220 μL of phosphate buffer (75 mM; pH 7.24), 20 μL of AAPH solution and 20 μL of sample or standard. Each assay was carried out with two blanks: one containing indicator and buffer and the other with indicator, buffer and the AAPH solution. Absorbance was measured every 60 seconds in kinetic mode and the absorbance values used were those obtained after 60 minutes of reaction. The calibration curve was obtained using Trolox, ranging from 1.00 mg/mL to 0.01 mg/mL, and the results are expressed as μmol Trolox equivalent per mL of sample.

$GEAC_{RED}$ reducing capacity method (gallic acid equivalent antioxidant capacity-reduction): This method was performed to analyse the global reducing capacity of the samples, using indigo carmine as a redox indicator. The assay was carried out using a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37^0C) and measuring the absorbance at 400 nm. Each well of the transparent 96-well polystyrene microplate used (Biogen Científica, Spain) contained 15 μL of indigo carmine indicator (1 mM), 265 μL of phosphate buffer (0.5 M; pH 7.24) and 20 μL of

sample, although this volume could be increased for very-low-reducing-activity samples, but always reducing the buffer volume by the same quantity. Absorbance was measured every 60 seconds in kinetic mode and the absorbance values used were those obtained after 60 minutes of reaction. The calibration curve was obtained using gallic acid, ranging from 0.1 mg/mL to 2 mg/mL, and the results are expressed as μmol gallic acid equivalent per mL of sample.

$TEAC_{ABTS}$ *(Trolox equivalent antioxidant capacity against ABTS radicals)*: The antioxidant capacity was estimated in terms of radical scavenging activity following the procedure described by Roberta, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, (1999). Briefly, ABTS was produced by reacting ABTS stock solution (7 mM) with potassium persulphate (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS solution was diluted with an ethanol: water (50:50) mixture to an absorbance of 0.70 ± 0.02 at 730 nm. After placing 20 μL of sample or Trolox standard and 280 μL of diluted ABTS solution on a transparent 96-well polystyrene microplate (Biogen Científica, Spain), absorbance readings were taken every 60s for 20 min on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37^0C). Calibration was performed with a Trolox stock solution ranging from 0.01 to 1.00 mg/mL. Results are expressed as μmol Trolox equivalent per mL of sample.

$TEAC_{FRAP}$ (Trolox equivalent antioxidant capacity coupled with the ferric reducing capacity of plasma): The ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie & Strain (1996), adapted to a microplate reader. Briefly, 280 μL of FRAP reagent, prepared freshly and warmed at 37^0C, was mixed in each well of a transparent 96-well polystyrene microplate (Biogen Científica, Spain) with 20 μL of sample or water to provide appropriate blank reagent. The FRAP reagent contained 2.5 mL of a TPTZ solution (10 mM) in HCl (40 mM), plus 2.5 mL of $FeCl_3 \cdot 6H_20$ (20 mM), and 25 mL of acetate buffer (0.3 M; pH 3.6).

Readings of maximum absorbance (595 nm) were taken every 60s using a FLUOStar Omega microplate reader (BMG Labtech, Germany). Temperature was maintained at 37^0C and the reaction was monitored for 30 min. Trolox stock solutions ranging from 0.01 to 1.00 mg/mL were used to obtain the calibration curves. Results are expressed as μmol Trolox equivalent per mL of sample.

$TEAC_{ORAC}$ (Trolox equivalent antioxidant capacity against AAPH radicals, fluorimetric method): The antiradical activity against AAPH was estimated according to the procedure reported by Dávalos, Gómez-Cordobés & Bartolomé (2004), slightly modified. The reaction was carried out in phosphate buffer (75 mM; pH 7.40) and the final reaction mixture was 200 μL. Sample or Trolox (20 μL) and fluorescein (120 μL; 70 mM, final concentration) solutions were placed in each well of a black 96-well polystyrene microplate (Biogen Científica, Madrid, Spain). The mixture was pre-incubated for 15 min at 37^0C on a FLUOStar Omega microplate reader (BMG Labtech, Germany). AAPH solution (90 μL; 12 mM, final concentration) was added rapidly using the plate reader syringe, the microplate was shaken for 15 seconds and the fluorescence was recorded every minute for 90 min at 485 and 528 nm excitation and emission wave lengths, respectively. The blank (fluorescein + AAPH) was obtained by using phosphate buffer instead of the sample solution. Raw data were processed by the microplate reader and the area under the curve (AUC) was calculated according to the equation:

$AUC = F_0/F_0 + F_0/F_1 + F_0/F_2 + \ldots + F_0/F_n$

where F_0 = Fluorescence intensity at time 0, F_1 = Fluorescence intensity at time 1 minute, and so forth. Finally, the net AUC was calculated by subtracting the AUC of the blank from that of each sample. ORAC values are expressed as Trolox equivalents by using the standard curve (ranging from 0.01 to 1.00 mg/mL) calculated for each assay and the final results are expressed as μmol Trolox equivalent per ml of sample.

2.4. Statistical analysis

The statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($p < 0.05$). The relationships among the different assays were evaluated by computing the relevant correlation coefficient (Pearson linear correlation) at the $p < 0.05$ confidence level. All statistical analyses were performed using Statgraphics Plus software, version 5.1.

3. Results and discussion

3.1. $TEAC_{OH}$ method

Hydroxyl radicals, which are among the most harmful radicals produced in the human body, were generated in our study through the Fenton reaction (Lee et al., 2004). This was done under different pH conditions (neutral or acidic), with different concentrations of Fenton reagents (H_2O_2 and Fe^{3+}), different sources of Fe^{3+} (free or chelated with EDTA or phosphotungstate) and with different redox indicators (methylene blue, indigo carmine and neutral red). The parameters studied with which to determine the most suitable redox indicator were the amount of absorbance change, absorbance differences with blank and the reaction speed (**Table 3**).

Table 3. Behaviour of the different indicators depending on pH conditions.

		Absorbance differences					
		Absorbance change		with blank		Reaction speed	
	Indicator	**pH < 7**	**pH > 7**	**pH < 7**	**pH > 7**	**pH < 7**	**pH > 7**
Fe^{3+}	**Methylene blue**	+++	-	+++	-	+++	-
	Indigo carmine	+++	+++	+++	+++	+++	++
	Neutral red	-	-	-	-	-	-
Fe^{3+}+EDTA	**Methylene blue**	+++	-	+++	-	++	-
	Indigo carmine	+++	+++	+++	+++	++	++
	Neutral red	-	-	-	-	-	-
Fe^{3+} + Phophotungstate	**Methylene blue**	+++	+++	+++	+++	+++	+++
	Indigo carmine	+++	+++	+++	+++	++	+++
	Neutral red	++	++	++	++	+	++

Methylene blue: In acidic conditions, methylene blue showed good properties as a redox indicator, whether or not the iron was chelated. This finding could be related to the high solubility of Fe^{3+} in acidic conditions resulting in a high production of oxidants through the Fenton reaction. However, at physiological pH (7.24), methylene blue did not show any response as a redox indicator, either with free iron or chelated with EDTA. According to Lee et al. (2009), this may have resulted from the low solubility of Fe^{3+} in neutral pH, leading to an inefficient production of oxidants, or because, although EDTA increases iron solubility, this chelating agent also captures the hydroxyl radicals produced, preventing them from reacting with the indicator. Therefore, phosphotungstate was added as a chelating agent to increase iron solubilisation. This chelating agent increases iron solubilisation in a wide range of pH through complexation and, at the same time, allows hydroxyl radicals to react with the redox indicator (Lee & Sedlak, 2009). Thus, a good response at physiological pH was obtained (**Table 3**). In view of these results, phosphotungstate was chosen as the chelating agent at neutral pH.

Various concentrations of H_2O_2, ranging from 10 M to 100 μM, were then assayed. Hydrogen peroxide at concentrations of 10 M, 1 M and 100 mM gave a good visual colour change, with all experiments being carried out with Fe^{3+} and phosphotungstate 3.4 mM in 1:1 (v/v) proportion. However, two problems were observed under these conditions: (i) methylene blue only presented fast performance as an indicator (around 50 minutes) with the highest concentrations of H_2O_2; (ii) this indicator precipitated after a few minutes, and so the readings obtained were not useful, even when the mixing mode of the microplate reader was used. Therefore, methylene blue was discarded as an indicator.

Indigo carmine: The same assays were carried out to determine the suitability of this new redox indicator. Indigo carmine showed good properties in acidic conditions whether or not the iron was chelated. At physiological pH, only when iron was chelated with EDTA or phosphotungstate, indigo carmine also presented good activity. In addition, since this indicator did not suffer any interference during the absorbance readings or experience any precipitation, this was considered the leading candidate as a redox indicator for the new antioxidant capacity method.

Neutral red: The same assays were carried out with this indicator, but uneven results were obtained. Neutral red only showed activity as a redox indicator when iron was chelated with phosphotungstate, when the neutral red presented activity both at physiological and at acidic pH. However, the absorbance change, absorbance difference with the blank and the reaction speed were not as good as in the previous case. In addition, some precipitation still occurred. Therefore neutral red, too, was discarded as a redox indicator, and indigo carmine was selected.

Once the indicator had been established, various experiments were then carried out to optimise the other reagent concentrations. In the case of iron, different concentrations around 3.4 mM, a value that has been established in prior assays (Moore, Yin & Yu, 2006), were assessed. However, we found that this concentration caused interference in the absorbance readings due to the iron reacting with polyphenols (data not shown). Secondly, at the concentration of 3.4 mM, Trolox was not able to slow oxidation (**Figure 20A**). After several tests, we found that the best concentration, one that did not cause such interference, was 100 μM, and therefore this iron concentration was selected. Moreover, at this concentration Trolox exerted activity (**Figure 20B**). Several further assays were conducted to determine which chelating agent should be used (EDTA or phosphotungstate), although phosphotungstate had already presented

better behaviour than EDTA. For this purpose, the behaviour of the indicator was examined using iron chelated against tea, either with EDTA or with phosphotungstate (**Figure 18)**. The blank and the tea with EDTA-chelated iron presented similar outcomes, while the use of phosphotungstate obtained antioxidant behaviour with tea. Thus, iron was chelated with phosphotungstate 0.1 μM in a 1:1 proportion.

Next, the H_2O_2 concentration was established, using a range from 10 M to 100 mM. As shown in **Figure 19**, the best concentration was found to be 1 M, at which the colour change took place fast enough for us to obtain a quick kinetic reaction but not so fast that differences between samples could not be observed. In order to reduce the volume of H_2O_2 per microplate well, we finally opted for a concentration of 4 M.

Figure 18. Behaviour of tea using either iron chelated with EDTA or phosphotungstate (iron 100 μM, phosphotungstate/EDTA 100 μM, H_2O_2 1M, phosphate buffer 0.5M and carmine indigo 2 mM).

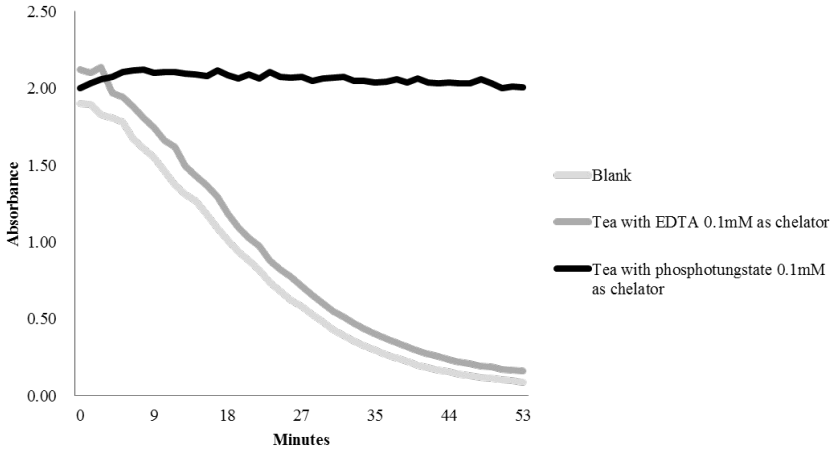
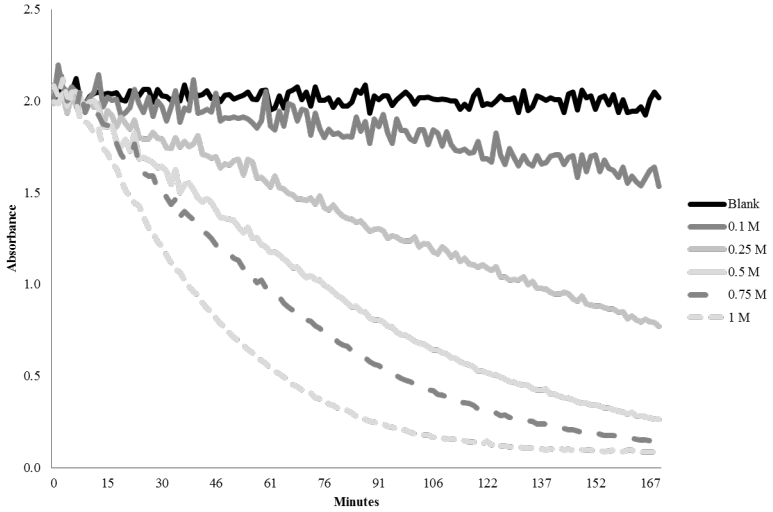


Figure 19. Kinetics curves for different H_2O_2 concentrations with phosphotungstate-chelated iron (iron 100 μM, phosphotungstate 100 μM, phosphate buffer 0.5M and carmine indigo 2 mM).



The standard used for the calculations was determined as follows. The first one assayed was Trolox, the traditional choice. Experiments were conducted using a wide range of concentrations, from 10 mg/mL to 0.0001 mg/mL (F**igures 20A and 20B**). As shown in **Figure 20A,** Trolox had no significant protective effect against oxidation mediated by hydroxyl radicals under the following conditions: iron (III) 3.4 mM chelated with phosphotungstate 3.4 mM, H_2O_2 4 M, carmine indigo 2 mM and phosphate buffer 0.5 M pH 7.24. However, under the appropriate conditions, namely 0.1 mM iron (III) chelated with phosphotungstate 0.1 mM (1:1), H_2O_2 4M, carmine indigo 1 mM and phosphate buffer 0.5 M pH 7.24 (**Figure 20B**), Trolox presented good antioxidant capacity. Ascorbic acid was also assayed, but as observed previously (Higuchi, Yonemitsu, Koreeda, & Tsunenari, 2003) ascorbic acid acts as a pro-oxidant in the presence of iron. Gallic acid and catechin were also considered, since these substances have been reported to show OH· scavenging activity (Ou et al., 2002). These compounds showed good response and good linearity. However, after some

experiments it was decided to use Trolox as the standard, for the following reasons: (i) Trolox is widely used in antioxidant assays; (ii) Trolox gives a transparent solution, so that possible interference by colour is avoided (**Figure 21A**); (iii) the calibration curve shows a good response in a wider range of concentrations than catechin (10 mg/mL-0.01mg/mL). Thus, Trolox was selected, and its kinetic curve as the standard is shown in **Figure 21B**. The final steps in establishing the method were to obtain the calibration curve, conduct recovery assays and determine intra and interday repeatability (**Table 4**). The method demonstrated excellent linearity and recovery, and the coefficient of variation was below 4% even when the analysis was repeated over five consecutive days.

Table 4. Analytical parameters of the antioxidant methods developed

Method	**Calibration curve**		**CV (%)**		**Recovery (%)**
	Equation	**R^2**	**Intraday**	**Interday**	
$TEAC_{OH}$	y = 0.0328x + 0.1792	0.9956	2.69	3.73	99.74
$TEAC_{AAPH}$	y = 7.7222x + 0.3747	0.9974	3.97	5.22	102.14
$GEAC_{RED}$	y = 0.3306x + 0.1253	0.9977	2.45	5.11	97.11

Figure 20. Trolox kinetics curves at physiological pH. 20A: (iron 3.4 mM, phosphotungstate 3.4 mM, H_2O_2 4M, phosphate buffer 0.5M and carmine indigo 2 mM). 20B: (iron 0.1 mM, phosphotungstate 0.1 mM, H_2O_2 4M, phosphate buffer 0.5M and carmine indigo 1 mM).

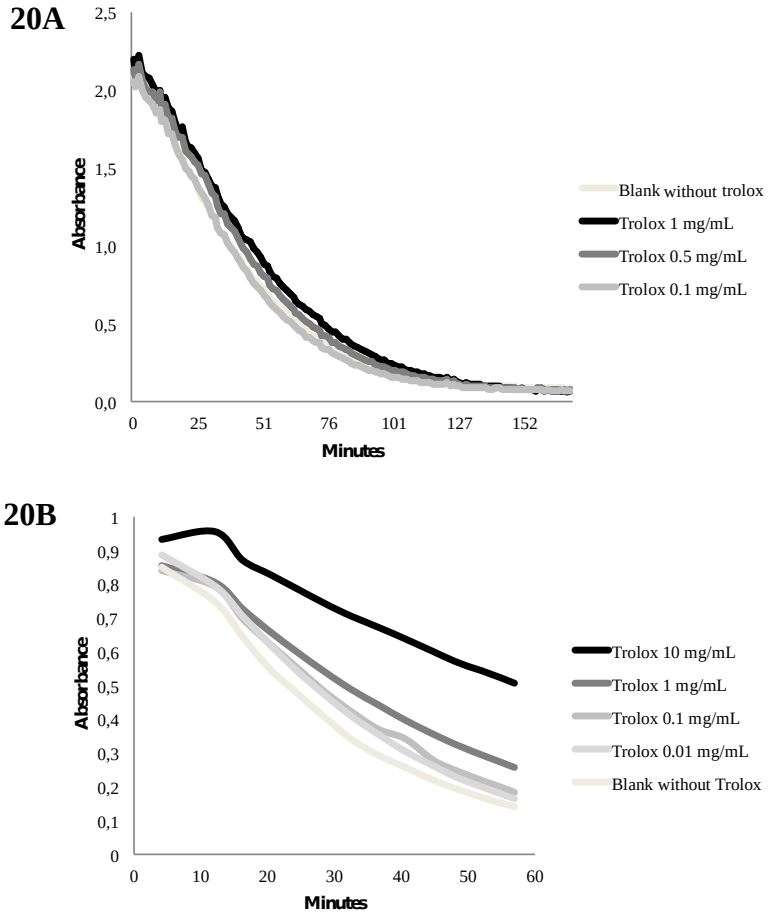
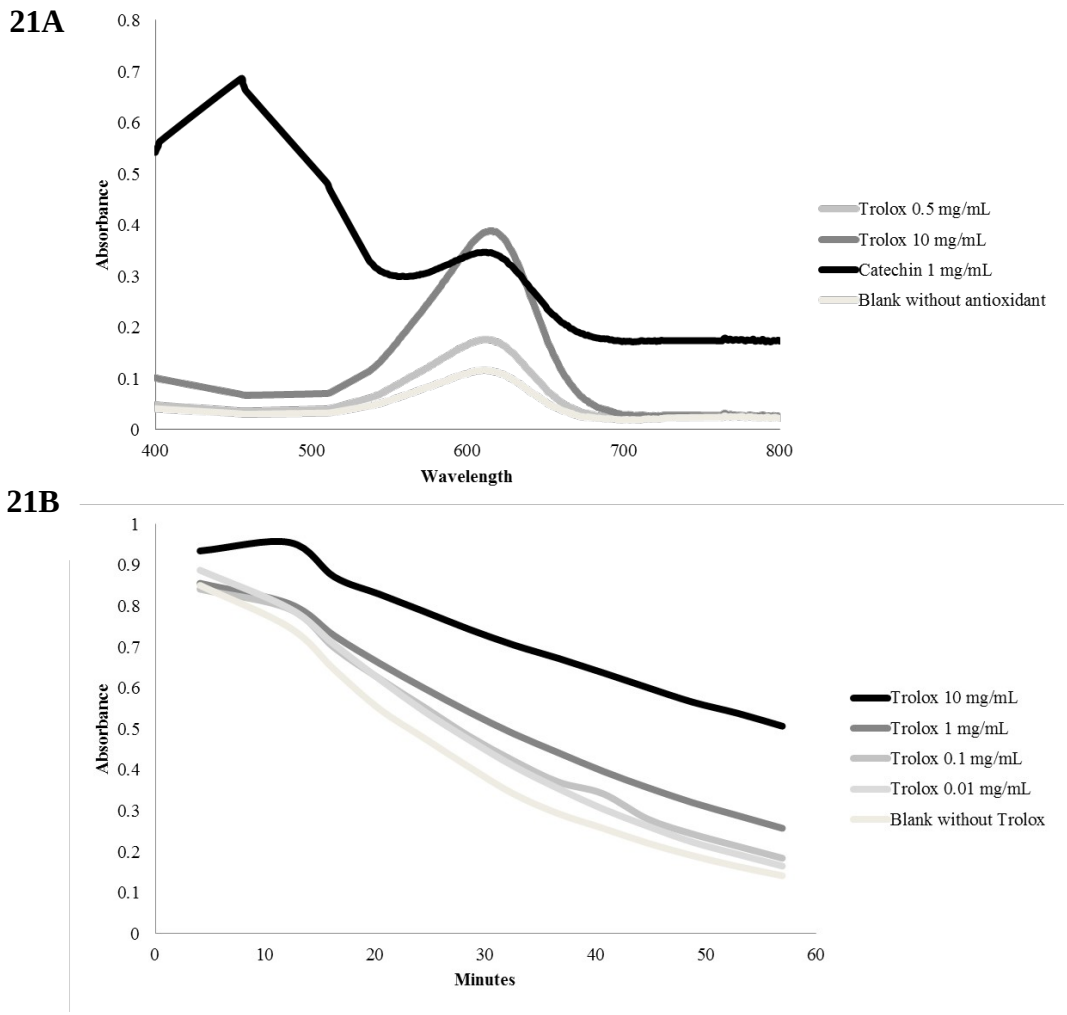


Figure 21. Absorbance spectra of catechin and Trolox (21A) and kinetics curves of Trolox (21B) as standard (iron 100 μM, phosphotungstate 100 μM, H_2O_2 4M, phosphate buffer 0.5M and carmine indigo 1 mM).



3.2. $TEAC_{AAPH}$ method

The first step in developing a method to measure antioxidant capacity against AAPH· radicals was to assess the performance of the above three indicators. Figures **22A-C** show the redox behaviour of each one, and the redox activity against the AAPH· radical is shown in **Figure 22**. In the case of methylene blue, no activity was observed (**Figure 22A**). However, antiradical reactivity was obtained both for neutral red (**22B**) and for indigo carmine (**22C**). The latter was selected because it presented larger

differences between the blank and Trolox concentrations, compared to those obtained with neutral red at physiological pH.

The next step was to standardise the AAPH concentration. To do so, an initial AAPH concentration of 32 mM was used, following prior research in this field (Prior et al., 2003). However, the concentrations used by other authors (32 mM) did not have any effect at physiological pH (blank absorbance = 2.012; AAPH absorbance = 1.946) and so the concentration was increased to 553 mM (1.5 g/10 mL), a level at which the best reaction speed was obtained. In the case of the standard, Trolox showed a linear pattern of behaviour, as shown in **Figure 22C** and as observed in previous studies (Prior et al., 2003). Finally, the calibration curve, repeatability and recovery were all found to be correct, as in the case of the $TEAC_{OH}$ method (**Table 4**).

3.3. $GEAC_{RED}$ method

This method was first developed as an alternative to FRAP in order to measure the reducing capacity against ferric ions of a food extract at physiological pH (instead of acidic pH with the FRAP method). Both indigo carmine and methylene blue were reduced (although neutral red was not), generating an absorbance increase at 400nm (green), which is different from the wavelength used in the other two assays (610 nm). Nevertheless, some difficulties appeared, since ferrous ions were quickly autooxidised at neutral pH conditions, making it difficult to measure the iron reduction, as reported previously (Yang & Chasteen, 1999; Welch, Davis & Aust, 2002). Therefore, iron was removed and the general reducing capability of the food extract was measured instead. Only methylene blue and indigo carmine showed activity as redox indicators in the assayed conditions. However, methylene blue was discarded because of precipitation problems and in view of the higher sensitivity and linearity obtained with indigo

carmine. The concentration of the latter was the same as was used with the $TEAC_{OH}$ method, since any higher volume of indicator might cause interference with the absorbance readings (absorbance exceeding 2000 mAU).

The next step was to decide the standard to be used for the calculations (Trolox, ascorbic acid, catechin or gallic acid). Trolox did not show any reducing activity in the range from 0.01 to 10 mg/mL and ascorbic acid only showed some activity with a very slow kinetic reaction. However, catechin and gallic acid presented good reducing activity in the whole range from 0.1 to 50 mg/mL within a suitable period of time. Accordingly, gallic acid was chosen as the standard. Gallic acid was chosen over catechin because, first, it is widely used as a standard in the Folin-Ciocalteu method and, second, because it gives a transparent solution thus avoiding interference that may be caused by coloured solutions.

Finally, to demonstrate the linearity and sensitivity of the method, increasing volumes of tea and grape juice ranging from 2 μl to 100 μl were added. Good linearity was obtained (**Figure 23**), with a Pearson linear correlation coefficient of 0.9983 (**Table 4**). In addition, both recovery and repeatability were in a suitable range.

Figure 23. Kinetic curves for tea and grape juice obtained with the $GEAC_{RED}$ method. (phosphate buffer 0.5 M pH 7.24, carmine indigo 1 mM)

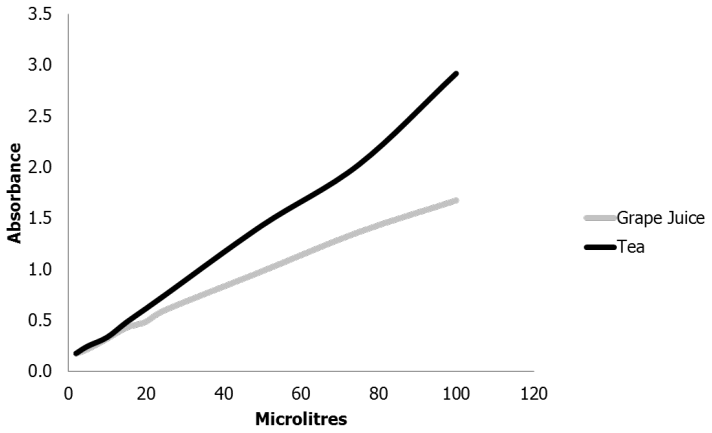
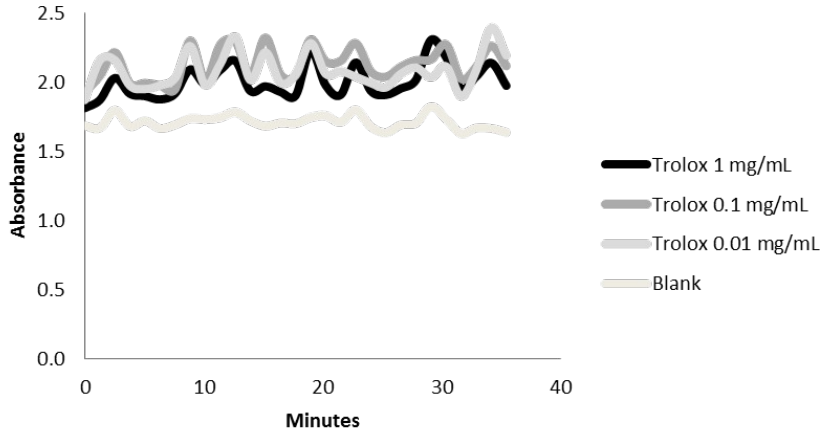
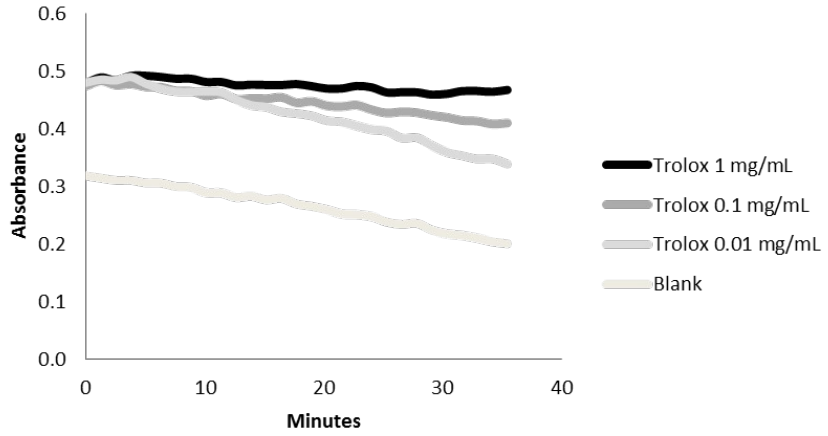


Figure 22. Kinetics curves for different Trolox concentrations with methylene blue (22A), neutral red (22B) and indigo carmine (22C) as indicators (iron 100 μM, phosphotungstate 100 μM, H_2O_2 4M, phosphate buffer 0.5M and dye 1 mM).

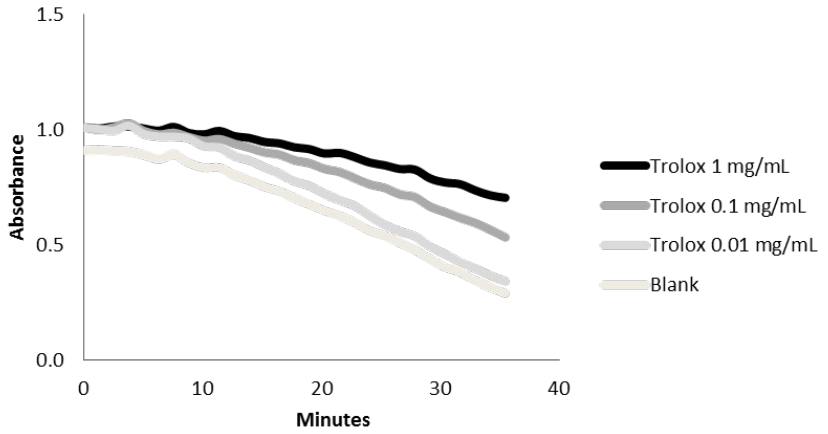
22A



22B



22C



3.4. Evaluation of the antioxidant capacity of tea and of tea-based beverages

The methods described above were applied to different samples of green, white, red and black tea and also to some beverages claiming to have tea as a major ingredient. The assays were carried out applying the protocols described above. Every sample was measured six times, with each method, since three different dilutions of each one were made and measured twice. The results obtained are expressed in gallic acid Equivalent Antioxidant Capacity (GEAC) for the reducing method and in Trolox Equivalent Antioxidant Capacity (TEAC) for the AAPH, OH·, ABTS, FRAP and ORAC methods. The results clearly show that tea is 3-10 times more antioxidant ($p < 0.05$) than tea-based drinks, irrespective of the method used to assess the antioxidant capacity (**Table 5**). Only in a few cases (the samples that included pineapple and pomegranate extract in addition to tea extract) was the activity of these beverages comparable to that found in tea.

According to the results obtained (**Table 5**), green and white tea had the highest OH· scavenging activity, with a value equivalent to 13 mmol Trolox/L, whereas black and red tea had a statistically ($p < 0.05$) lower activity (11 and 8 mmol Trolox/L, respectively). This higher activity of green and white tea against OH· radicals could be related to their higher content of compounds such as phenols (Cabrera et al., 2003), while in black and red tea these compounds could have been degraded during the fermentation process, resulting in a lower level of OH· scavenging activity.

The peroxyl (AAPH·) scavenging activity of white tea was almost three times ($p < 0.05$) that of the other teas (14.61 vs. 5.50, respectively). These results are in line with those obtained with the ORAC method (Table 2) except for green tea, which showed an antioxidant capacity similar to that of white tea with ORAC. With the reducing capacity method, our results show that black tea had the strongest reducing

effect ($p < 0.05$), with a mean activity of 413 meq gallic acid/L. The greater reducing activity of black and red tea could be due, in part, to the polymeric compounds generated during the fermentation process (Aoshima et al., 2007). Thus, red tea had a lower reducing capacity than black tea due to the partial fermentation of phenolic compounds in the former.

As stated above, our analysis of the antioxidant capacity of teas was also performed with standard methods (ABTS, FRAP and ORAC) in order to compare our results with those obtained by other authors and to determine whether the highest and lowest capacities obtained previously were in line with our new protocols. The ABTS method showed that green and white teas, which were equivalent to 13.20 and 10.55 meq Trolox/L, had the highest antioxidant capacity ($p < 0.05$), as with the $TEAC_{OH}$ method (**Table 5**). These results concur with those of Gorjanovic et al. (2012), who observed that the fermentation process could have an impact on the antioxidant capacity of fermented teas (black and red).

The results obtained with the FRAP method were similar to those obtained with the ABTS and $TEAC_{OH}$ protocols and in line with those reported previously by Gorjanovic et al. (2012), with green and white teas being the most strongly antioxidant samples (**Table 5**). Nevertheless, our reducing method found that black tea was the strongest reducing sample. This difference could be related to the analysis of global reducing capacity that is performed with the $GEAC_{RED}$ method, whereas the FRAP method only takes iron reduction into account. In this respect, the fermentation process could generate new substances with reducing power that are not taken into account by the FRAP method. Again, the ORAC results were similar to those of the classical methods and $TEAC_{OH}$, being in the same range as those reported previously by Alarcón et al. (2008).

Table 5. Antioxidant capacity of teas and tea-based beverages.

Samples	$TEAC_{OH}$	$TEAC_{AAPH}$	$GEAC_{RED}$	$TEAC_{ABTS}$	$TEAC_{FRAP}$	$TEAC_{ORAC}$
Black tea	$9.99^{a} \pm 1.12$	$5.64^{a} \pm 0.89$	$413^{a} \pm 33$	$4.82^{a} \pm 0.83$	$5.20^{a} \pm 0.43$	$657^{a} \pm 47$
Green tea	$13.24^{b} \pm 1.43$	$5.24^{a} \pm 1.70$	$298^{b} \pm 24$	$13.20^{b} \pm 1.16$	$11.43^{b} \pm 1.09$	$1324^{b} \pm 98$
Red tea	$8.23^{a} \pm 0.97$	$6.07^{a} \pm 2.08$	$355^{b} \pm 14$	$2.98^{c} \pm 0.34$	$3.69^{c} \pm 0.38$	$784^{a} \pm 52$
White tea	$13.01^{b} \pm 2.01$	$14.61^{b} \pm 1.56$	$250^{b} \pm 20$	$10.55^{b} \pm 1.06$	$11.60^{b} \pm 1.32$	$1298^{b} \pm 88$
Tea beverages	$5.36^{c} \pm 5.21$	$1.48^{c} \pm 1.56$	$75^{c} \pm 48$	$4.84^{a} \pm 4.78$	$1.30^{d} \pm 0.32$	$224^{c} \pm 17$

Different letters indicate statistically significant differences $p < 0.05$. Antioxidant capacity expressed as mmol Trolox/L ($TEAC_{OH}$) mmol Gallic acid/L ($GEAC_{RED}$) and mmol Trolox/L ($TEAC_{AAPH}$, $TEAC_{ABTS}$, $TEAC_{DPPH}$, $TEAC_{ORAC}$). For each tea type n = 4 and for tea beverages n = 13.

3.5. Evaluation of the antioxidant capacity of common foodstuffs

Since the main antioxidant compounds present in tea (catechins) are fairly water-soluble and react readily due to the absence of a real food matrix, the validity of the above-described methods was tested using other liquid and solid foods submitted to *in vitro* digestion with the GAR method (Pastoriza et al., 2011). As shown in **Table 6**, for all three new methods, the highest antioxidant capacity was obtained for coffee while the lowest corresponded to chamomile infusion. Intermediate values were obtained for different vegetable foods (green pepper, onion, spinach and lettuce) while a lower (but still interesting) antioxidant capacity was obtained for animal foods (salami and fermented milk).

In the case of regular antioxidant methods (ABTS and FRAP) the values obtained presented the same distribution as that obtained for the new methods. In general, these data are in line with those reported by Pastoriza et al. (2011) for vegetable and animal products. In the case of ORAC, green pepper was 4-5 times more antioxidant than coffee, which is in line with previous reports (Prior et al., 2003; USDA, 2010). Whatever the order of the antioxidant capacity of the foods measured with the ORAC method (**Table 6**), the values obtained are similar to those reported in the USDA database for the ORAC of selected foods (USDA, 2010).

Table 6. *In vitro* antioxidant capacity of several standard compounds and foodstuff extracts.[1]

Standard Compound	**mg/mL**	**$TEAC_{OH}$**	**$TEAC_{AAPH}$**	**$GEAC_{RED}$**	**$TEAC_{ABTS}$**	**$TEAC_{FRAP}$**
Catechin	10	45.26 ± 2.65	40.25 ± 0.41	29.22 ± 1.68	50.12 ± 3.69	33.69 ± 1.22
	1	6.25 ± 0.54	4.78 ± 0.09	4.06 ± 0.52	7.12 ± 1.11	5.66 ± 0.11
	0.5	3.56 ± 0.21	2.36 ± 0.09	2.01 ± 0.11	2.99 ± 0.41	3.22 ± 0.40
Gallic acid	10	24.45 ± 0.25	11.98 ± 1.11	-	47.36 ± 1.36	21.36 ± 1.89
	1	2.56 ± 0.16	1.36 ± 0.10	-	5.69 ± 0.25	5.78 ± 0.98
	0.5	1.03 ± 0.07	0.98 ± 0.05	-	2.12 ± 0.55	3.71 ± 0.25
Naringenin	1	9.37 ± 0.46	5.02 ± 0.25	13.56 ± 0.63	11.10 ± 1.04	4.78 ± 0.74
Resveratrol	1	13.07 ± 0.07	7.01 ± 0.58	18.74 ± 0.10	15.23 ± 1.23	11.45 ± 1.03
Genistein	1	7.42 ± 0.03	4.23 ± 0.31	9.23 ± 0.21	8.99 ± 1.11	6.87 ± 0.87
Foodstuff		**$TEAC_{OH}$**	**$TEAC_{AAPH}$**	**$GEAC_{RED}$**	**$TEAC_{ABTS}$**	**$TEAC_{FRAP}$**
Coffee		67.54 ± 4.23	60.24 ± 5.56	70.89 ± 6.36	102.15 ± 9.56	55.69 ± 4.12
Chamomile infusion		0.83 ± 0.07	1.23 ± 0.10	5.12 ± 1.02	25.36 ± 2.87	5.23 ± 1.44
Green pepper		8.45 ± 0.56	8.03 ± 0.21	10.58 ± 0.99	32.12 ± 4.56	10.58 ± 1.47
Onion		6.56 ± 0.14	7.12 ± 0.14	12.69 ± 1.12	29.63 ± 4.21	11.25 ± 1.98
Spinach		4.12 ± 0.23	3.56 ± 0.12	6.23 ± 0.54	17.23 ± 3.69	6.36 ± 1.01
Lettuce		3.78 ± 0.12	2.41 ± 0.09	5.78 ± 0.65	16.56 ± 1.99	5.99 ± 0.47
Fermented milk		0.25 ± 0.01	0.36 ± 0.04	1.47 ± 0.07	14.56 ± 2.01	4.12 ± 0.74
Salami		1.79 ± 0.09	2.01 ± 0.08	2.87 ± 0.06	20.36 ± 2.14	15.23 ± 1.32
Peanuts		32.42 ± 2.02	8.77 ± 0.54	8.34 ± 0.69	71.08 ± 5.26	16.26 ± 1.56

[1]Results are expressed in mmol trolox ($TEAC_{OH}$, $TEAC_{AAPH}$, $TEAC_{ABTS}$, $TEAC_{FRAP}$, $TEAC_{ORAC}$) or mmol gallic acid ($GEAC_{RED}$) per Kg of sample (for solid foods) or L of infusion (for liquid samples).

3.6. Evaluation of the antioxidant capacity of plasma after acute intake of tea

After the successful use of $TEAC_{OH}$, $TEAC_{AAPH}$ and $GEAC_{RED}$ with different food matrixes, the new methods, together with the classical ones of ABTS, FRAP and ORAC, were tested *in vivo* to determine their physiological relevance and whether they were sensitive to changes in the antioxidant capacity of plasma. In this process, the overall antioxidant capacity of plasma was measured in thirty human subjects two hours after the acute intake of tea (green *vs.* black tea). This delay was established in the view that two hours is the time required to obtain the maximum absorption of tea catechins (Takahashi et al., 2014). As shown in **Table 7**, the antioxidant capacity of plasma in the

persons who drank black tea was double that of the control group ($p < 0.05$) while the intake of green tea increased the antioxidant capacity up to six times ($p < 0.05$). The results obtained were similar irrespective of the method used to measure the antioxidant capacity in plasma, and so the new methods presented good performance *in vivo*. The results obtained for the increase in antioxidant capacity were in line with those reported previously for the FRAP method after the acute intake of green tea (Benzie, Szeto, Strain & Tomlinson, 1999), for ABTS after the acute intake of dark chocolate (Radosinska, Horvathova, Frimmel, Muchova, Vidosovicova, Vazan & Bernatova, 2017) and for the ORAC method for healthy women with a diet rich in antioxidant compounds (Wang, Yang, Lee, Davis, Kenny, Koo & Chun, 2012).

Table 7. *In vivo* antioxidant capacity of plasma after 2-hours acute intake of one cup of green or black tea.[1]

Group	**$TEAC_{OH}$**	**$TEAC_{AAPH}$**	**$GEAC_{RED}$**	**$TEAC_{ABTS}$**	**$TEAC_{FRAP}$**	**$TEAC_{ORAC}$**
Control	$0.87^{a} \pm 0.12$	$0.61^{a} \pm 0.09$	$0.16^{a} \pm 0.05$	$2.86^{a} \pm 0.09$	$0.28^{a} \pm 0.07$	$1.87^{a} \pm 0.17$
Green tea	$2.19^{b} \pm 0.18$	$2.24^{b} \pm 0.17$	$1.27^{b} \pm 0.11$	$4.05^{b} \pm 0.16$	$0.79^{b} \pm 0.09$	$6.44^{b} \pm 0.43$
Black tea	$1.43^{c} \pm 0.08$	$1.31^{c} \pm 0.10$	$0.53^{c} \pm 0.07$	$3.44^{c} \pm 0.05$	$0.51^{c} \pm 0.11$	$4.03^{c} \pm 0.21$

[1]Different letters indicate statistically significant differences $p < 0.05$. Results are expressed in mmol trolox ($TEAC_{OH}$, $TEAC_{AAPH}$, $TEAC_{ABTS}$, $TEAC_{FRAP}$, $TEAC_{ORAC}$) or mmol gallic acid ($GEAC_{RED}$) per L of plasma.

4. Conclusions

This paper presents three novel methods developed to measure the antioxidant capacity of foods and plasma at physiological pH, using the dye indigo carmine as a redox indicator. Two of these methods measure scavenging capacity against OH· and AAPH· radicals and the third measures the global reducing capacity of the food. These methods offer reliable measurement of the antioxidant compounds that are obtained from foods, thus enabling more accurate predictions of their activity within the human body. The simplicity, use of absorbance readings and physiological-resembling conditions (i.e. pH and radicals) of these assays make them suitable for any laboratory. A possible limitation could be the time needed to complete the reaction (50 minutes) although this could be shortened or extended depending on the sample activity.

Green and white teas were found to be the most antioxidant foods, by all methods except $GEAC_{RED}$, with which black and red tea presented the highest values. The new methods were also shown to be useful for *in vitro* digestion-fermentation performed on solid and liquid foods. Finally, the three methods developed in this study enabled us to measure the global antioxidant capacity of plasma after the acute intake of tea, showing them to be useful *in vivo*.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Towards an improved Global Antioxidant Response method (GAR+): physiological-resembling *in vitro* digestion-fermentation method

Este trabajo muestra la puesta a punto de un método de digestión gastrointestinal *in vitro* al que se le acopla un método de fermentación *in vitro* para simular el efecto de la microbiota intestinal sobre la fracción no digerida de alimento. Así, este trabajo está íntimamente ligado a la temática de la presente tesis doctoral, además de ser uno de sus objetivos principales.

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Towards an improved Global Antioxidant Response method (GAR+): physiological-resembling *in vitro* digestion-fermentation method

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ABSTRACT

The antioxidant capacity of food influences its shelf life and human health. In order to determine this parameter, the corresponding antioxidant chemical species must first be extracted. This paper presents an improved protocol to determine the global antioxidant response of foods (GAR+), which includes *in vitro* digestion and fermentation. The latter process, in conjunction with new, physiological-resembling antioxidant methods, provides values for antioxidant capacity (calculated for seven different foods) that are close to their *in vivo* activity. The fermentation step was analysed in order to determine the main factors influencing the extraction of antioxidant compounds. The use of a pool of faeces from healthy donors is strongly recommended in order to improve repeatability and to extrapolate results to the general population. Although most antioxidant capacity is achieved after digestion, in some foods fermentation plays a role. Therefore, the effects of gut microbiota should be taken into account in future studies.

KEYWORDS: Antioxidant capacity, digestion, fermentation, gut microbiota, ABTS, FRAP.

1. Introduction

Antioxidant capacity has drawn a great deal of research attention due to the vital importance of protecting biological tissues from oxidation. This concern arises from an increasingly clear understanding of the negative impact of oxygen-derived free radicals on chronic disorders such as cancer, cardiovascular diseases, neurological diseases and aging (Moon & Shibamoto, 2009). Internal antioxidants (glutathione, vitamin E, etc.) are depleted in such pathologies and foods are the main source of exogenous antioxidant compounds. Thus, it is important to determine the antioxidant capacity that is supplied through nutrition.

Although many methods exist for measuring antioxidant activity, most of them rely on the use of organic solvents to extract target compounds or on physical-chemical conditions (pH, temperature, pressure) that in many cases differ greatly from those found inside the human body (Perez-Burillo, Rufian-Henares, Molino, & Delgado-Andrade, 2015). A more physiological approach is to use *in vitro* digestion and fermentation, which more realistically represents the processes taking place within the gut (Saura-Calixto & Goñi, 2004). *In vitro* digestion methods are widely used to study food behaviour among other purposes. It is well-known that though in nutrition is far better the use of *in vivo* studies, *in vitro* ones have some advantages that make them of choice most times: they are rapid, less expensive, conditions are better controlled, no ethical problems, etc. Usually *in vitro* digestion protocols include an oral phase, a gastric phase and an intestinal phase. In each one of them conditions such as enzyme and salts concentrations, pH, temperature, etc. are well controlled (Minekus et al., 2014). The antioxidant capacity of foods is derived from the synergic effect of vitamins, phenolic compounds, Maillard reaction products and many other compounds released

during gastrointestinal digestion (Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011).

Moreover, many compounds that are not digested (resistant starch, plant cell wall polysaccharides, proteins such as elastin or collagen, phenolic compounds, etc.) reach the small intestine (Power, O'Toole, Stanton, Ross, & Fitzgerald, 2014). The microbial fermentation of these macronutrients within the gut yields many chemical species, including thiols, indols and phenols. Larger structures such as tannins are degraded into smaller phenolic compounds whose chemical nature varies among individuals (Selma, Espín, & Tomás-Barberán, 2009). Apart from the high antioxidant activity of polyphenol metabolites, intra-individual (David et al., 2013; Power et al., 2014, Trosvik, de Muinck, & Stenseth, 2014) and inter-individual (Schloissnig et al., 2012) variability in gut microbiota activity could give rise to differences in antioxidant capacity among individuals even if they present the same dietary pattern.

However, the solid residue that is obtained after the classic extraction or digestion step is usually discarded, despite the fact that this residue can retain antioxidant capacity (Gökmen, Serpen, & Fogliano, 2009). In such conditions, it is difficult to ascertain accurate values of the antioxidant capacity derived from foods.

Classical methods tend to underestimate the antioxidant capacity of samples because they only consider the supernatant of foodstuffs obtained after digestion (which would be absorbed through the large intestine). However, the supernatant obtained after fermentation (releasing soluble compounds like polyphenol metabolites that are readily absorbed in the small intestine) and the solid residue (which can trap radicals produced during fermentation within the small intestine) are systematically excluded. Accordingly to Pastoriza et al. (2011) the solid residue obtained after digestion contains antioxidant species such as undigested proteins, tannins or melanoidins; this can be particularly

important in fibre-rich foods. To derive a better overall measure of antioxidant activity, these authors developed the GAR – Global Antioxidant Response – method. This approach involves determining the antioxidant capacity of both the supernatant and the solid residue obtained after *in vitro* digestion. However, the GAR does not include a fermentation step, which would release more antioxidant species, due to gut microbiota activity. To overcome this shortcoming, we modify the GAR method to include an *in vitro* fermentation step, and thus obtain a more physiological-resembling system to extract antioxidant compounds from foods. This new method, which we term GAR+, provides a better understanding of the antioxidant potency of raw and cooked foods, when combined with relevant antioxidant methods like those developed in our previous paper (Perez-Burillo, Pastoriza, & Rufián-Henares, in press). In addition, this new method takes into consideration the importance of the gut microbiota metabolism in relation to the bioactivity derived from foods and their health-giving properties.

Objective: to develop a new extraction method based on an *in vitro* digestion followed by and *in vitro* fermentation aiming to mimic physiological conditions *in vivo*. This new methodology would take into account compounds that are released during gastrointestinal digestion and compounds that are released during gut microbiota fermentation along with the residue that is left after the whole process. Though we have use it to determine antioxidant capacity, this method could be used to shed some light into different activities biologically relevant.

2. Materials and methods

2.1. Chemicals

For the antioxidant assays, the following reagents were used: 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®), methanol, 2,2'-azobis(2-methylpropionamide) (AAPH), sodium dihydrogen phosphate monohydrate, ferric chloride hexahydrate, hydrogen peroxide, carmin indigo, gallic acid and phosphotungstate, all obtained from Sigma-Aldrich (Germany). For the *in vitro* digestion and fermentation, the following reagents were used: potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, hydrochloric acid, all obtained from Sigma-Aldrich (Germany). The enzymes – salivary alpha-amylase, pepsin from porcine, and bile acids (bile extract porcine) – were purchased from Sigma-Aldrich, and pancreatin from porcine pancreas was purchased from Alpha Aesar (United Kingdom). The fermentation reagents – sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide and resazurin – were obtained from Sigma-Aldrich (Germany).

2.2. Samples

Grilled chicken, peanuts, whole grain bread, boiled lentils, raw tomato, raw orange and whole milk yoghurt were selected as representative foods for the study. All of the foods were bought in local supermarkets (Granada, Spain) and prepared in the laboratory. All the samples were ground using an Ultraturrax (model T25, IKA, Spain) at 13000 rpm, aliquoted and stored at -80^{o}C until analysis.

2.3. In vitro digestion

The *in vitro* digestion method followed was an adaptation of a previously-described technique (Minekus et al., 2014), composed of an oral phase, a gastric phase and an intestinal phase (**Figure 24**). The extended protocol, together with the preparation of the simulated salivary, gastric and intestinal fluids, is fully described in the supplementary information. Briefly, in the oral phase, 5 mL of SSF with alpha-amylase and 25 μL of $CaCl_2$ were added to 5 g of ground food and the mix was incubated at 37ºC for 2 minutes. Then, 10 mL of SGF with pepsin and 5 μL of $CaCl_2$ were added and the pH was lowered to 3.0 by adding 1N HCl; the mix was then incubated at 37ºC for 2 hours. Finally, 20 mL of SIF with pancreatin, bile salts and 40 μL of $CaCl_2$ were added and the pH was raised to 7.0 with 1N NaOH, after which the mix was incubated at 37ºC for 2 hours. The enzymatic reactions were halted by immersing the tubes in iced water. The samples were then centrifuged at 6000 rpm for 10 minutes at 4ºC and the supernatants stored at -80ºC until further analysis. 10% of the supernatant was added to the solid residue in order to mimic the fraction that is not readily absorbed after digestion. Then, the mixed fractions were frozen for further freeze-drying.

2.4. In vitro fermentation

The *in vitro* fermentation method was adapted from a previously-described technique (Ludwig, IA, Paz de Peña, M, Concepción, C, & Alan, C, 2013). The extended protocol, together with the solution preparation, is fully described in the supplementary information. Faecal samples from healthy donors (not taking antibiotics, people with body mass index within the “normal weight range” (mean Body Mass Index = 21.3)). were obtained in the morning, placed in sterile containers and stored at 4ºC

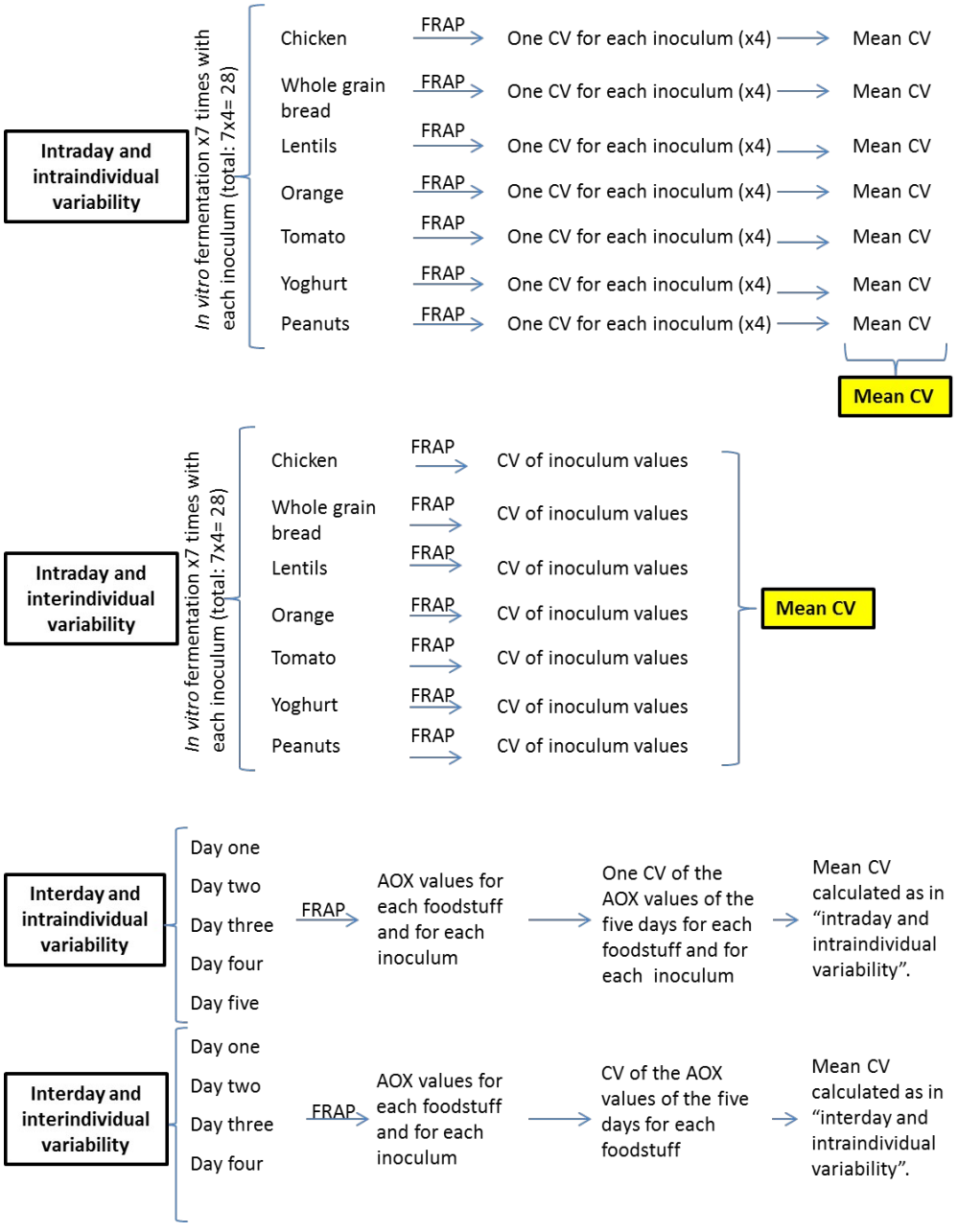
until the inoculum was prepared. Briefly, 500 mg of digested wet-solid residue were placed in a screw-cap tube, making sure that enough digested residue remained in order to determine its water content. Then, as said above, 10% of the supernatant was added but only in proportion to the digested residue to be fermented (i.e. if the supernatant were 36 mL and the total digested residue were 5 g, the 10% would be 3.6 mL to be added to 5g of residue, but as the amount to be fermented was only 500 mg, the volume to be added was 0.36 mL). Then, 7.5 mL of fermentation final solution and 2 mL of inoculum (consisting of a solution of 32% faeces in phosphate buffer 100 mM, pH 0 7.0) were added, to reach a final volume of 10 mL + supernatant volume. Nitrogen was bubbled through the mix to produce an anaerobic atmosphere and the mix was then incubated at 37ºC for 20 hours under oscillation. Immediately afterwards, the samples were immersed in ice, to stop microbial activity, and centrifuged at 6000 rpm for ten minutes. The supernatant was collected as a soluble fraction potentially absorbed after fermentation and stored at -80ºC. The solid residue, representing the non-absorbed fraction after fermentation, was also stored in order to measure the direct antioxidant capacity. The following five experiments were performed in order to obtain the optimal conditions for *in vitro* fermentation and set up the method.

- *Amount of solid residue to be fermented and inoculum volume to be added*: in order to stablish the best amount of solid residue as fermentation substrate, several amounts were fermented (100 mg, 250 mg and 500 mg). Each amount was fermented using 1 mL and 2 mL of inoculum. Antioxidant capacity of each assay was measured through FRAP method.

- *Intraday variability*: seven different foods were fermented seven times with four different inoculum (three from healthy donors and the fourth consisting of a mix of equal weight of the three individual different faeces) and the antioxidant capacity was

measured by the FRAP method. With this data we assessed two variables: a) intraday and interindividual variation due to the use of different inoculum (coming from different people); b) intraday and intraindividual variation due to the methodology which would be fermenting the same sample using the same inoculum (coming from the same individual) 7 times the same day (**Figure 25)**.

Figure 25. Schematic representation of the experiment carried out to determine variability.



- *Interday variability*: the experiment described above was carried out 5 consecutive days. With this experiment we obtained two variables: a) interday and intraindividual variation due to the use of fecal samples from the same person during several different days; b) interday and interindividual variation due to the use of different fecal samples (coming from different people) during several days (**Figure 25**).

In order to assess the coefficient of variation (CV) we carried out the following calculations:

-*Intraday and intraindividual variability*: CVs of the antioxidant capacity of each foodstuff fermented with each inoculum were first calculated separately for each inoculum/individual and then a mean of these CVs was performed. This CV represents the variability of fermenting food several times the same day using inoculum from the same person.

-*Intraday and interindividual variability*: we performed a CV among the antioxidant capacity values obtained with each inoculum and thereafter, a mean of these CVs was calculated expressing variability among individuals.

-*Interday and intraindividual variability*: we performed a mean of the CVs obtained each day (during five days) for each inoculum/individual obtaining a CV that says the variation of using faecal material from the same person during several days.

-*Interday and interindividual variability*: a CV of the antioxidant capacity values obtained with each inoculum along the five days was performed obtaining a CV that indicates the variability of using faecal material from different people in different days.

- *Effect of freezing storage of faecal samples*: The effect of freezing and storing on microbial activity during fermentation was also assessed. Fresh faeces from three healthy donors were aliquoted in eppendorf tubes, frozen and stored at -80ºC for 5, 8 and 11 days respectively. After the corresponding period, the faecal samples were defrosted at room temperature and the daily inocula were prepared. Peanuts and whole grain bread were fermented with the different inocula and the antioxidant capacity was measured by the FRAP method.

- *Use of lyophilised vs. non-lyophilised solid residue*: The effect of using as fermentation substrate either the digestion solid residue lyophilised or the solid residue obtained right after digestion (wet) was also studied. In both cases 10% of the supernatant was included. For this purpose, peanuts and whole grain bread were first digested and then fermented performing two experiments; in one lyophilized solid residue was used as fermentation substrate whereas in the other the one used was wet solid residue. After that, antioxidant capacity was measured by the FRAP method.

2.5. Antioxidant capacity

The antioxidant capacity of three fractions was assessed: the supernatant obtained after gastrointestinal digestion, the supernatant derived from fermentation and the solid residue remaining after fermentation. Five different methods were used to analyse the antioxidant capacity of foods. All antioxidant capacity values for all five methods were corrected taking into account their respective blanks (enzymes, chemicals and inoculum).

$TEAC_{OH}$ method: In this method, performed to unravel the scavenging activity against OH· radicals, carmin indigo was used as the redox indicator, following Pérez-Burillo et al. (*in press*). For this purpose, OH· radicals were generated by the Fenton reaction

using H_2O_2 and ferric ions. The assay was carried out using a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37ºC) and measuring the absorbance at 610 nm. Each well of the transparent 96-well polystyrene microplate (Biogen Científica, Spain) contained 15 μL of carmin indigo indicator (1 mM), 150 μL of phosphate buffer (0.5 M; pH 7.24), 30 μL of ferric chloride hexahydrate (0.1 μM) chelated with phosphotungstate (0.1 μM) in proportion 1:1 (v/v), 15 μL of freshly prepared H_2O_2 (4M) and 90 μL of sample or standard. Each assay was carried out with two blanks: one containing indicator and buffer and the other with indicator, buffer and the Fenton reagents. Absorbance was measured every 60 s in kinetic mode and the absorbance values used were those obtained at 80 minutes. The calibration curve was performed with Trolox ranging from 0.1 mg/mL to 10 mg/ml and the results obtained are expressed as μmol Trolox equivalents per mL of sample.

$TEAC_{AAPH}$ method: This method was performed to determine the scavenging activity against AAPH· radicals, and again carmin indigo was used as the redox indicator. The reagent solution contained 1.5 g of AAPH in 10 mL of distilled water. The assay was carried out using a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37ºC) and the absorbance was measured at 610 nm. Each well of the transparent 96-well polystyrene microplate (Biogen Científica, Spain) contained 40 μL of carmin indigo indicator (1 mM), 220 μL of phosphate buffer (75 mM; pH 7.24), 20 μL of AAPH solution and 20 μL of sample or standard. Each assay was carried out with two blanks: one containing indicator and buffer and the other with indicator, buffer and the AAPH solution. Absorbance was measured every 60 s in kinetic mode and the absorbance values used were those obtained after 65 minutes of reaction. The calibration curve was performed with Trolox ranging from 1.00 mg/mL to 0.01 mg/mL and the results obtained are expressed as μmol Trolox equivalents per mL of sample.

$GEAC_{RED}$ reducing capacity method: This method was developed to analyse the global reducing capacity of the samples, using carmin indigo as the redox indicator. The assay was carried out using a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37ºC) and measuring the absorbance at 400 nm. Each well of the transparent 96-well polystyrene microplate (Biogen Científica, Spain) contained 15 μL of carmin indigo indicator (1 mM), 265 μL of phosphate buffer (0.5 M; pH 7.24) and 20 μL of sample, although this volume could be increased if the samples presented very little reducing activity; in this case, the buffer volume was always reduced by the same quantity. Absorbance was measured every 60 s in kinetic mode and the absorbance values used were those obtained after 60 minutes of reaction. The calibration curve was performed with gallic acid ranging from 0.1 mg/mL to 50.0 mg/ml and the results obtained are expressed as μmol gallic acid equivalents per mL of sample.

$TEAC_{ABTS}$ assay: The antioxidant capacity was estimated in terms of radical scavenging activity, following the procedure described by Roberta, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans (1999). Briefly, ABTS was produced by reacting ABTS stock solution (7 mM) with potassium persulphate (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS solution was diluted with an ethanol: water (50:50) mixture to an absorbance of 0.70 ± 0.02 at 730 nm. After placing 20 μL of sample or Trolox standard and 280 μL of diluted ABTS solution on a transparent 96-well polystyrene microplate (Biogen Científica, Spain), absorbance readings were taken every 60 s for 20 minutes on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37ºC). Calibration was performed with a Trolox stock solution ranging from 0.01 to 1.00

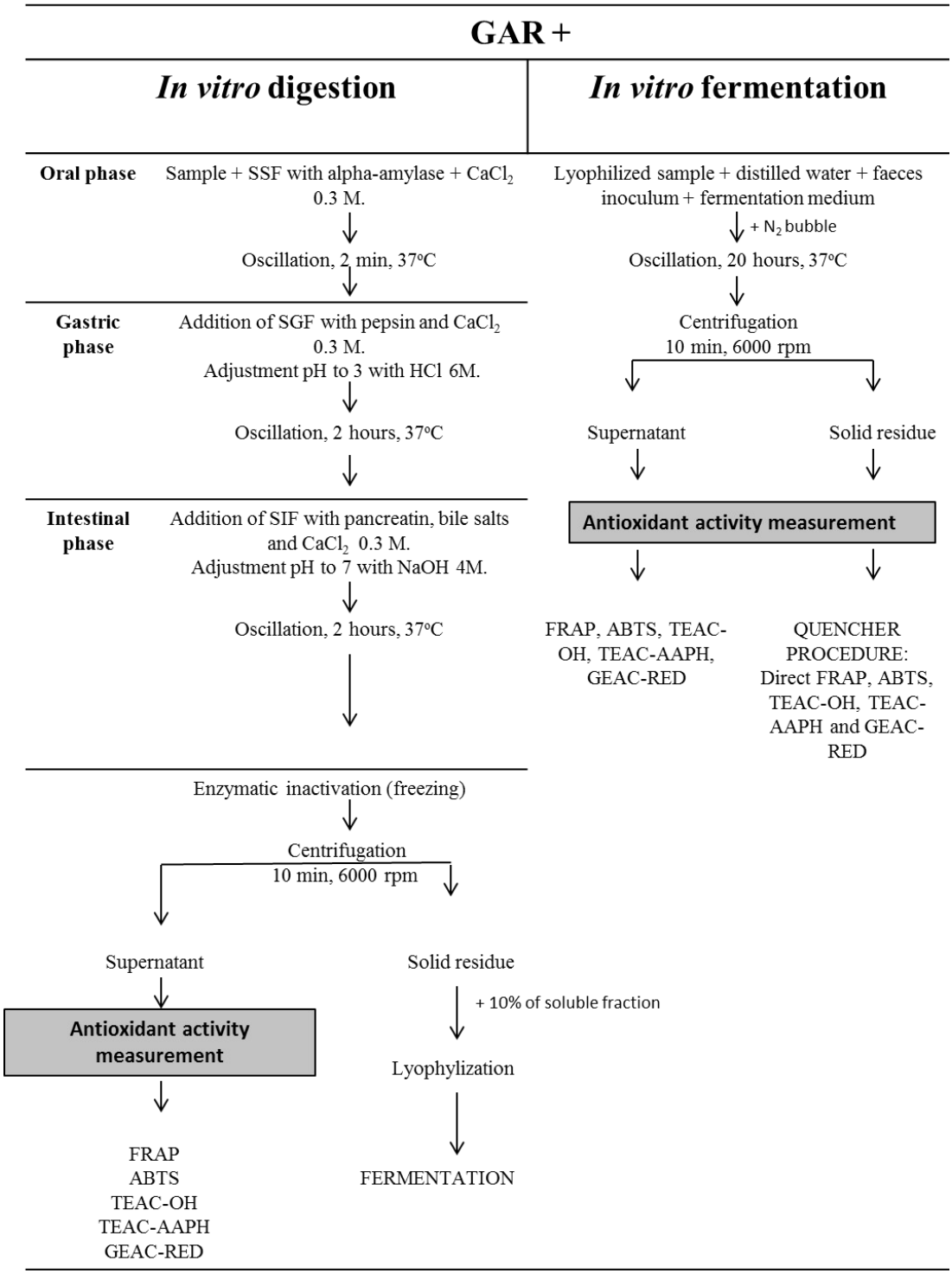
mg/mL. The results obtained are expressed as μmol Trolox equivalents per mL of sample.

$TEAC_{FRAP}$ assay: The ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie & Strain (1996) and adapted to a microplate reader. Briefly, 280 μL of FRAP reagent, prepared freshly and warmed at 37ºC, were mixed in each well of a transparent 96-well polystyrene microplate (Biogen Científica, Spain) with 20 μL of sample or water to provide appropriate blank reagent. The FRAP reagent contained 2.5 mL of a TPTZ solution (10 mM) in HCl (40 mM), plus 2.5 mL of $FeCl_3 \cdot 6H_20$ (20 mM), and 25 mL of acetate buffer (0.3 M; pH 3.6). Readings of maximum absorbance (595 nm) were taken every 60 s using a FLUOStar Omega microplate reader (BMG Labtech, Germany). The temperature was maintained at 37ºC and the reaction was monitored for 30 min. Trolox stock solutions ranging from 0.01 to 1.00 mg/mL were used to perform the calibration curves. The results obtained are expressed as μmol Trolox equivalents per mL of sample.

2.6. Statistical analysis

The statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($p<0.05$). All the statistical analyses were performed using Statgraphics Plus software, version 5.1.

Figure 24. Schematic description of the *in vitro* digestion and fermentation process.



3. Results and discussion

In order to develop a more physiological-resembling method to measure the antioxidant capacity of foods, we combined our GAR method for digestion with an *in vitro* fermentation step and our previously developed antioxidant methods (Perez-Burillo et al., in press). The first stage of this process involved studying different parameters of the fermentation step, such as the amount of sample and faecal inoculum, variability among faecal donors and samples, among others. The antioxidant capacity of different foods was then studied, both by well-established antioxidant methods and by the new approach presented in this paper.

3.1. In vitro fermentation: set up

3.1.1. Amount of sample and faecal inoculum

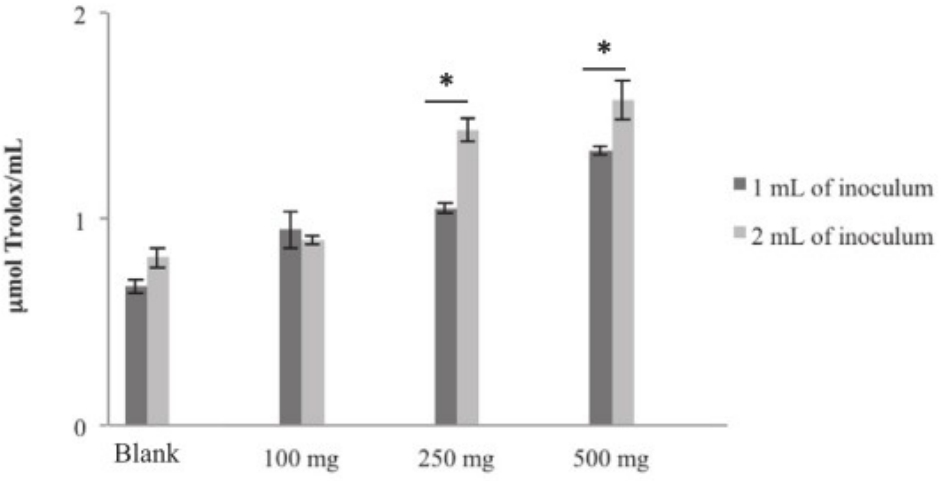
The *in vitro* fermentation step used in this study was based on the method described by (Ludwig et al., 2013) but extending the fermentation time to 20 hours in order to perform a 24-hour process, including the *in vitro* digestion of the GAR method. This modification was adopted because batch cultures need a long incubation time due to factors such as possible composition changes in the medium, or the production of microbial metabolites that could inhibit growth (Venema & van den Abbeele, 2013).

Subsequently, different amounts of sample and inoculum volumes were assessed in order to evaluate the amount of sample that the gut microbiota are able to assimilate. A further aim of this study was to determine whether a greater volume of inoculum raises the level of fermentative activity (and hence of antioxidant activity). Accordingly, 100, 250 and 500 mg of the solid residue obtained after the *in vitro* digestion of bread (with the corresponding supernatant volume) were added to 1 and 2 mL of faecal inoculum (obtained from the fresh, morning faeces of a healthy adult donor). As shown

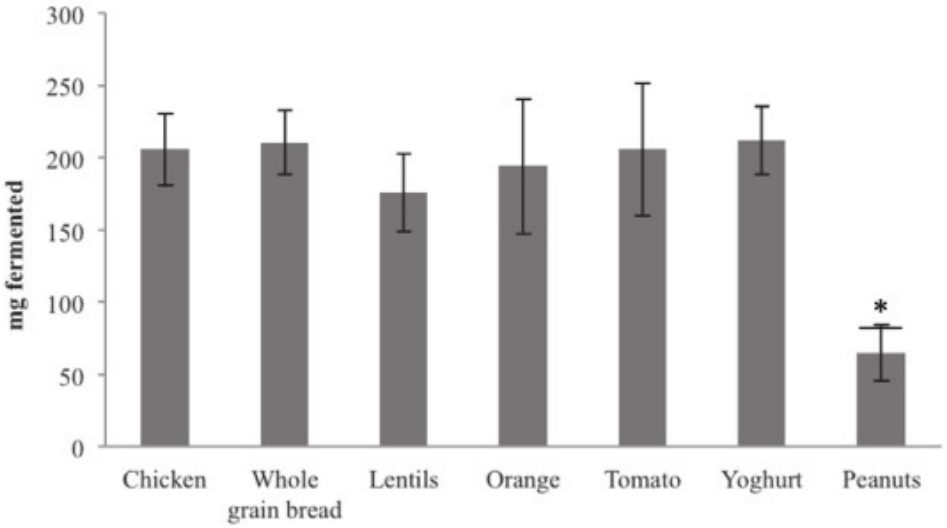
in **Figure 26A**, an increase in the amount of sample gave rise to higher antioxidant activity (p<0.05), according to measurement with the FRAP method, when 250 or 500 mg of inoculum were used for fermentation. Doubling the volume of inoculum also increased the antioxidant activity released due to greater fermentative activity. Finally, as depicted in **Figure 26B**, two thirds of the foods were effectively fermented, except in the case of peanuts, which presented a statistically lower amount fermented (p<0.05). With this trial (**Figure 26B**) we wanted to see gut microbiota behaviour against different food matrixes. As result we observed that protein rich food such as chicken or yoghurt as well as fibre rich food such as lentils, tomato or orange can be well fermented. The only exception was peanuts, which were fermented only a 10%. This could be due to their low water content making them more difficult to be attacked than other types of food. The amount of inoculum (2 mL) used was able to ferment around 200 mg of the sample except for peanuts. Since in most foods the maximum amount fermented is similar (not statistically significant differences), we can probably say that 200 mg is the limit that the amount of bacteria (added with 2 mL) can ferment. In view of these considerations, the final fermentation proportion adopted was 500 mg of digested sample + 2 mL of faecal inoculum due to: i) 500 mg is a representative amount of sample avoiding errors in this sense, ii) using 500 mg of sample allow us to digest only 5 g of food which is an affordable amount allowing us to process several samples in the same experiment, iii) with 500 mg, there is sufficient solid residue after fermentation allowing us to measure antioxidant capacity there. Regarding inoculum volume, we chose 2 mL because with this volume higher amount of sample is fermented so greater differences among them could be seen.

Figure 26. Effect of sample amount and inoculum on antioxidant activity released (26A) and final amount of fermented sample with the selected final proportion (26B).

26A



26B



3.1.2. Use of freeze-dried solid residue vs. wet solid residue

The next step in the development of the fermentation step was to assess the effect of using the solid residue as a freeze-dried powder (solid residue obtained after digestion + 10% of the supernatant) or the wet one directly obtained after digestion plus 10% of the supernatant. The results obtained with the FRAP method, after the digestion-fermentation of peanuts and whole grain bread, indicated a variability of 1.3% between the two methods (data not shown). Since the freeze-drying step takes longer than the humidity analysis of the wet residue obtained after digestion, and a freeze-drying system is not found in all laboratories, for further analysis it was decided to use the wet residue obtained after digestion.

3.1.3. Fermentation variability

We next studied the influence of inter- and intra-individual variability of the microbiota metabolic activity on food fermentation and, consequently, on the extraction of antioxidant compounds from food. To do so, the solid residue that remained insoluble after the *in vitro* digestion (plus 10% of the supernatant) of each food item (chicken, bread, lentils, orange, tomato, yoghurt, peanut) was fermented. Fermentation variability experiment was carried out as depicted in section 2.4 of materials and methods (**Figure 25**). The results obtained are depicted in **Table 8.**

Table 8. General variation coefficients (%) of the antioxidant activity (measured with the FRAP method) obtained after fermentation[1].

	Same day		Different day	
	Same inoculum	**Different inoculum**	**Same inoculum**	**Different inoculum**
Supernatant obtained after fermentation	4.0 ± 0.2	30.6 ± 9.2	30.2 ± 9.0	30.8 ± 9.7
Solid residue obtained after fermentation	7.4 ± 1.3	29.2 ± 7.6	17.5 ± 4.7	39.6 ± 8.3

[1] Each result is the mean value of 7 different foods, each one fermented in triplicate. The antioxidant activity is also measured in triplicate.

3.1.3.1. Fermentation variability of the supernatant

Intraindividual variability: Each of seven food items was subjected to fermentation seven times in the same day, using an inoculum provided by the same person. From this process, we calculated a mean variability of 3.95% for the seven foods analysed (7 foods x 7 times/each x 4 faecal inoculum/food); this value is the inherent variability of the method. Since the composition of gut microbiota and metabolic functionality are both known to vary from day to day (David et al., 2013), the above experiment was repeated on five consecutive days (7 foods x 7 times/each x 4 faecal inocula/food x 5 days) in order to determine the day-by-day variation (Figure 3), producing a variability of 30.2% (**Table 8**). This value could be related to changes in the diet consumed by the faecal donors, triggering variations in the microbiota metabolic pathways and thus in the compounds released during fermentation (Power et al., 2014). According to David et al. (2013), the composition of gut microbiota, at species-level, can change within 24 hours when a high-fibre diet is replaced by a high-protein diet. Changes in nutrient intake can also modify the gene expression modulated by microbiota, due to adaptive changes within the microbiota provoking alterations in

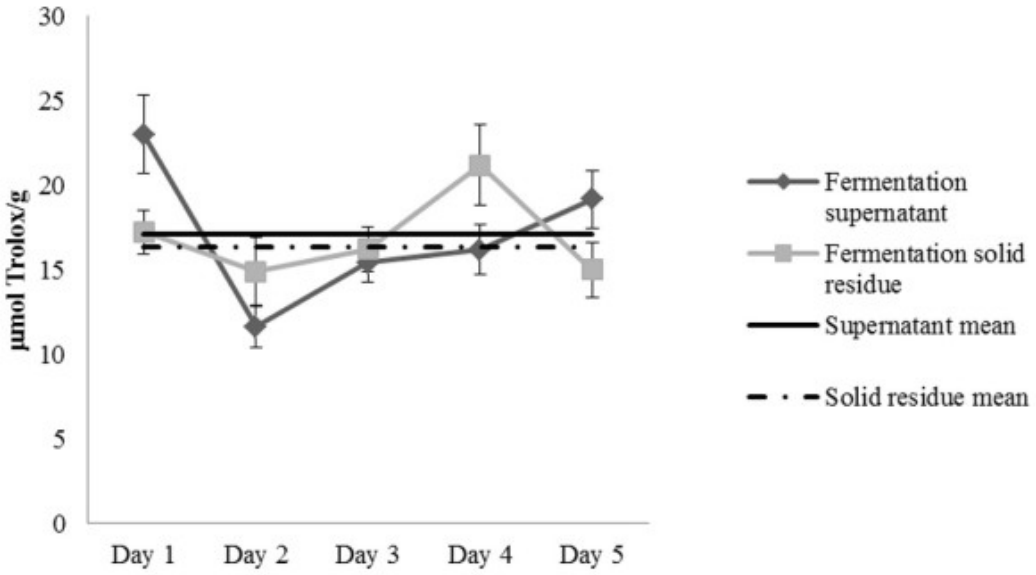
metabolic pathways. In this respect, the stability of microbial communities, within a healthy population, has been monitored in a long-term study (443 days) (Trosvik et al., 2014). These authors found that the main phylum (*Bacteroidetes*) is fairly stable over time, but further examination, at the order level (*Bacteroidales*), revealed two well-differentiated groups: one composed of relatively stable genera and the other composed of highly variable genera. The authors also described an abrupt and persistent change within the *Proteobacteria* phylum at around day 100, together with two spikes in the abundance of two different taxonomic classes belonging to the phylum *Tenericutes*. Moreover, microbial populations vary along the length of the small intestine, making it very improbable that bacterial communities would be evenly distributed within the volume of the faeces (Wang, Ahrna, Jeppsson, & Molin, 2005, Wesolowska-Andersen et al., 2014). This circumstance might not present any special difficulty in cross-sectional studies, but in longitudinal ones it could be an issue, requiring the homogenisation of the sample.

When each sample was fermented on the same day, including residues from foods of a different nature, the variability found using the same inoculum was 104%. This high value is due to the different nature and composition of the samples tested. Different substrates will yield different products; thus, the products released by microbiota from carbohydrate fermentation differ from those derived from proteolytic fermentation (Power et al., 2014). This variability is even higher when inter-day variation is taken into account (111%).

Interindividual variability: Next, we studied the effects produced by faecal inocula from three different individuals, considered both individually and jointly. For the faecal samples as a whole, a variability of 30.6% was obtained (**Table 8**). This significant variability could be due to the considerable variation among gut microbiota

obtained from different subjects, although it is not statistically different (p>0.05) from the CV obtained for the foods that were fermented on five different days. The gut microbiota are known to be quite variable among different individuals (Schloissnig et al., 2012). For instance, the metabolism of phenolic compounds by the gut microbiota in human beings varies notably among individuals (Selma et al., 2009). In this respect, the latter authors observe that equol production from isoflavones such as daidzein is related to specific bacteria that are only present in some individuals. Furthermore, the faecal donors in our study did not follow the same diet (the volunteers freely choose their diet), and this question, too, can significantly influence the behaviour of the gut microbiota (Power et al., 2014). The variability found was higher in protein-rich foods (28.8% ± 4.3) than in fibre-rich ones such as whole grain bread or lentils (20.0% ± 5.9). Because of this high inter-individual variability, the faecal inoculum used must comprise a mixture, obtained from different people, in order to obtain more accurate, applicable results. When the experiment was repeated on five consecutive days, almost the same CV was obtained (30.8%). These results reinforce the idea that variations in the fermentative process are related to changes in the activity of the gut microbiota of each donor, which in turn resemble those found among different faecal donors.

Figure 27. Daily antioxidant capacity evolution (FRAP method) over five days using the same faecal inoculum.



3.1.3.2. Fermentation variability of the solid residue

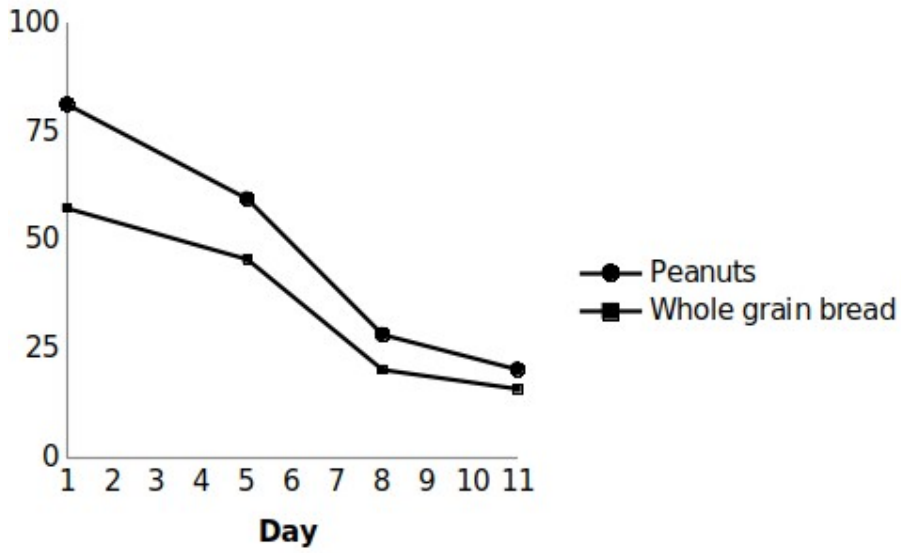
Fermentation of the solid residue obtained after the *in vitro* digestion and fermentation of each foodstuff, performed 7 times in the same day, using the inoculum from the same persons (three inocula from three healthy donors and a fourth consisting of a mix of the three faeces) gave a mean variability of 7.42% (**Table 8**). This variability represents the inherent variability of the method. This CV was slightly higher than that of the supernatant, a difference probably related to the amount and chemical nature of the insoluble non-fermentable compounds that formed part of this solid residue, which in turn could display a broader antioxidant activity depending on the food composition, while the compounds released to the supernatant after fermentation could present greater similarity. The use of a different inoculum or the repetition of the fermentation process on five consecutive days increased the variability from 17.5% to 39.6%. The reasons for this increase are similar to those for the supernatant, concerning

mainly the differing compositions of the gut microbiota and different types of biochemical activity.

3.1.3.3. Effect of freezing and storage of faecal samples

Taking into account the need to obtain repeatable results from the *in vitro* fermentation, the above results clearly show that a fresh faecal inoculum must be used, composed of faeces obtained from different, healthy donors on the same day. In the next stage of this analysis, we assessed the effect of freezing and storage on microbial activity related to the release of antioxidant compounds, after the fermentation of peanuts and whole grain bread. As can be seen in **Figure 28**, the antioxidant capacity released from the frozen inocula decreased in a linear fashion over time. This outcome could be related to a diminished viability of certain bacterial taxa when frozen, which in turn alters the fermentative activity on foods. We conclude, therefore, that the use of a frozen inoculum is not a good alternative, as regards achieving repeatability.

Figure 28. Effect of frozen inoculum over the release of antioxidant capacity of peanuts and whole grain bread ($TEAC_{FRAP}$ method). Results expressed as mM.



3.2. Antioxidant capacity

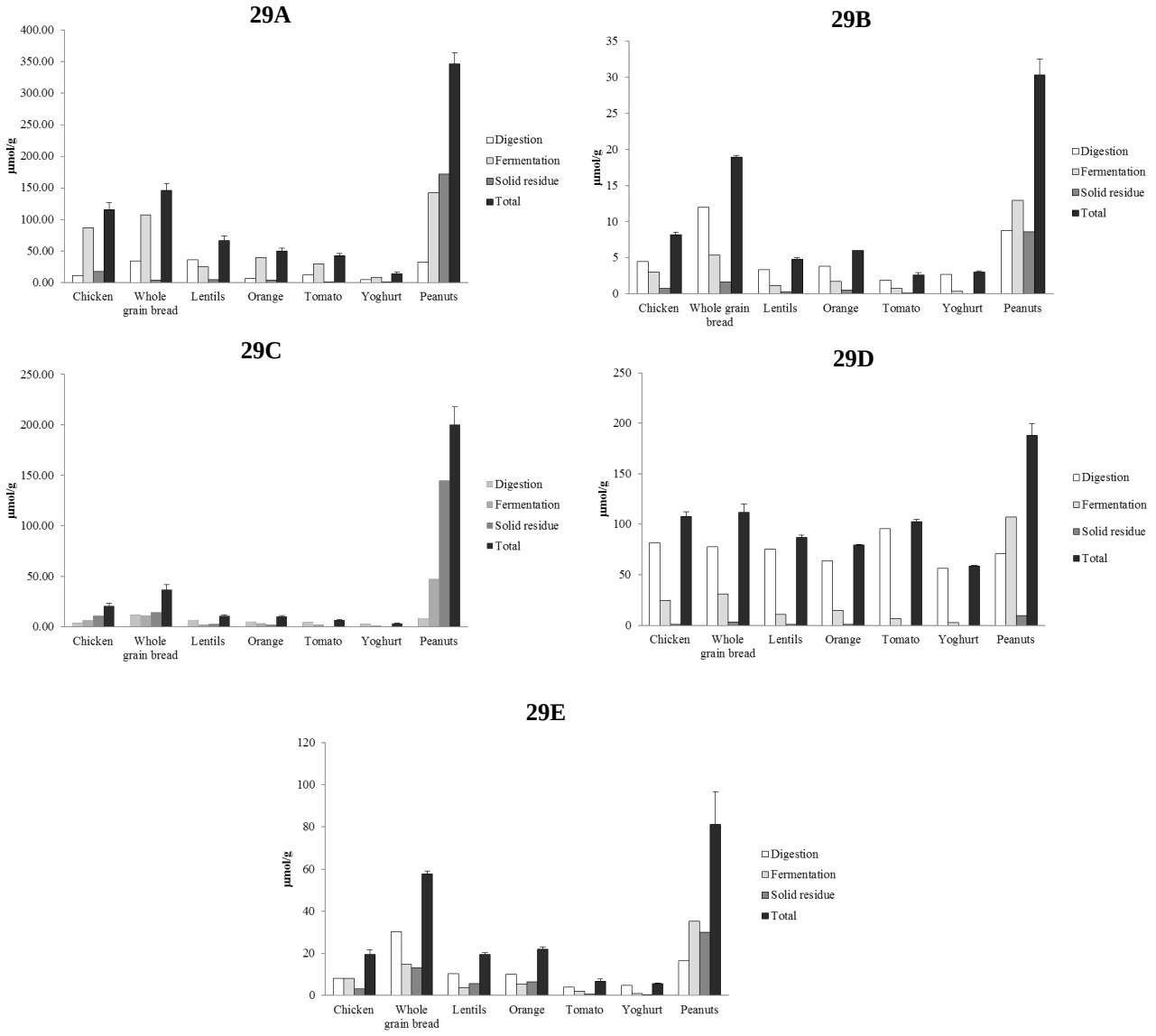
The antioxidant capacity of foods is determined by that of many different substances that are released during the digestion process. Moreover, the digestion residue that is left after absorption in the large intestine undergoes a fermentation process carried out by the gut microbiota. *In vitro* digestion achieves the enzymatic hydrolysis of macronutrients present in the food, releasing many small molecules. Consequently, *in vitro* fermentation yields many different chemical species derived from the metabolization of proteins, carbohydrates and phenolic compounds by the gut microbiota. The latter compounds play an essential role in antioxidant activity in the human body. Thus, the *in vitro* fermentation step is essential to our analysis, since the solid residue of food (fibre), non-digested proteins or lipids and phenolic compounds all reach the small intestine, where they are modified to some extent by the gut microbiota (Power et al., 2014). Vegetables, which are rich in polysaccharides that cannot be hydrolysed by mammalian enzymes, yield molecules related to carbohydrate fermentation when they are attacked by the gut microbiota. On the other hand, protein-rich foods supply the gut microbiota with proteins (such as collagen or elastin, which are chemically modified by heat treatment during cooking) that are fermented by proteolytic microorganisms and which yield thiols or indols. Phenolic compounds are also metabolised by the gut microbiota, resulting in the formation of many different metabolites. Even complex tannins are degraded by the gut microbiota, releasing smaller phenolic compounds which obviously would increase the antioxidant activity (Selma et al., 2009). In short, the antioxidant capacity of any food results from the total activity of all these molecules that are released during digestion and fermentation, although some antioxidant capacity is lost with the residue expelled with faeces.

3.2.1. Antioxidant capacity obtained with the GAR+ method

GAR+ measures the global antioxidant capacity of three different food fractions: the supernatant obtained after digestion (potentially bioaccessible), the supernatant obtained after fermentation (potentially bioaccessible) and the solid residue remaining after fermentation (antioxidant activity lost with the faeces). This physiological-resembling approach provides more accurate data about the antioxidant capacity of foods than the GAR method alone. Global antioxidant capacity of each food would be the sum of these three values. The global antioxidant capacity results determined by each method are shown in **Table 9** and **Figure 29**. The $TEAC_{OH}$ method measures the antioxidant capacity of foods against hydroxyl radicals, and produced results ranging from 14 to 347 μmol of Trolox per g of sample for yoghurt and peanuts, respectively. The range of antioxidant capacity was as follows: peanuts >> whole grain bread > chicken >> lentils > orange > tomato >> yoghurt. It should be emphasised that chicken was more active against hydroxyl radicals than some vegetables, such as lentils, orange and tomato. Pastoriza et al. (2011) reported the high antioxidant activity of protein-rich foods (grilled beef steak or fried sausages) compared with certain vegetables (boiled red beans, steamed spinach or fresh carrots, among others). The antioxidant capacity of the supernatant obtained after the digestion of chicken was higher than that for orange or tomato, but lower than that for lentils (**Figure 29**). However, the fermentation by gut microbiota is a significant factor, since proteolytic fermentation could release compounds with strong antioxidant activity such as thiols and indols. In addition, the chicken was cooked with spices (curry and olive oil), which increased its content in phenolic compounds that enhance the antioxidant power of chicken. Thus, although the digestion process accounted for 54.1% of the antioxidant capacity in lentils and for 9.4% of that in peanuts, most of the antioxidant capacity of the foods in our analysis

was released by gut microbiota fermentation, which reinforces the idea that microbial metabolism releases compounds with strong activity against hydroxyl radicals. Thus, in our study, peanuts, whole grain bread and chicken released a large amount of antioxidant capacity in the supernatant obtained after fermentation, which is potentially bioaccessible (**Figure 25**). The percentage of antioxidant capacity lost with the solid residue ranged from 1.6% to 49.5% for tomato and peanuts, respectively. Although considerable antioxidant capacity may be excreted with the faeces, this action may at the same time counteract the formation of radicals during the fermentation process, thus protecting epithelial cells in the colon.

Figure 29. Antioxidant capacity of foods fractions (29A: $TEAC_{OH}$; 29B: $TEAC_{AAPH}$; 29C: $GEAC_{RED}$; 29D: $TEAC_{ABTS}$; 29E: $TEAC_{FRAP}$).



Regarding the $TEAC_{AAPH}$ method, which measures the reactivity of samples against peroxyl radicals, the global antioxidant capacity ranged from 2.6 to 30.3 μmol Trolox per g of sample for tomato and peanuts, respectively (**Table 9**). The antioxidant activity was much higher for peanuts and whole grain bread, while the other foods presented similar levels of

activity: peanuts > whole grain bread >> chicken > orange > lentils > yoghurt = tomato. As in the case of the TEAC-OH method, the antioxidant capacity of chicken was higher than that of many vegetables. The digestion process contributed to the global antioxidant capacity with percentages ranging from 28.9% for peanuts to 88.7% for yoghurt (**Figure 29**) while the supernatant obtained after fermentation produced values ranging from 11.3% to 42.7% for yoghurt and peanuts, respectively. Again, peanuts seem to be less digestible than foods like yoghurt, although the overall contribution of the supernatant obtained after digestion was higher than that obtained for the TEAC-OH method (**Figure 29**). The values obtained for yoghurt are readily comprehensible since this food is easily digested and most of its antioxidant compounds would be absorbed in the large intestine (Gaudichon et al., 1995). Finally, the percentage of antioxidant activity released with the faeces was negligible for yoghurt but rose to 28.4% for peanuts.

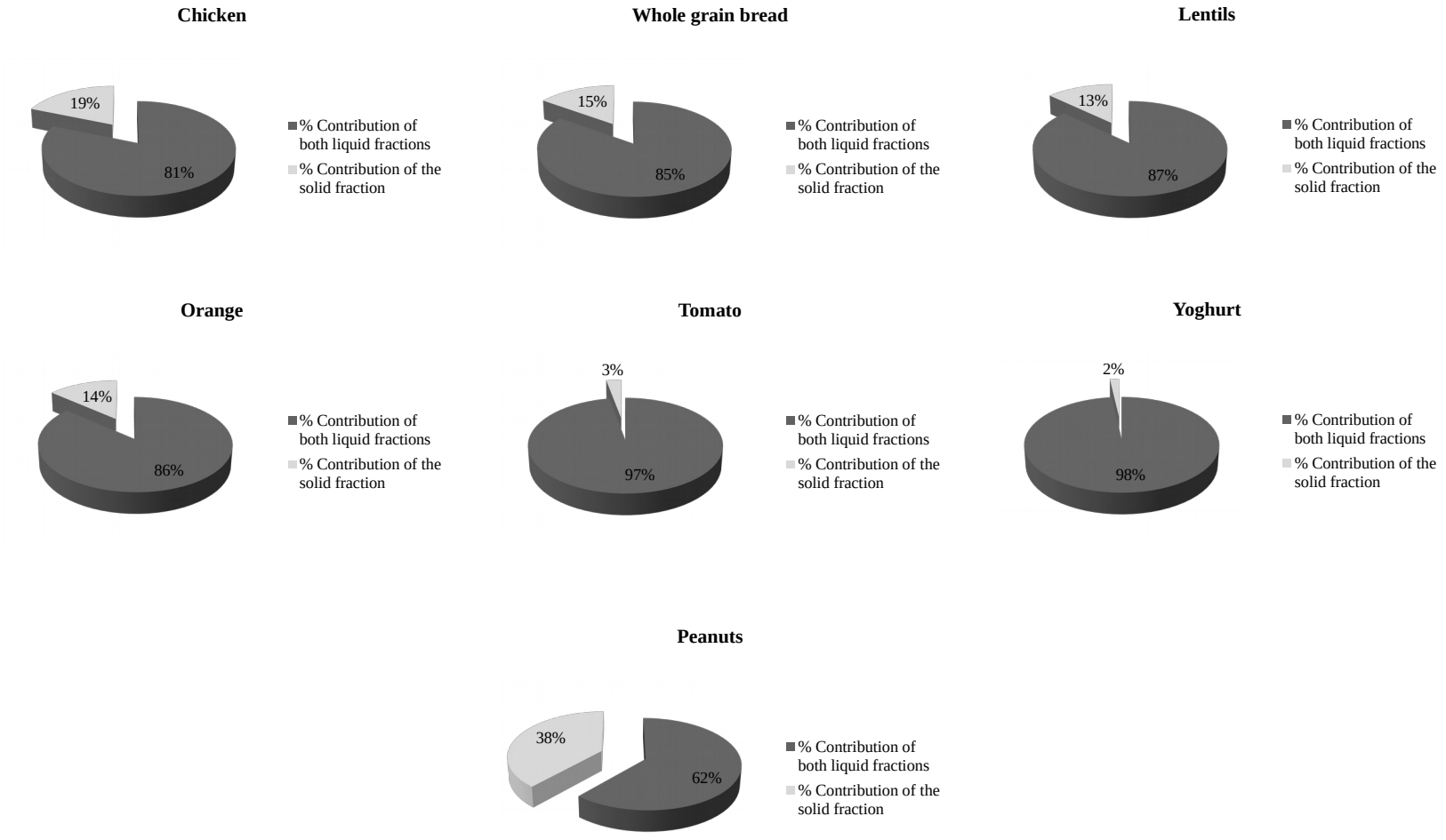
The $GEAC_{RED}$ assay measures the overall reducing capability of the food analysed. The results obtained ranged from 3.4 to 200 μmol of gallic acid per g of sample for yoghurt and peanuts, respectively (**Table 9**). The order in reducing capability was similar to that obtained for the TEAC-OH and TEAC-AAPH methods: peanuts >>> whole grain bread >> chicken > lentils = orange > tomato = yoghurt. The supernatant obtained after digestion contributed more to the reducing capacity than did the supernatant after fermentation, which is in line with the results of the TEAC-AAPH method but contrasts with those of the TEAC-OH assay (**Figure 25**). Thus, the values obtained for digestion ranged from 4.2% to 72.0% for peanuts and yoghurt, respectively, while the supernatant released after fermentation ranged from 17.7% to 30.4% for lentils and tomato, respectively. In addition, the reducing capacity released in the solid residue after fermentation for peanuts was the highest (72.3%) obtained with the three physiological-resembling antioxidant methods assayed (**Figures 25A to 25C**).

The antioxidant capacity of foods was also assessed by the ABTS and FRAP methods. Although these are not conducted under physiological-resembling conditions, they are very commonly used and thus provide a suitable basis for comparison with the results of other authors. With the $TEAC_{ABTS}$ method, the antioxidant capacity ranged from 59 to 188 μmol Trolox per g of sample for yoghurt and peanuts, respectively (**Table 9**). These values are 5-30 times higher than those reported by other authors (**Table 10**), a discrepancy that might be related to the use of less efficient extraction methods (chemical extraction or the GAR method) to detach the antioxidant compounds from the food matrix. Again, the order of antiradical activity was similar to that found with the other methods, although no statistically significant differences (p>0.05) were obtained for whole grain bread, chicken or tomato: peanuts >> whole grain bread = chicken = tomato > lentils = orange > yoghurt. We stress that most of the antioxidant capacity was released for all the samples during the digestion process (37.8% to 95.8% for peanuts and yoghurt, respectively) compared with the previous methods (**Figure 29D**). This could mean that most of the compounds released after digestion are more reactive against the ABTS radical than hydroxyl or peroxyl radicals, which in turn could overestimate the antioxidant power of foods if measured with the ABTS reagent. The supernatant obtained after fermentation also presented a high level of activity against the ABTS radical (ranging from 57.0% to 4.2% for peanuts and yoghurt, respectively), leaving less than 5% of antioxidant capacity accounted for by the solid residue (**Figure 29D**). This contrast is explicable in view of the high reactivity of the blanks (digestion enzymes and faecal inoculum) against the ABTS reagent. This explanation reinforces the idea that the ABTS method is not relevant under physiological conditions, since many compounds react with the ABTS radical, which, however, does not react with physiologically important radicals (like hydroxyl or peroxyl radicals).

The $TEAC_{FRAP}$ method presented a similar pattern of results to the other methods, with peanuts providing the most antioxidant sample (81 μmol Trolox per g of sample) and yoghurt the least (6 μmol Trolox per g of sample) (**Table 9**). These values are 5-10 times higher than those reported by other authors (**Table 10**), possibly due to the use of different extraction methods (chemical extraction or the GAR method) or even a different means of calculating the reducing capacity of the FRAP method (expressed in mmol Fe^{2+}/ Kg). These differences in approach make it difficult to draw clear conclusions from the comparison. The highest values were found in peanuts, whole grain bread, lentils and orange. Digestion contributed 20-85% (peanuts and yoghurt, respectively) of the global reducing capacity of the samples, while fermentation accounted for 42% to 14% of the capacity, for the same foods. The contribution of the solid excreted residue ranged from 1% to 37% for yoghurt and peanuts, respectively.

Finally, the contribution of the different food fractions (supernatant *vs.* solid residue) to the overall antioxidant capacity was compared (**Figure 30**). The mean antioxidant capacity of the supernatants (obtained after digestion and fermentation) was 85.2% ± 12.2, ranging from 61.6% to 97.1% for peanuts and tomato, respectively. In the solid residue, the mean contribution to the antioxidant capacity was 14.8% ± 12.1, ranging from 1.8% to 38.4% for yoghurt and peanuts, respectively. The antioxidant capacity of the supernatant was statistically higher ($p<0.05$) than that of the solid residue, an indication that irrespective of the foodstuff, the amount of antioxidant compounds potentially absorbed is higher than that remaining in the solid residue.

Figure 30. Contribution of liquid and solid fractions to the antioxidant capacity of foods.



3.2.2. Comparison of extraction methods

In order to obtain reliable values for the antioxidant capacity of foods, we must use both physiologically-important radicals as antioxidant probes and also appropriate methods with which to extract the antioxidant compounds. Most researchers in this field use chemical extraction methods (aqueous-organic solvents) to isolate antioxidant chemical species from the food matrix (Ou et al., 2002, Michalska et al., 2007, Han & Baik, 2008). However, the insoluble part or solid residue is systematically discarded and these methods fail to take into account many molecules that are released, and others that may be modified, during the digestion-fermentation process. Some authors have evaluated the antioxidant capacity of foods without performing the extraction step, and this omission means that, again, the physical and chemical changes produced during digestion are not taken into account (Gökmen et al., 2009). In view of these considerations, we decided to compare the results obtained with the GAR+ method with

Table 9. Antioxidant capacity of foods.

Sample	TEAC-OH[2]	TEAC-AAPH[2]	GEAC-RED[1]	$TEAC_{ABTS}$[2]	$TEAC_{FRAP}$[2]
Chicken	115 ± 11^{a}	8.2 ± 0.3^{a}	21 ± 3.0^{a}	108 ± 5.1^{a}	19 ± 2.3^{a}
Whole grain bread	146 ± 11^{b}	19.0 ± 0.3^{b}	37 ± 5.2^{b}	112 ± 8.3^{ab}	58 ± 1.1^{b}
Lentils	66 ± 8^{c}	4.8 ± 0.3^{c}	11 ± 0.7^{c}	87 ± 2.0^{c}	20 ± 1.7^{c}
Orange	50 ± 4^{d}	6.0 ± 0.0^{d}	10 ± 1.0^{c}	80 ± 3.1^{c}	22 ± 1.6^{c}
Tomato	43 ± 4^{d}	2.6 ± 0.4^{e}	6.5 ± 0.4^{d}	103 ± 3.6^{a}	7.5 ± 1.2^{d}
Yoghurt	14 ± 3.1^{e}	3.0 ± 0.1^{e}	3.4 ± 0.1^{e}	59 ± 1.1^{d}	6.6 ± 0.6^{d}
Peanuts	347 ± 17^{f}	30.3 ± 2.2^{f}	200 ± 18^{f}	188 ± 12^{e}	81 ± 16^{e}

[1] Results expressed as μmol of gallic acid per g of fresh sample.
[2] Results expressed as μmol of Trolox per g of fresh sample.
Different letters within the same column indicate statistically significant differences ($P < 0.05$).

Table 10. Comparison of antioxidant capacity (ABTS and FRAP methods) depending on the extraction method.

	$TEAC_{ABTS}$[e]				$TEAC_{FRAP}$[e]			
Sample	**GAR+**	**GAR**	**Chemical extraction**	**QUENCHER**	**GAR+**	**GAR**	**Chemical extraction**	**QUENCHER**
Chicken	-	20-25[i]	-	-	-	2.5-3[i]	-	-
Whole grain bread	-	2.6[h]**	0.3[h]**	-	-	64[h]***	12.1[h]***	-
Lentils	-	-	-	-	-	-	9.2[f]*	-
Orange	-	-	8.8[e]	-	-	-	20.5[e]*	-
Tomato	-	174[j]****	1.7[e]	-	-	-	5.1[e]*, 1.6[f]*, 5.9[f]*	-
Yoghurt	-	-	-	-	-	-	1.0[g]*	-
Peanuts	-	-	-	-	-	-	-	-

[a] Data obtained from (1)
[b] Data obtained from (2)
[c] Data obtained from (3)
[d] Data obtained from (4)
[e] Data obtained from (5)
[f] Data obtained from (6)
[g] Data obtained from (7)
[h] Data obtained from (8)
[I] Data obtained from (9)
[j] Data obtained from (10)
(-) Data not available
*mmol Fe2+/ Kg
**μg TE/ dry weight
***μg TE/ dray weight
****μg TE/ dray weight as a mean of three different cultivars

those derived by three other techniques for extracting antioxidant compounds: the GAR method developed by Pastoriza et al. (2011) chemical extraction, and the QUENCHER method (**Table 11**). In all three cases, the antioxidant capacity was assessed by ABTS and FRAP.

The results obtained with GAR+ for the $TEAC_{ABTS}$ method were very similar to those obtained by GAR (Pastoriza et al., 2011). However, in the cases of chicken and peanuts, the antioxidant capacity obtained with GAR+ was 2-3 times higher than that obtained by the GAR method. The reason for the higher antioxidant capacity determined by GAR+ could be that during the fermentation of chicken and peanuts, a large number of antioxidant chemical species are released (**Figure 29D**), which are not analysed by GAR. In the chemical extraction, the results obtained by GAR+ were much higher (10-500 times higher) than those previously obtained (**Table 11**) by other authors (Marathe, Rajalakshmi, Jamdar, & Sharma, 2011, Pastoriza et al., 2011, Gironés-Vilaplana, Moreno, & García-Viguera, 2014, and (Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & de la Serrana, 2015). It is clear that enzymatic digestion plus fermentation generates many changes in the food ingested. During digestion, starch and glycogen are hydrolysed, together with proteins, lipids and other compounds that reach the stomach and the large intestine. This process yields smaller molecules, and some new ones also appear as a result of the hydrolysis. Moreover, when the non-digested residue reaches the small intestine, gut microbiota feed on it, generating an even more complex pool of new substances, which further increases the antioxidant activity, and has been demonstrated to play an important role during fermentation (**Figure 29**). This plethora of new compounds is not taken into account when measuring the enriched fractions obtained after a chemical extraction,

which only addresses the soluble compounds already present in food. The QUENCHER method does take into account the solid residue, but is only able to measure the compounds already present in food. Moreover, it does not take into account the new chemical species formed after digestion-fermentation. **Table 11** shows that only the results found for whole grain bread with the QUENCHER method approached, but were still lower than, those found with the GAR+ assay (Pastoriza et al., 2011). The reasons for these differences could lie in the effect of digestion-fermentation: i.e. the fact that many different chemical species not present in the original food are released, and many others are generated.

With the $TEAC_{FRAP}$ method, the results found by GAR+ were – for whole grain bread, lentils, peanuts and orange – at least twice as high as those previously reported by the GAR method (Pastoriza et al., 2011). This difference could be related to the large amount of compounds that are released by the gut microbiota during fermentation, which is a step not included in the GAR method. Regarding the chemical extraction, the same behaviour was observed as with the ABTS method: a much greater antioxidant capacity is recorded by the GAR+ method, probably due to the metabolic activity of the gut microbiota. With respect to the QUENCHER method, up to 30-times higher values were obtained with GAR+, which we assume is due to the presence of new chemical species generated during the processes of digestion and fermentation.

Table 11. Antioxidant capacity of foods (μmol Trolox per g of fresh sample) obtained with four different extraction methods.

	$TEAC_{ABTS}$[e]				$TEAC_{FRAP}$[e]			
Sample	**GAR+**	**GAR**	**Chemical extraction**	**QUENCHER**	**GAR+**	**GAR**	**Chemical extraction**	**QUENCHER**
Chicken	108 ± 5	35 ± 3[a]	3 ± 1[a]	7 ± 1[a]	19 ± 2	27 ± 3[a]	4 ± 1[a]	5 ± 1[a]
Whole grain bread	112 ± 8	109 ± 3[a]	7 ± 0[a]	83 ± 3[a]	58 ± 1	19 ± 2[a]	6 ± 0[a]	12 ± 1[a]
Lentils	87 ± 2	105 ± 10[a]	7 ± 0[c]	11 ± 1[a]	20 ± 1	11 ± 1[a]	1 ± 0[a]	1 ± 0[a]
Orange	80 ± 0	-	10 ± 1[b]	-	22 ± 1	-	12 ± 1[b]	-
Tomato	103 ± 3	99 ± 7[a]	4 ± 0[d]	10 ± 1[a]	7 ± 1	7 ± 1[a]	1 ± 0[d]	0 ± 0[a]
Yoghurt	59 ± 1	65 ± 5[a]	0.1 ± 0.1[a]	2 ± 0[a]	6 ± 0	15 ± 2[a]	1 ± 0[a]	0 ± 0[a]
Peanuts	188 ± 12	100 ± 9[a]	12 ± 1[c]	65 ± 5[a]	81 ± 16	37 ± 3[a]	3 ± 1[a]	3 ± 1[a]

[a](Pastoriza et al., 2011)
[b](Gironés-Vilaplana et al., 2014)
[c] Data obtained from (Marathe et al., 2011)
[d] Data obtained from (Ramírez-Anaya et al., 2015)
(-) Data not available

3.2.3. Antioxidant intake

The antioxidant capacity of any food is derived from the synergetic effect of many of its constituents, such as phenolic compounds, Maillard reaction products, vitamins and the products of enzymatic digestion or of fermentation by gut microbiota. Thus, dietary antioxidant capacity is the total antioxidant capacity of all food and beverages consumed daily (Saura-Calixto, Pérez-Jiménez, & Goñi, 2009). Therefore, the amount of antioxidant units (either Trolox or gallic acid equivalent, depending on the method) that are ingested daily and, in theory, are available to exert their activity inside the human body, can be calculated. In our study, the antioxidant intake was calculated using two different approaches: first, by estimating the amount of antioxidant capacity consumed per day and per person. To do so, the average food consumption in Spain by the adult population, obtained from the annual survey carried out by the Spanish Ministry of Agriculture, Food and the Environment (MAGRAMA, 2015) was taken into account. In the second approach, the amount of antioxidant units per serving is calculated, taking as the usual serving size that commonly found in Spain (Carbajal & Sánchez-Muniz, 2003).

The results obtained for the daily intake of antioxidant capacity are shown in **Table 12**. These values were calculated excluding the antioxidant capacity of the solid residue released after fermentation, since it is assumed to be excreted together with the faeces. The highest contribution to the diet came from oranges, according to almost all the methods assayed, due to their high level of consumption in Spain (56 g/day) and relatively high antioxidant capacity. Chicken is also an interesting food, since it provides a large intake of antioxidant compounds, thanks to high levels of consumption and of antioxidant activity, according to all analytic methods. On the contrary, the lowest values were obtained for peanuts and lentils, due to their low levels of

consumption, although their antioxidant activity is quite high (especially in the case of peanuts). If all the foods studied are included in the daily diet, the antioxidant capacity intake ranges from 976 to 16116 μmol Trolox/day or 19452-34067 μmol gallic acid/day. Saura-Calixto et al. (2009) reported that the mean intake of antioxidant capacity in the Spanish diet was 3459 μmol Trolox/day according to the ABTS assay and 6014 μmol Trolox/day by the FRAP method. In our case, the daily intake calculated with the GAR+ method, for only seven foods, was 16116 and 2344 μmol Trolox/day by the ABTS and FRAP methods, respectively. The daily antioxidant capacity calculated by the FRAP method is only 39% of that reported by Saura-Calixto et al. (2009) but the ABTS assay is 466% of the latter value. This high contribution, by only seven foods, could be explained by reference to the chemical extraction method used by Saura-Calixto et al. (2009). For example, Pastoriza et al. (2011) calculated a daily antioxidant intake of 7327 and 1161 μmol Trolox/day for the ABTS and FRAP methods, which are considerably lower than those we obtained, due to the absence of the fermentation step from the GAR method.

The results for antioxidant intake per serving are shown in **Table 13**. The foods that contributed most in this respect, in an average diet, were chicken, whole grain bread and tomatoes, due to their high antioxidant capacity and large serving size (in the case of chicken). The lowest values were obtained for peanuts, yoghurt and lentils, due to their modest serving size and limited antioxidant capacity. In general, according to both ABTS and FRAP, the antioxidant capacity per serving is higher with the GAR+ method than with GAR. Again, this difference is related to the absence of a fermentation step in the GAR method, a step that releases chemical species that are not present in the solid residue obtained after digestion.

Table 12. Intake of antioxidant capacity per person/day.[1]

	Intake	$TEAC_{OH}$	$TEAC_{AAPH}$	$GEAC_{RED}$	$TEAC_{ABTS}$	$TEAC_{FRAP}$
Food	(g/inh/day)	(μmol/day)	(μmol/day)	(μmol/day)	(μmol/day)	(μmol/day)
Chicken	38.82	3814	290	380	4130	629
Whole grain bread	7.15	1016	124	160	778	320
Lentils	2.55	156	12	22	219	35
Orange	56.05	2598	309	444	4404	867
Tomato	39.18	1641	102	246	4015	232
Yoghurt	42.05	560	127	140	2472	233
Peanuts	0.55	96	12	30	98	28
Whole diet	-	**9882**	**976**	**1422**	**16116**	**2344**

[1] Units referred to Trolox for TEAC-OH, TEAC-AAPH, $TEAC_{ABTS}$ and $TEAC_{FRAP}$. Units referred to gallic acid for GEAC-RED. Calculations were performed excluding the solid fraction obtained after fermentation.

Table 13. Intake of antioxidant capacity per serving.[1]

	Serving size	$TEAC_{OH}$	$TEAC_{AAPH}$	$GEAC_{RED}$	$TEAC_{ABTS}$	$TEAC_{FRAP}$
Food	(g)	μmol/portion	μmol/portion	μmol/portion	μmol/portion	μmol/portion
Chicken	125	12282	935	1224	13299	2022
Whole grain bread	60	8531	1039	1342	6523	2689
Lentils	70	4285	315	600	6003	974
Orange	150	6953	827	1187	11784	2316
Tomato	180	7539	467	1129	18445	1065
Yoghurt	125	3329	378	834	7347	693
Peanuts	20	3503	435	1110	3566	1028

[1] Units referred to Trolox for TEAC-OH, TEAC-AAPH, TEACABTS and TEACFRAP. Units referred to gallic acid for GEAC-RED. Calculations were performed excluding the solid fraction obtained after fermentation

4. Conclusions

This paper presents a new model to assess the *in vitro* antioxidant capacity of foods. This model has two novel features: i) a digestion-fermentation step to extract relevant antioxidant chemical species from the food matrix; ii) physiological-resembling antioxidant methods that better represent what actually takes place within the human body. The enzymatic hydrolysis of macronutrients allows the release of many low molecular weight substances that can be absorbed in the upper gastrointestinal tract. Moreover, the fermentation carried out by the gut microbiota yields a plethora of small molecules derived from non-digested macromolecules such as polysaccharides, proteins and tannins. The GAR+ method takes into account all the phases experienced by ingested food, from intake until it has been transformed into a residue, and therefore provides a very useful means of measuring the biologic activities derived from the food, such as antioxidant, antihypertensive and prebiotic processes. However, due to the natural variation found among individuals during fermentation, a pool of faeces (from different healthy donors) should be used in the assay in order to obtain more accurate and translatable results to the general population.

Regarding antioxidant capacity, enzymatic digestion released most of the antioxidant power present in the foods, according to most of the methods tested. However, in the case of hydroxyl scavenging activity, most antioxidant capacity was exerted by the supernatant released after fermentation. These findings could mean that the majority of anti-hydroxyl activity is exerted by phenolic metabolites derived from gut microbiota activity. Further research in this respect is needed to determine the real contribution of gut microbiota to antioxidant capacity and how variations in gut microbiota (both among individuals and within the same person over time) can affect its health-giving properties.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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How brewing parameters affect the healthy profile of tea

Uno de los objetivos principales de la presente tesis doctoral es la evaluación de la capacidad antioxidante de los componentes de la dieta española. En este review se evalúan los factores que afectan a la capacidad antioxidante del té (como el tiempo y temperatura de infusión, lugar de procedencia, tipo de procesado, etc.), una de las bebidas más consumidas en el mundo.

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How brewing parameters affect the healthy profile of tea

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Abstract

Green and white teas are some of the most consumed beverages in the world, especially due to their inherent health properties such as prevention of cardiovascular and neurodegenerative pathologies, diabetes, body weight control, among others. Such properties are related with the presence on tea beverage of different bioactive compounds like catechins, caffeine, theophylline or minerals. The extraction of catechins is related with the final antioxidant capacity of the beverages, which in turn correlates with many parameters such us time/temperature of infusion, particle size, number of extractions, storage time, exposition to light, etc.

Introduction

Tea is the most widely consumed beverage in the world aside from water, and is highly appreciated due to its pleasant sensory properties [1,2], health properties [3] and socio-cultural characteristics [4]. Tea beverage is obtained after infusion of *Camellia sinensis var. sinensis* leaves of four different varieties: black, oolong, green and white. Black and oolong teas are totally or partially- fermented [5] while green and white teas have a lower oxidation degree [6,7]. Thus, white tea is supposed to have a higher antioxidant capacity due to a higher content of tea catechins and the lowest oxidation degree among teas [8–10]. There are many studies in the literature reporting the beneficial properties of tea, especially against oxidative stress, due to the large amount of antioxidant catechins [11–16]. The availability of tea catechins is associated with many different factors such as cultivar conditions and drying process [16] or even the brewing conditions [17–19]. Thus, there is a debate about the best or improved conditions to prepare tea infusions in order to extract as many antioxidant compounds as possible [20]. In fact, the extraction of catechins (the main responsible compounds of tea antioxidant capacity) is related with many parameters such us time/temperature of infusion, particle size after grinding, number of extractions, storage time, exposition to light, among others.

Therefore, it is time to deep into the factors affecting tea brewing in an updated review. The present paper wants to shed light about recent literature which studies the relationship between infusion conditions and antioxidant capacity of green and white teas, paying special attention to new brewing strategies compared with the traditional way of preparing teas.

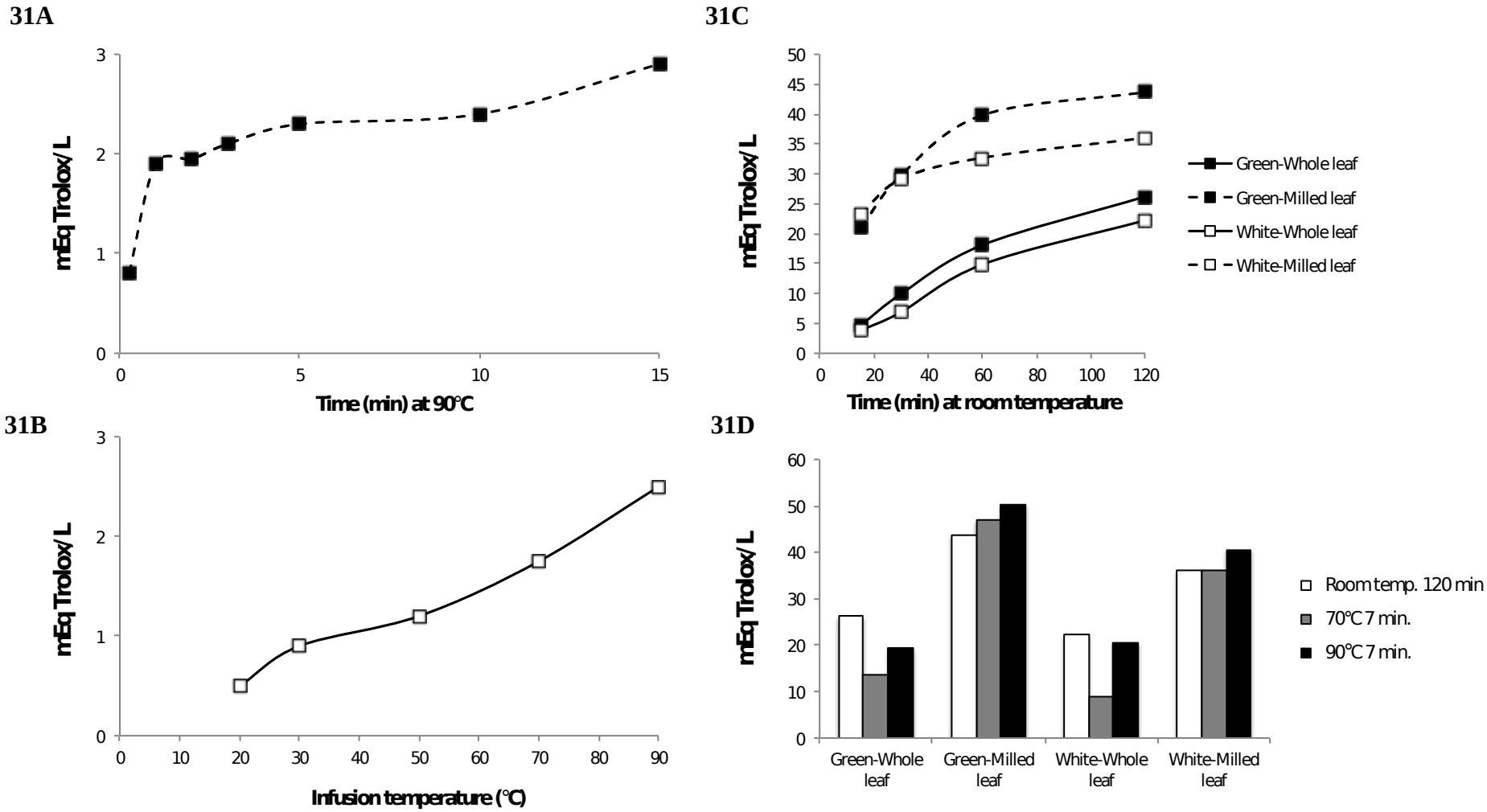
Brewing time/temperature

It has been reported that both time and temperature of tea infusion have pronounced effects on the extraction of bioactive compounds and antioxidant capacity[21, 22, 23]. Thus, green tea is typically prepared as an infusion by immersing dried green tea leaves in hot water at around 80–90ºC for a period of 3–4 min, while 5 min at 60ºC are recommended for white tea [24]. Langley-Evans [25] studied the effect of time (15 s to 15 min) and temperature (20–90ºC) on the antioxidant activity of green tea measured with the FRAP method. As depicted in **Figure 31A**, the antioxidant capacity of green tea beverage increase with brewing time, reaching the highest activity at 5 min. Although after 15 min of brewing a slightly higher antioxidant capacity was found, the sensory properties of the beverage (too much astringency) did not allow its consumption [25]. Other authors found the same effect [26, 27] or even discovered decreased antioxidant activity at very long infusion times due to oxidation and epimerization of catechins [27, 28]. Brewing temperature also increased the antioxidant activity of green tea (**Figure 31B**) since temperature increases the solubility of tea catechins. These results are in line with those reported by other authors [27, 29, 30, 31].

In the last four of years some studies point out to a new way to obtain tea by means of cold water steeping [22, 26, 29 ,32]. Daminani et al. [22] found that teas brewed at room temperature for 120 min had up to 70% more catechins and antioxidant capacity (assessed with the ABTS method) than those brewed at 70ºC for 7 min. In order to shed light about these results, Castiglioni et al. [26] studied the effect of brewing at room temperature up to 120 min and found an increase on the antioxidant activity (measured with the ABTS method) with infusion time (**Figure 31C**). Interestingly, these authors found a relationship between the physical state of tea leaves (whole leaves versus milled ones): when tea leaves are milled (broken as they are

usually found in tea bags) the highest antioxidant activity was obtained with hot water for both white and green tea (**Figure 31D**); however, when whole tea leaves were used for brewing the maximum antioxidant capacity was obtained at room temperature (with infusion times 20-times longer than those used for hot extraction). In addition, both the antioxidant activity (**Figure 31D**) and amount of total phenols (data not shown) were always higher with milled leaves. Under the sensory point of view, hot and cold teas were almost indistinguishable although cold water steeping provided lower bitter taste and higher aroma. Although cold infusion seems to be a promising technology to maximize potential health benefits, it requires long infusion times. In order to overcome it, Lantano et al. [32] proposed a modification involving an infusion step with hot water followed by ice addition, which avoids the slow cooling process responsible for changes in the contents of functional compounds [30, 33]. Hot-iced tea was prepared by placing 30 g of green tea in 600 mL of water at 80ºC and adding 400 g of ice after 5 min of infusion. Although these authors found that the antioxidant activity (measured with the FRAP method) of hot-iced tea was lower than that of cold tea (obtained after infusion of 8 g of green tea at 4ºC for 12 hours), the amount of individual catechins was higher in hot-iced tea. In the case of Oolong and black teas, not only the amount of catechins was higher in hot-iced tea but also the antioxidant capacity increased.

Figure 31. Influence of infusion time/temperature on the antioxidant activity of tea (calculated from the data presented in Ref. [25,26]).



As stated above, the main beneficial health effects of green and white teas have been attributed to their high content of catechins since they can act as antioxidants by donation of a hydrogen atom, as acceptors of free radicals and interrupters of chain oxidation reactions [1]. Tea epicatechins (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate) can change to their epimers (gallocatechin gallate, catechins gallate, gallocatechin and catechin) that show lower antioxidant capacity [34]. As infusion time is prolonged catechins may undergo epimerization, being tea catechins converted to their corresponding non-epi isomers [35–39,40]. In this sense, Saklar et al. [30] studied the effect of both time (1, 2, 3, 5, 10, 20, 30 and 45 min) and temperature (75, 85 and 95ºC) on the extraction and epimerization of catechins in green tea. These authors found that brewing at 85ºC for 3 min was the optimal condition for the extraction of epigallocatechin gallate. In addition, they found that increasing brewing time and temperature increased the amount of non-epi isomers up to a 50%, being infusion time the parameter with a deeper effect on epimerization.

Figure 32. Evolution of antioxidant capacity of tea beverages after storage of tea leaves at room temperature (calculated from the data presented in Ref. [40]).

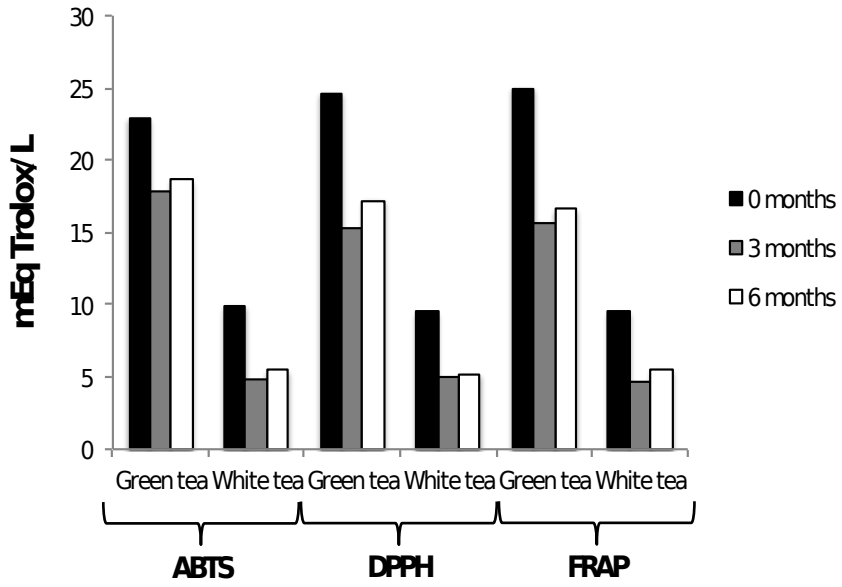
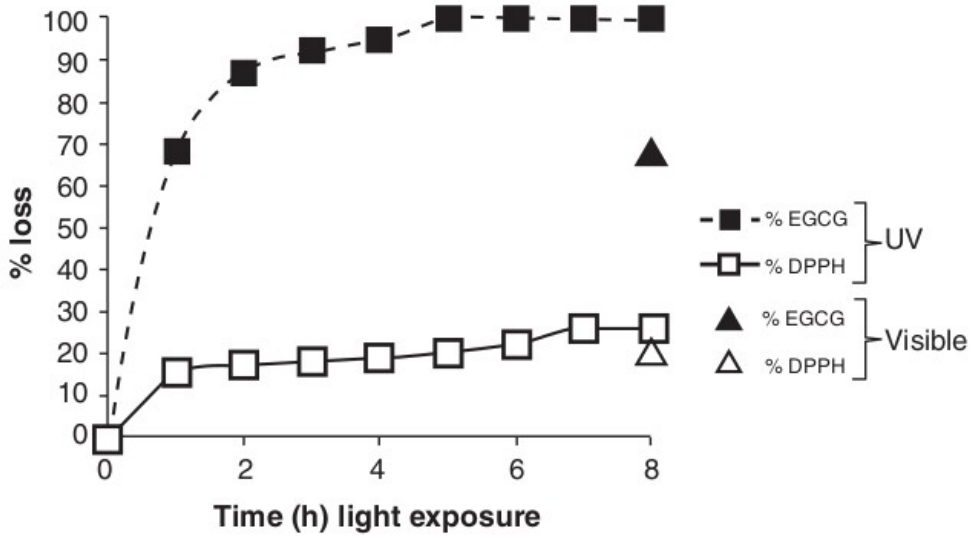


Figure 33. Response of antioxidant capacity and amount of epigalocatechin gallate to exposition of light (calculated from the data presented in Ref. [44]).



Storage time

Storage time (for both infusion and tea leaves) influences also the amount of bioactive compounds as well as the antioxidant capacity of the tea beverage. In this sense, Komes et al. [27] studied the changes in the composition of polyphenols during 24 hours storage of green tea extracts (at room temperature). High variations of total flavan-3-ols and phenolic acids were observed, but finally after 24 hours storage the content of polyphenols decreased in all tea extracts. The variation might be due to a great abundance and variability of all tea constituents, which participate in various reactions during storage of a tea infusion in the presence of oxygen, such as polymerization, or even degradation of some tea compounds.

The maximum storage time for infusion plants like tea should not exceed two years [39]. During a prolonged storage, the antioxidant capacity of the plants may be impaired. Related to this, Jiménez-Zamora et al. [40] evaluated the antioxidant stability of plants used to obtain infusions (including different types of teas) during their storage at room temperature for 6 months. As depicted in **Figure 32**, irrespective to the method used to measure the antioxidant activity (ABTS, DPPH or FRAP) it decreased 20–50% after the storage of white and green teas at room temperature. Such decrease could be related with the oxidation of antioxidant compounds along time [41,42].

However, the extension of the storage up to 6 months did not induce additional changes. It can be hypothesized that the oxidation of the more exposed phenolic compounds take place during the first 3 months, but those phenols firmly attached to the plant matrix are protected and then active for longer periods.

Exposition to light

Recently, it has been demonstrated that polyphenolic compounds such as gallic acid can act as pro-oxidant and generate reactive oxygen species (ROS) and hydrogen eroxide upon UV irradiation [43]. Thus, it is important to check the stability of antioxidant capacity and catechins in teas after light exposure. Abd-ElSalam et al. [44] checked the loss of scavenging activity (measured with the DPPH methods) and epigalocatechin gallate (EGCG) of a green tea beverage after 8 hours exposure to visible or UV irradiation. As depicted in **Figure 33**, there was almost a complete degradation of EGCG within 4 hours of UV irradiation time and almost 70% under light exposure.

Surprisingly, this complete degradation in EGCG content was not reflected on the antioxidant capacity. Abrupt increase in the percentage degradation of antioxidant

capacity was obtained after 1 hours of UV irradiation time followed by a slight increase until the end of the incubation period; almost the same degradation was found under light exposure. These authors explained such behavior taking into account a lack of correlation between EGCG content and antioxidant capacity, which was best correlated with the total phenolic content.

Number of extractions

Tea brewing can be also performed with re-infusion of used tea leaves, since they retain many different bioactive compounds that has not been exhausted [27, 31]. Thus, Komes et al. [27] found that a second and third infusion performed at 80ºC for 3 min extracted up to 25% of antioxidant activity. Very recently, Sharpe et al. [31] studied the effect of different brewing conditions over the antioxidant capacity of 24 commercial green tea varieties. Among them, these authors checked the effect of six successive extractions at 80ºC for 5 min. They observed that certain teas do not release significant levels of active antioxidants beyond their first brew. However, some teas continue to release active catechins throughout six or more brews. Thus, other factors affect the ability to be reused, such as growing location, initial drying method), harvest season and whether the tea was loose-leaf or bagged. All these parameters will be described in the subsequent section.

Other parameters affecting antioxidant capacity

The growing region of teas has an impact on their antioxidant capacity [45]. In this sense, Sharpe et al. [31] found that teas grown in China had significantly higher antioxidant capacity than those grown in other region, probably related with the ideal environment for growth and development of *C. sinensis*, creating tea plants with increased catechin content that can be released even if infused repetitively. In the case

of first-brew antioxidant capacities, the teas from Kenya also scored high. On the other hand, the harvest season is also a limiting factor for the antioxidant capacity of teas [46]. Spring harvested teas were more antioxidant in the first brew compared to fall harvest green teas [31]. This could be related with the motion that tea leaves just appear in spring, having robust antioxidant stores for preservation.

Another factor with a deep impact on the catechin content of tea is the drying method. During tea manufacture different a first drying step is applied in order to reduce moisture from 80% to 70% [31]. Such heating prevents oxidation of catechins by internal polyphenol oxidase enzyme, esterases, glycosidases and decarboxylases, which catalyze transformations and degradations of polyphenolic compounds [47]. A second drying step (reducing moisture to 3–4%) is performed before packaging. Sharpe et al. [31] found that fan drying was correlated with increased first-brew antioxidant capacity, while steam-drying teas was correlated with increased total antioxidant capacity. The proposed explanation relies on a higher preservation of catechins in the initial drying process of steam dried teas, compared with fan dried ones. This could be related with the extended amount of time (12–17 hours) required for fan drying. Fan drying is the method of choice for the majority of Kenyan teas studied by Sharpe et al. [31], likely explaining why Kenyan teas are also correlated with high first-brew antioxidant capacity.

The use of bagged tea has an impact on the antioxidant capacity depending whether the tea is brewed once or several times. Sharpe et al. [31] found that loose-leaf teas had measurable antioxidant activities even after six brews while only bagged teas exerted antioxidant capacity after two brews. However, a similar total antioxidant capacity was obtained for both types of tea, assuming that bagged teas must release increased catechins in their early brews. Similar results were obtained previously by

Komes et al. [27]. Such behavior could be related with the use of very finely ground leaf particles in bagged teas, having increased surface area as compared to loose-leaves and resulting in a close contact with water during infusion. This increased surface area of ground leaves could cause most catechins to be released in the first-brew, while loose-leaf teas may release catechins in a more sustainable manner over the course of multiple infusions in which whole leaves would gradually open and become hydrated. These results have been confirmed very recently by other researchers [48], who studied the effect of grinding on particle size (ranging from 20 mm to 500 mm), catechins release and antioxidant capacity (measured with the ABTS and DPPH methods). Thus, the fraction of 100–180 mm showed the maximum catechin release and antioxidant capacity, while less activity for those particles with a size lower than 50 mm and longer than 200 mm.

Finally, other factors have less impact on the antioxidant capacity of green tea beverages. For example, neither decaffeination nor the use of organic practices or sale price has an effect of the antioxidant capacity [31]. Tap water does not interfere with the antioxidant capacity of green tea. However, as stated above there is a time-dependent epimerization of catechins [30, 35, 36, 38] in tap water that could be prevented by adjusting pH with lemon juice [31]. Thus, Komes et al. [27] found that the antioxidant capacity of green tea increases with the addition of lemon juice, which could be directly attributed to the synergistic effect of ascorbic acid on the polyphenols present in tea extract [49]. In the case of milk addition, controversial results have been reported. Hollman et al. [50] claimed that the addition of milk does not alter the antioxidant capacity of tea, while Langley-Evans [25] and Komes et al. [27] stated the opposite, a decrease on the antioxidant capacity due to the flavonoid-binding capacity of milk proteins.

Conclusions

Green and white teas are a good source of antioxidants within the human diet, although the way that tea brews are prepared influences the final content of catechins and antioxidant capacity. Many parameters may affect the sensory and nutritional quality of tea infusions such as maturity of the leaves, processing parameters and expected sensory quality. Taking into account the literature concerning all these factors, for optimal antioxidant extraction a combination of 5–10 min at temperatures 80ºC will result the most antioxidant brews compared with shorter brewing time and lower temperatures (60– 70ºC). In addition, teas prepared during long-tem infusions at room temperature will be a future trend to prepare tea beverages very rich in antioxidant compounds. On the other hand, tealeaves can be re-used, although higher extraction efficiencies are obtained with grinded and bagged teas. In order to protect the antioxidant capacity of tea leaves, it should not be stored for longer periods of time, while tea infusions should be stored refrigerated, even with some drops of lemon juice, in order to retard catechins epimerization. Therefore, taking together the most recent scientific literature and the own experience of authors, to obtain a tea infusion with a high antioxidant capacity while maintaining good sensorial properties, 7 min of brewing time at 90ºC are enough for white and green teas. If the tea beverage is not going to be consumed exactly after brewing, immediate storage under refrigeration is need. In addition, tea leaves should not be stored more than two months at room temperature since they became oxidized.

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Effect of brewing time and temperature on antioxidant capacity and phenols of white tea: relationship with sensory properties

En este trabajo se describe cómo el tiempo y la temperatura de infusión influyen en la capacidad antioxidante de la infusión y en sus sensoriales. Puesto que uno de los objetivos principales de la presente tesis doctoral es la evaluación de la capacidad antioxidante, es necesario conocer cómo puede potenciarse dicha actividad en el té. En este paper se demuestra que ambos factores son importantes y van a influir en gran medida en las propiedades beneficiosas del té.

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Effect of brewing time and temperature on antioxidant capacity and phenols of white tea: relationship with sensory properties

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ABSTRACT

White tea is highly consumed due to its sensory properties and health benefits, although most scientific reports don't include the analysis of both properties. Therefore, the objective of the present study was to unravel the best brewing conditions for optimal extraction of the bioactive compounds and antioxidant capacity, while realising the best sensory properties. Infusions of eighty commercial teas (sold in bags or leaves) were obtained at different time-temperature ratios, studying bioactive compounds (caffeine and individual catechins), antioxidant capacity and sensory analysis. Brewing at 98ºC for 7 min was the best condition to obtain a high content of antioxidant polyphenols and pleasant sensory properties. Those teas sold in bags give rise to tea brews with almost double antioxidant capacity. In conclusion, it is very important to link sensory and chemical data to obtain optimal sensorial quality and the highest healthy properties in white tea infusions.

KEYWORDS: White tea, green tea, antioxidant capacity, brewing conditions, catechins.

1. Introduction

Green tea is one of the most consumed beverages around the world due to its sensory attributes and socio-cultural factors, especially in Asia (Hilal & Engelhardt, 2007). However, white tea (an unfermented tea made from the new growth buds and young leaves of the plant) is well recognized by its higher sensorial quality and health properties (Cabrera, Artacho & Giménez, 2006). Thus, tea has been related to beneficial effects on several diseases such as neurodegenerative and cardiovascular diseases, diabetes, obesity and basically, to every pathology involving oxidative stress (Higdon & Frei 2003). Such protection is most probably due to a wide range of bioactive compounds in tea beverage such as flavonoids, other polyphenols, caffeine or theanine (Vuong, 2014). Flavan-3-ols, commonly called catechins, can account for up to 30% of the dry weight of white and green tea leaves, being epigallocathin-gallate (EGCG) the major component (Cabrera et al., 2006). Consumption of 200-300 mg of EGCG (5-6 tea cups/day) has beneficial effects on cardiovascular health (da Silva Pinto, 2013) since EGCG and other catechins are very effective scavengers of radical oxygen species (ROS) and radical nitrogen species (RNS) both *in vitro* and *in vivo*.

The presence of these bioactive molecules depends on several factors. On one hand, the industrial process to obtain the commercial product from fresh leaves affects the amount of these compounds (Gorjanović et al., 2012). Thus, white and green teas suffer several drying steps, being less aggressive in white tea (Cabrera, Giménez & López, 2003; Pastoriza, Mesías, Cabrera & Rufián-Henares, 2017). On the contrary, black and red tea suffer an oxidation process where phenolics and other substances are oxidized (Vuong, 2014). On the other hand, preparing the beverage by infusion becomes another critical point since this process allows the extraction of bioactive compounds from tea (Damiani, Bacchetti, Padella, Tiano & Carloni, 2014). The

extraction of catechins depends on time and temperature, so monitoring these parameters while making the infusion is of great importance to get all the benefits from tea (Komes, Horžić, Belščak, Ganić & Vulić, 2010). This extraction process affects not only the antioxidant capacity of the tea beverage but also its organoleptic characteristics, since the extracted molecules also play a role in taste (Pastoriza, Pérez-Burillo & Rufián-Henares, 2017). Finally, the physical state of tea leaves also play a role on sensory properties. In this sense, Castiglioni, Damiani, Astolfi & Carloni (2015) found that milled leaves (usually sold in bags) have a more astringent taste than those obtained from whole leaves (those found in high-quality teas).

Different researchers have studied the extraction kinetics of catechins from white tea, based on water temperature and extraction time (Dai et al., 2017; Lin, Xia, Hsieh, Liu & Mau, 2017; Tan, Engelhardt, Lin, Kaiser & Maiwald, 2017) but they usually lack the study of sensory analysis. On the other hand, there are scientific reports that centered on the effect of water temperature and extraction time on sensory properties (Lin et al., 2014; Castiglioni et al., 2015; Lantano, Rinaldi, Cavazza, Barbanti & Corradini, 2015) or even antioxidant capacity, but lacked analysis of the extraction of bioactive compounds. Therefore, the aim of this research was to perform a deep study on the effect of the extraction time and temperature on the release of healthy molecules and antioxidant capacity of white tea (Chinese Pai Mu Tan) infusion, in relation to their sensory attributes. After deciding the best time-temperature binomial for optimal sensory properties, the antioxidant capacity and bioactive compounds content of white and green teas commercialized in Spain were then measured. In addition to these studies, the influence of commercial presentation of teas (whole leaves Vs. bagged teas) was also assessed.

2. Materials and methods

2.1. Reagents, standards and solvents

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ), Folin-Ciocalteu reagent, iron (III) chloride hexahydrate, sodium acetate, potassium persulphate, sodium hydroxide, sodium carbonate, caffeine, gallic acid (GA), epicatechin (E), epicatechin gallate (EG), epigallocatechin (EGC) and epigallocatechin galate (EGCG) were from Sigma-Aldrich (Germany). All solvents were of HPLC quality (Sigma-Aldrich, Madrid, Spain). Doubly distilled deionized water was obtained from a Milli-Q system (Millipore, Milford, MA). Bronchales mineral water (Bronchales, Teruel, Spain) was used for tea infusion. It was a mineral water with very low mineral content: bicarbonates 8 mg/L, chloride 2.52 mg/L, sulphate 9.97 mg/L, silica 8 mg/L, calcium 2.71 mg/L, magnesium 2.75 mg/L, sodium 1.05 mg/L and potassium 1.21 mg/L.

2.2. Tea samples

White teas [n = 13 for white bagged teas (WTB); n = 21 for white tea leaves (WTL)] and green teas [n = 27 for green bagged teas (GTB); n = 19 for green tea leaves (GTL)] were purchased from 16 local tea shops (Granada, Spain). Tea infusions were prepared as follows: Two grams of tea leaves (or a tea bag) was put into 150 mL of water. Several infusions were made using in each occasion a different pair of time-temperature. Mineral water was heated at 60, 70, 80, 90 and 98ºC and the tea sample was left for 3, 5, 7, 10 and 15 min to obtain the corresponding infusion. The samples were then stored at -80ºC until they were analyzed. For comparative analysis of the

white-green tea samples, tea samples were prepared at 98ºC for 7 min. Each sample was prepared in triplicate and each one was analyzed three times.

2.3. Antioxidant assays

2.3.1. ABTS method

The ABTS assay was conducted as described by Roberta, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans (2009) with slight modifications (Jiménez-Zamora, Delgado-Andrade & Rufián-Henares, 2016). The $ABTS^{+\cdot}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{+\cdot}$ working solution (stable for 2 days) was diluted with a mixture of ethanol:water (50:50) to an absorbance of 0.70 ± 0.02 at 730 nm. After adding 20 µL of sample or trolox standard to 280 µL of diluted $ABTS^{+\cdot}$ solution, the absorbance reading was taken at 20 min. by using a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Aqueous solutions of Trolox were used for calibration (0.15-1.15 mM). The results are expressed as mmol equivalents of Trolox per L of infusion.

2.3.2. FRAP method

The ferric reducing ability of the extract of each sample was estimated following the procedure described by (Benzie & Strain, 1996) with some modifications. Briefly, 280 µL of FRAP reagent, freshly prepared and warmed at 37ºC, was mixed with 20 µL of sample. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \cdot H_20$ and 25 mL of 0.3 M acetate buffer, pH 3.6. The samples were incubated at 37ºC and readings at the absorption maximum (595 nm)

were taken at 30 minutes by using a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Trolox solutions were used to perform the calibration curve. The results are expressed as mmol equivalents of Trolox per L of infusion.

2.3.3. Folin-Ciocalteu method

The total phenolic content was measured following the procedure Folin-Ciocalteu with some modifications (Moreno-Montoro, Olalla-Herrera, Giménez-Martínez, Navarro-Alarcón & Rufián-Henares, 2015). Briefly, 15 μL of Folin-Ciocalteu reagent, 60 μL of sodium carbonate (10%), 190 μL of distilled water and 30 μL of sample were mixed. The samples were incubated at 37ºC and readings at the absorption maximum (595 nm) were taken at 60 minutes by using a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Gallic acid solutions were used to perform the calibration curve. Results are expressed as mg gallic acid equivalents per L of infusion.

2.4. Catechins and caffeine determination

The determination of catechins and caffeine in white tea was performed by using an UPLC (Accela 600 UPLC, Thermo-Fisher Scientific, Bremen, Germany) equipped with a diode array detector (DAD) and a reversed-phase column (Hypersil ODS C_{18}, 3 μm, 2.1 mm × 100 mm, Thermo Scientific, Bremen, Germany) according to Cabrera *et al.* (2003). Gradient mobile phase consisted on a solution of acetic acid (3 mL) in 100 mL water (v/v) (eluent A) and methanol (v/v) (eluent B). The gradient was as follows: 0–5 min, 20 % B; 5–7 min, linear gradient from 20 to 24 % B; 7–10 min, 24 % B; 10–20 min, linear gradient from 24 to 40 % B; 20–25 min, linear gradient from 40 to 50 %

B; 25–28 min linear gradient from 50 to 20 % B; 28–30 min 20 % B. Elution was performed at a solvent flow rate of 0.6 ml/min. Chromatograms were recorded at 280 nm. An external calibration line was used for quantification. Catechin and caffeine amounts in white tea infusions were calculated as mg/L.

2.5. Sensory evaluation

Sensory evaluation was carried out using both a consumer panel and a trained panel. Consumer panel was composed of 51 consumer panelists from Granada, who performed the Consumer Preference Test, which aimed to establish the best brewing conditions (time-temperature) for white teas. Consumers were recruited from green-white tea drinkers who were willing to taste white tea. The first test consisted of the analysis on the overall likeness for tea samples obtained after 5 min in water at 60, 70, 80, 90 and 98ºC. After selection of the preferred temperature (98ºC), the likeness for tea samples based on the infusion time (7, 10 and 15 min) was also evaluated. Every consumer evaluated 4 of the 8 samples each time and 5 min were given to evaluate each sample. Consumers cleansed their palates between evaluations by eating apple and rinsing their mouths with the mineral water used to prepare the tea infusions.

The trained panel was composed of 12 trained panelists who performed a Descriptive Sensory Analysis (taste and aroma). The panelists had completed 50 h of sensory training and had a minimum of 200 h of general sensory testing including olive oil, coffee, vegetables, and tea. All panelists were given a 4-h reorientation to white tea and the green tea lexicon previously developed by Lee & Chambers (2007). The panelists were familiar with tea drinks and were not allowed to use drinks, smoke or drink-eat anything (except water) one hour before the session. Each member was offered one sample at a time, cleaning their palate between evaluations by rinsing it

with mineral water and apple (Castiglioni et al., 2015). The panelists recorded orthonasal smell, retronasal aroma (brown, citrus, floral, fruity, green, seaweed, spinach) and taste (astringent, bitter, persistent and sweet). The intensities of sensory attributes were expressed on a 0-5 scale (0 = absent, 1 = barely, 2 = fairly, 3 = rather, 4 = highly, 5 = extremely).

2.6. Antioxidant capacity and polyphenols intake calculations

The dietary antioxidant capacity and polyphenols intake was calculated as the individual contribution of each juice, taking into consideration both the amount of food per serving and the daily consumption (Mercasa, 2017). Thus, the antioxidant capacity and polyphenols of each tea infusion was compared with the usual Spanish serving size (Salvador i Castells, 2000). The contribution to the daily intake of antioxidant capacity and polyphenols of each juice was referred to the results previously published by Saura-Calixto & Goñi (2006).

2.7. Statistical analysis

Statistical significance of the data obtained was tested by one-way analysis of the variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($p < 0.05$). Evaluation of the relationship between different assays was carried out by computing the relevant correlation coefficient (Pearson linear correlation) at $p < 0.05$ confidence level. Multivariate analysis was performed by principal component analysis (PCA) with those means computed from aforementioned analyses. All statistical analyses were performed using Statgraphics Plus software, version 5.1, 2001 (Statgraphics Technologies Inc., The Plains, VI, USA).

3. Results and discussion

3.1. Time-temperature relation with bioactive compounds extraction and antioxidant capacity.

3.1.1. Catechins and caffeine.

The influence of water temperature and infusion time on the extraction of catechins and caffeine was assessed with different time-temperature pairs as described in the materials and methods section. The content of gallic acid, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate and caffeine were determined and depicted in **Table 14**. In general, increase in the infusion time amounted to extraction of more bioactive compounds, but temperatures from 60 to 80ºC did not exert any significant effect. At such temperatures, only 15 min produced a statistically significant ($p < 0.05$) higher concentration of gallic acid, epigallocatechin and caffeine. At temperatures higher than 80ºC, the content of gallic acid, epigallocatechin gallate and caffeine increased greatly (almost at an exponential increase) when infusion time was above 10 min (**Table 14**). On the contrary, epicatechin, epicatechin gallate and epigallocatehcin showed a lineal increase. Little changes were obtained after 3-5 min of extraction, while 7 min of infusion gave significant increases ($p < 0.05$) for all the assessed bioactive compounds, always higher at 98ºC.

The results reported by other researchers are in accordance with the results obtained in this study, both the amount of catechins and caffeine extracted and the effect of heating temperature and infusion time, being the main factors when extracting bioactive compounds (Komes *et al*., 2010; Saklar, Ertas, Ozdemir & Karadeniz, 2015). Some of these researchers (Saklar *et al*., 2015; Lin *et al*., 2017) also reported that

Table 14. Evolution of catechins and caffeine of white tea infusions with heating time and brewing temperature.

Bioactive compound	**Temperature** (°C)	**Infusion time** (minutes)				
		3	5	7	10	15
Gallic acid	60	12.1 ± 1.2 ^{a,1}	12.4 ± 1.0 ^{a,1}	19.2 ± 1.0 ^{a,2}	22.2 ± 2.1 ^{a,2}	26.8 ± 2.0 ^{a,3}
	70	12.8 ± 1.0 ^{a,1}	20.2 ± 2.0 ^{b,2}	21.7 ± 2.0 ^{a,2}	26.1 ± 2.0 ^{a,3}	28.3 ± 2.0 ^{a,3}
	80	13.2 ± 0.9 ^{a,1}	23.1 ± 2.0 ^{b,2}	31.3 ± 2.5 ^{b,3}	39.5 ± 3.1 ^{b,4}	42.6 ± 3.6 ^{b,4}
	90	13.7 ± 0.8 ^{a,1}	34.2 ± 2.6 ^{c,2}	42.2 ± 3.2 ^{c,3}	82.7 ± 5.7 ^{c,4}	126 ± 10 ^{c,5}
	98	25.1 ± 1.5 ^{b,1}	43.6 ± 3.2 ^{d,2}	43.7 ± 4.0 ^{c,2}	105 ± 10 ^{d,3}	160 ± 10 ^{d,4}
Epicatechin	60	1.42 ± 0.21 ^{a,1}	1.56 ± 0.12 ^{a,1}	1.72 ± 0.11 ^{a,1}	1.79 ± 0.11 ^{a,1}	1.45 ± 0.12 ^{a,1}
	70	1.94 ± 0.65 ^{a,1}	2.74 ± 0.11 ^{a,1}	2.88 ± 0.12 ^{a,1}	2.67 ± 0.12 ^{a,1}	2.19 ± 0.32 ^{a,1}
	80	2.04 ± 0.45 ^{a,1}	2.81 ± 0.32 ^{a,1}	3.72 ± 0.45 ^{a,1}	3.30 ± 0.23 ^{a,1}	3.61 ± 0.54 ^{a,1}
	90	3.36 ± 0.36 ^{a,1}	6.90 ± 0.99 ^{a,2}	8.04 ± 1.21 ^{a,2}	12.6 ± 1.2 ^{a,3}	20.8 ± 2.0 ^{a,4}
	98	7.46 ± 0.99 ^{a,1}	9.72 ± 0.87 ^{a,1}	22.9 ± 2.0 ^{a,2}	38.5 ± 3.0 ^{a,3}	62.9 ± 4.1 ^{a,4}
Epicatechin gallate	60	3.46 ± 0.45 ^{a,1}	3.55 ± 0.32 ^{a,1}	4.03 ± 0.56 ^{a,1}	4.52 ± 0.45 ^{a,1}	4.54 ± 0.54 ^{a,1}
	70	3.98 ± 0.62 ^{a,1}	4.43 ± 0.56 ^{a,1}	4.48 ± 0.42 ^{a,1}	4.63 ± 0.46 ^{a,1}	4.90 ± 0.56 ^{a,1}
	80	4.37 ± 0.33 ^{a,1}	4.24 ± 0.45 ^{a,1}	4.53 ± 0.23 ^{a,1}	4.73 ± 0.54 ^{a,1}	4.85 ± 0.45 ^{a,1}
	90	4.74 ± 0.01 ^{a,1}	16.4 ± 1.3 ^{b,2}	27.1 ± 2.6 ^{b,3}	48.6 ± 4.2 ^{b,4}	55.8 ± 5.0 ^{b,4}
	98	6.84 ± 0.09 ^{b,1}	18.3 ± 1.2 ^{b,2}	47.4 ± 4.0 ^{c,3}	64.6 ± 5.1 ^{c,4}	87.0 ± 7.1 ^{c,5}
Epigallocatechin	60	0.15 ± 0.10 ^{a,1}	0.32 ± 0.10 ^{a,1}	0.37 ± 0.10 ^{a,1}	0.68 ± 0.11 ^{a,2}	0.98 ± 0.22 ^{a,3}
	70	1.59 ± 0.50 ^{b,1}	1.65 ± 0.10 ^{b,1}	1.76 ± 0.11 ^{b,1}	2.04 ± 0.33 ^{b,1}	2.66 ± 0.11 ^{b,2}
	80	1.97 ± 0.40 ^{b,1}	2.41 ± 0.20 ^{c,1}	4.91 ± 0.55 ^{c,2}	7.94 ± 0.99 ^{c,3}	5.49 ± 0.45 ^{c,2}
	90	4.90 ± 0.56 ^{c,1}	5.44 ± 0.41 ^{d,1}	23.8 ± 3.6 ^{d,2}	53.9 ± 4.2 ^{d,3}	103 ± 10 ^{d,4}
	98	24.5 ± 2.4 ^{d,1}	29.5 ± 2.1 ^{e,1}	47.5 ± 4.0 ^{e,2}	100 ± 9 ^{e,3}	160 ± 11 ^{e,4}
Epigallocatechin gallate	60	5.05 ± 0.56 ^{a,1}	5.29 ± 0.56 ^{a,1}	5.31 ± 0.54 ^{a,1}	5.47 ± 0.41 ^{a,1}	6.33 ± 0.60 ^{a,1}
	70	5.55 ± 0.45 ^{a,1}	5.59 ± 0.45 ^{a,1}	5.64 ± 0.74 ^{a,1}	5.84 ± 0.32 ^{a,1}	8.02 ± 0.70 ^{a,2}
	80	7.31 ± 0.46 ^{b,1}	8.92 ± 0.88 ^{a,2}	14.4 ± 1.0 ^{a,3}	19.9 ± 2.03 ^{b,4}	52.0 ± 4.2 ^{b,5}
	90	9.73 ± 0.56 ^{c,1}	10.1 ± 1.2 ^{a,1}	22.4 ± 2.2 ^{a,2}	60.1 ± 7.03 ^{c,3}	107 ± 10 ^{c,4}
	98	13.3 ± 1.2 ^{d,1}	18.4 ± 1.5 ^{a,2}	52.4 ± 5.4 ^{a,3}	231 ± 31 ^{d,4}	298 ± 16 ^{d,5}
Caffeine	60	1.19 ± 0.11 ^{a,1}	1.46 ± 0.12 ^{a,1}	2.70 ± 0.25 ^{a,2}	2.91 ± 0.23 ^{a,2}	3.94 ± 0.30 ^{a,3}
	70	4.82 ± 0.56 ^{a,1}	5.74 ± 0.89 ^{a,1}	6.11 ± 0.56 ^{a,1}	10.2 ± 1.5 ^{b,2}	13.7 ± 0.11 ^{b,3}
	80	5.76 ± 0.23 ^{a,1}	7.58 ± 0.47 ^{a,2}	13.7 ± 1.2 ^{a,3}	16.0 ± 1.0 ^{c,3}	18.3 ± 1.2 ^{c,3}
	90	12.1 ± 0.8 ^{a,1}	16.4 ± 0.9 ^{a,2}	25.0 ± 2.9 ^{a,3}	51.3 ± 4.1 ^{d,4}	119 ± 7 ^{d,5}
	98	37.3 ± 2.6 ^{a,1}	41.2 ± 4.2 ^{a,1,2}	45.0 ± 3.1 ^{a,2}	58.5 ± 5.1 ^{d,3}	125 ± 8 ^{d,4}

Different letters indicate statistically significant differences $p < 0.05$ within the same column (and chemical compound).
Different superscript numbers indicate statistically significant differences $p < 0.05$ within the same line.

catechins extraction reaches a plateau after 10-15 min of extraction. On the contrary, Braud, Peyre, de Sousa, Armand, Rahmani & Maixent (2015) stated that catechins did not increase after 5 min of extraction, finding no significant differences among infusing for 5 or 15 min. (Damiani *et al*., 2014) compared cold extraction versus hot extraction, and found out that infusion at 20-25ºC for two hours increased total phenolic content and individual catechins. Moreover, the results obtained in this study show higher values for most of the individual catechins reported by these authors, except for EGC. Lantano *et al*. (2015) compared the extraction of bioactive compounds with three alternative extraction methods: 4ºC for 12 hours, 75ºC for 4 min and 80ºC for 5 min adding ice to the resulting infusion; these researchers also found out that temperature is the main factor to extract catechins, although extraction time also plays a role since cold extraction (4ºC) yielded high amounts of catechins. Finally, it has been also reported that cold and hot extractions yield different profiles of catechins; hot extraction releases higher amounts of larger molecules while decreasing smaller ones (Lin, Xia & Liu, 2014). This could be related to oxidation during the longer extraction times of catechins performed along cold extraction.

3.1.2. Antioxidant capacity.

The effect of time-temperature brewing conditions on the overall antioxidant capacity of white tea was also assessed. The ABTS method showed that the antiradical capacity of white tea gradually grew in a lineal manner with infusion time and water temperature (**Table 15**). Statistically significant changes ($p < 0.05$) were obtained from 7 min brewing with all the temperatures assessed except 60ºC. In the case of the reducing capacity of white tea brew (FRAP assay) the kinetics obtained were still lineal. However, temperatures below 98ºC had very low reducing activity with significant increases only after 15 min. Such behavior was similar to that found for the main part of

bioactive compounds studied (**Table 14**). The extraction of total phenolics (Folin-Ciocalteu assay, **Table 15**) was similar to that of the FRAP method and bioactive compounds. Therefore, a water temperature of 98ºC with brewing times from 7-15 min could increase the extraction of bioactive compounds and obtaining a good antiradical and reducing capacity on the tea brew.

Langley-Evans (2000) and Braud *et al.* (2015) studied the influence of time and temperature on the antioxidant capacity through the FRAP and DPPH methods, respectively. These authors found out that antioxidant capacity reached a maximum within 5-7 min of infusion compared to 15 and 30 min of extraction, while the maximum activity was obtained at temperatures of 90ºC (range 20-90ºC). The results stated by Komes *et al.* (2010) and Castiglioni *et al.* (2015) also show how total phenolics and antioxidant capacity increases significantly with water temperature, which is in agreement with the findings of this study. Other authors (Damiani *et al.*, 2014; Lin *et al.*, 2014) found a higher antioxidant capacity of total phenols in white teas extracted with the cold extraction method versus hot extraction. This could be related to the high brewing time (12-24 hours depending on the researcher).

Table 15. Evolution of antioxidant capacity and total phenols of white tea infusions with heating time and brewing temperature.

Antioxidant capacity	**Temperature**	**Infusion time** (minutes)				
	(ºC)	3	5	7	10	15
TEAC$_{ABTS}$	60	0.90 ± 0.09 ^{a,1}	0.80 ± 0.10 ^{a,1}	1.67 ± 0.32 ^{a,2}	1.31 ± 0.20 ^{a,2}	3.60 ± 0.34 ^{a,3}
	70	1.17 ± 0.14 ^{b,1}	1.50 ± 0.21 ^{b,2}	1.82 ± 0.12 ^{a,2}	1.80 ± 0.60 ^{a,2}	5.03 ± 0.56 ^{b,3}
	80	1.44 ± 0.14 ^{b,c,1}	2.70 ± 0.12 ^{c,2}	2.54 ± 0.33 ^{b,2}	3.02 ± 0.16 ^{b,2}	6.13 ± 0.65 ^{b,3}
	90	1.48 ± 0.13 ^{b,c,1}	3.80 ± 0.22 ^{d,2}	5.09 ± 0.62 ^{c,3}	4.19 ± 0.23 ^{c,2,3}	9.22 ± 0.98 ^{c,4}
	98	1.50 ± 0.12 ^{c,1}	4.10 ± 0.44 ^{d,2}	7.23 ± 0.88 ^{d,3}	8.40 ± 0.99 ^{d,3}	12.3 ± 1.2 ^{d,4}
TEAC$_{FRAP}$	60	0.14 ± 0.01 ^{a,1}	0.38 ± 0.02 ^{a,2}	0.29 ± 0.11 ^{a,2}	0.47 ± 0.13 ^{a,2}	2.02 ± 0.23 ^{a,3}
	70	0.21 ± 0.10 ^{a,1}	0.50 ± 0.13 ^{b,2}	0.52 ± 0.06 ^{b,2}	0.51 ± 0.01 ^{a,2}	4.74 ± 0.45 ^{b,3}
	80	0.37 ± 0.06 ^{b,1}	0.65 ± 0.15 ^{b,2}	0.80 ± 0.15 ^{c,2}	0.73 ± 0.06 ^{b,2}	5.96 ± 0.55 ^{c,3}
	90	0.39 ± 0.07 ^{b,1}	0.71 ± 0.14 ^{b,2}	1.05 ± 0.09 ^{d,3}	1.08 ± 0.12 ^{c,3}	6.54 ± 0.32 ^{c,d,4}
	98	0.58 ± 0.04 ^{c,1}	1.18 ± 0.09 ^{c,2}	1.20 ± 0.12 ^{d,2}	1.38 ± 0.13 ^{d,2}	7.01 ± 0.45 ^{d,3}
Folin-Ciocalteu	60	6.01 ± 0.61 ^{a,1}	26.7 ± 1.2 ^{a,2}	37.7 ± 2.1 ^{a,3}	41.9 ± 4.1 ^{a,3}	156 ± 10 ^{a,4}
	70	6.10 ± 0.54 ^{a,1}	47.5 ± 4.1 ^{b,2}	55.1 ± 4.5 ^{b,2}	50.8 ± 2.4 ^{b,2}	264 ± 21 ^{b,3}
	80	28.7 ± 2.1 ^{b,1}	60.4 ± 5.2 ^{c,2}	93.9 ± 9.0 ^{c,3}	77.6 ± 8.5 ^{c,3}	429 ± 33 ^{c,4}
	90	28.8 ± 2.0 ^{b,1}	74.8 ± 6.5 ^{d,2}	89.0 ± 7.2 ^{c,2}	135 ± 12 ^{d3}	520 ± 51 ^{d,4}
	98	58.3 ± 4.1 ^{c,1}	132 ± 11 ^{e,2}	143 ± 11 ^{d,2}	168 ± 10 ^{e,3}	619 ± 56 ^{d,4}

Different letters indicate statistically significant differences $p < 0.05$ within the same column and antioxidant capacity method.
Different superscript numbers indicate statistically significant differences $p < 0.05$ within the same line.
$TEAC_{ABTS}$: Trolox Equivalent Antioxidant Capacity measured with the ABTS method.
$TEAC_{FRAP}$: Trolox Equivalent Antioxidant Capacity measured with the FRAP method (Ferric Reducing Capacity of Plasma).

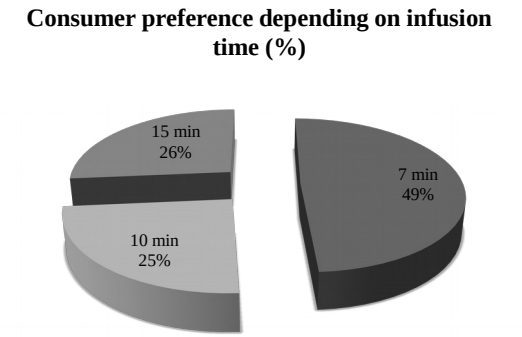
3.2. Sensory analysis

Previous chemical assays showed that a brewing temperature of 98ºC could be the best to obtain a tea beverage with superior antioxidant capacity and a high content of bioactive compounds (**Tables 14** and **15**). However, these temperatures don't preclude good organoleptic characteristics since white tea is usually brewed at 70ºC and green tea at 90ºC (Damiani *et al.*, 2014; Castiglioni *et al*., 2015). Thus, a consumer preference test was performed to establish the optimal conditions to obtain a pleasant white tea infusion while maintaining a high content of catechins. As depicted in **Figure 34A**, the percentage of panelists that liked tea brews increased along with extraction temperature. Brewing at 70ºC (the recommended temperature for white tea) only reached 13% of likes, similar to that obtained for 80ºC (14%). An increase in preference was obtained with the recommended temperature for green tea (27% at 90ºC) but brewing in boiled water (98ºC) was preferred by 43% of consumers.

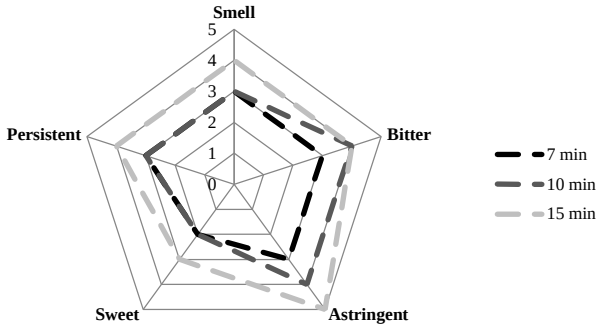
Since 98ºC was the preferred temperature for white tea and it was the temperature at which the maximum concentration of catechins and caffeine was extracted, 98ºC was selected to unravel the best extraction time (from 7 to 15 min) through the consumer and trained panelists. As depicted in **Figure 34B**, half of the consumer panelists preferred a tea infusion brewed for 7 min whereas only 24% and 25% of the panelists preferred those brewed for longer times (10 and 15 min, respectively). Consumers declared (data not shown) that such teas were quite astringent and "strong", which could be related to the large amount of individual (**Table 14**) and total polyphenols (**Table 15**) extracted at 98ºC.

Figure 34. Effect of brewing temperature and infusion time on consumer preference of white tea and sensory properties of white tea brewed at 98ºC. 34A: Consumer preference (% panellists) depending on brewing temperature (60, 70, 80, 90 and 98ºC). 34B: Consumer preference (% panellists) depending on brewing time (7, 10 and 15 min) at 98ºC. 34C: Descriptive Sensorial Analysis of smell and taste; 34D: Descriptive Sensorial Analysis of aroma attributes; Brewing time (7, 10 and 15 min) selected for improved antioxidant capacity and total phenols.

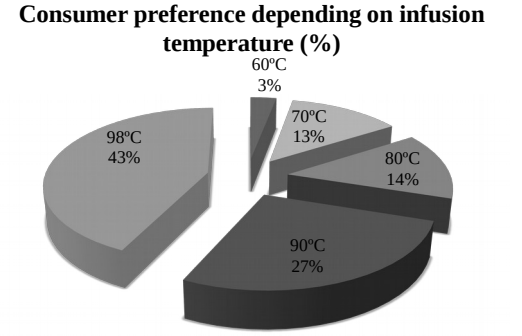
34A



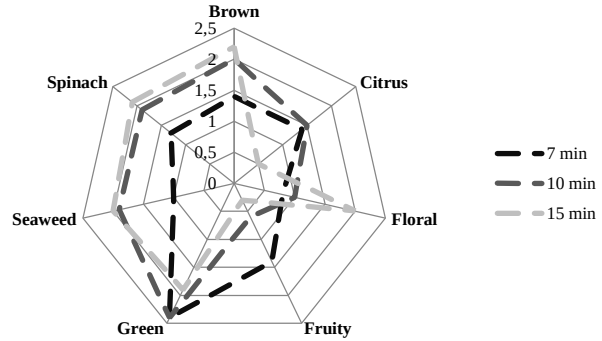
34C



34B



34D



As regards the Descriptive Sensory Analysis, the trained panel found a lower bitterness and astringency for the sample brewed for 7 min (**Figure 34C**). In the same way, sweetness and persistency was similar to the sample brewed for 10 min. Regarding orthonasal smell, teas brewed for 7 and 10 min obtained a “rather” mark (**Figure 34C**). In the case of the retronasal aroma seven descriptors were used: brown, citrus, floral, fruity, green, seaweed and spinach. These descriptors were selected from the lexicon of flavor descriptive analysis of green tea (Lee & Chambers, 2007) since white tea is a variety of green tea with better sensory properties (Cabrera, Artacho & Giménez, 2006). As depicted in **Figure 35D**, white tea brewed for 7 min was described mainly by “floral”, “fruity” and “green” attributes, which ranked with a similar score to green teas from Japan (Lee & Chambers, 2010) and Korea (Lee, Chamber & Chambers IV, 2013). On the contrary, the tea sample obtained after 15 min of brewing was described by “brown”, “seaweed” and “spinach” attributes, which ranked similar to those green tea samples from China (Lee & Chambers, 2010). Higher “seaweed” ranking of those teas obtained after longer brewing times could be a negative descriptor for Spanish panelists due to the low intake of seaweed in Spain, compared to countries like Japan or Korea. Therefore, the best brewing time-temperature was 98ºC for 7 min, which was selected for the profiling of Spanish white and green teas.

3.3. Relationship between sensory properties and chemical composition of white tea

The correlation between the composition of tea and its sensory profile is an issue due to the myriad of chemical species that play a role on taste and aroma. For example, there is a link between the sensory quality of Oolong tea and its different volatile compounds (Lee *et al*., 2013; Ziu, Chen, Wang, Niu & Xiao, 2017); many of which are detected in green tea during sensory analysis. However, only few research were focused on the relationship between taste active chemical species and probed bioactivity (like catechins or caffeine) or healthy properties (i.e. antioxidant capacity) with sensory attributes. This is the reason why the authors decided to study the linear relationship among all the assessed chemical variables obtained during optimization of infusion time of white tea brewed at 98ºC with the taste and smell attributes described by descriptive sensory analysis (**Figure 34C**). Statistically significant linear correlations ($p < 0.05$) ranging from 0.6020 to 0.8998 were obtained between total polyphenols, antioxidant capacity and catechins-caffeine content with taste attributes like bitterness or astringency (**Table 16**), when compared in pairs. These are logical results since polyphenols are the main antioxidant compounds found in tea. In addition, it is important to bear in mind that tea catechins and caffeine play a role on tea astringency and bitterness (Tokuşoğlu, Ünal & Balaban, 2008) so that the correlation of these bioactive compounds with such sensory attributes with increasing infusion time could be expected. To deepen the contribution of each type of chemical, the correlations were grouped depending on the chemical nature-bioactivity of the compounds: polyphenols versus caffeine versus antioxidant capacity. It was observed that linear correlations of bitterness ranged from 0.6020-0.6976 for individual polyphenols. Such range is similar to that found for antioxidant capacity and total polyphenols (**Table 16**). This could be explained by taking into account that tea polyphenols are the main players of tea

antioxidant capacity. However, a higher linear correlation between bitterness and caffeine was found (0.8875), which could be related with the stronger bitter sensation of caffeine compared to the milder one of tea polyphenols (Eschenauer & Sweet, 2006). On the contrary, correlations between astringency and polyphenols (individual or total) as well as antioxidant capacity was higher with phenolic compounds (ranging from 0.8614 to 0.8998) than in caffeine (0.6489). It is widely recognized that polyphenols have a strong astringent sensation (Soares, Brandao, Mateus & de Freitas, 2017) that plays a role on food acceptance by consumers.

Table 16. Linear correlation between sensory attributes and chemical species.

Chemical species	Bitter	Astringent	Sweet	Persistent	Smell
Gallic acid	0.6298*	0.8904*	0.3394	0.2798	0.0398
Epicatechin	0.6376*	0.8998*	0.2386	0.2622	0.1214
Epicatechin gallate	0.6020*	0.8749*	0.2729	0.2735	0.0857
Epigallocatechin	0.6976*	0.8614*	0.2638	0.2502	0.0894
Epigallocatechin gallate	0.6787*	0.8849*	0.3062	0.3039	0.0470
Total polyphenols	0.6316*	0.8996*	0.2680	0.2792	0.1487
Caffeine	0.8857*	0.6489*	0.1457	0.1392	0.1965
$TEAC_{ABTS}$	0.6985*	0.8973*	0.2748	0.2453	0.1104
$TEAC_{FRAP}$	0.6392*	0.8724*	0.2435	0.2349	0.1042

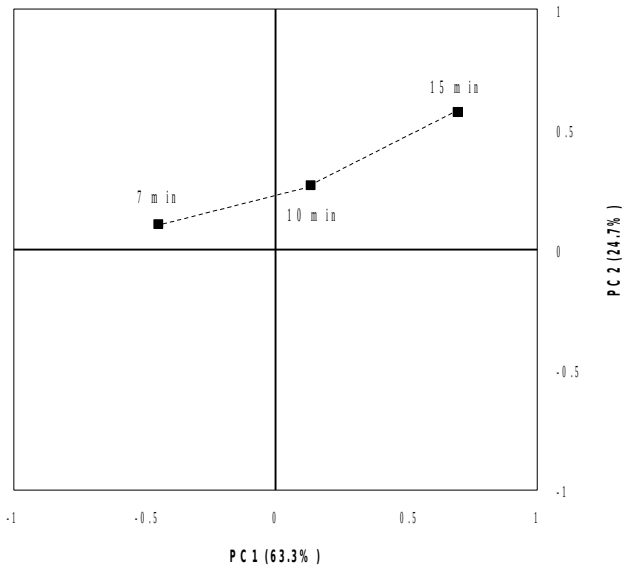
*Statistically significant correlation ($p < 0.05$)

On the other hand, no statistical correlation was obtained for smell attributes and persistency or sweetness. In the case of smell, this could be explained by taking into account that smell is related to volatile compounds (Ziu *et al*., 2017) and those measured in this study (catechins and caffeine) are not volatile. In addition, although the reason for the lack of correlation with persistency or sweetness is not fully understood, it can be assumed that this finding is related to lower differences of these sensory attributes, so that they cannot correlate with the concentrations of catechins and caffeine measured along the chemical analyses performed.

Other authors (Lee & Chambers, 2010) claimed that the correlation in pairs of sensory properties with chemical components of teas is difficult if such compounds are

not volatile. However, three years later, the same authors used PCA to describe the evolution of sensory profile of green tea (both aroma and taste descriptors) through five consecutive brewing steps, including data of volatile compounds obtained by GC-MS (Lee *et al*., 2013). Consequently, in order to solvent the lack of correlation of chemical compounds with sensory profile when compared by pairs during optimization of brewing time, the researchers decided to perform a PCA analysis including sensory attributes as well as chemical species. Such principal component analyses allowed for obtaining a small number of linear combinations of the 16 parameters assessed that explained as much as possible, data variability: The first component explained a 63.3% of sample variability while the second explained an additional 24.7% (total variability correlated was 88%). As depicted in the PCA biplot (**Figure 35**), the general trend of white tea with increasing infusion time was to intensify aroma, taste and bioactive compounds content. Although 7 min was selected as the optimum infusion time at 98°C, larger infusion times may be enjoyed by consumers who prefer stronger flavor, astringency and bitterness.

Figure 35. Principal component analysis of sensory properties and chemical composition of white teas depending on brewing time at 98ºC.



3.4. Antioxidant capacity differences between commercial white and green teas

The usual brewing temperature to obtain green tea infusions is 90-98ºC (boiling water) while that for white tea is around 70ºC (Cabrera *et al*., 2003; Damiani *et al*., 2014; Castiglioni *et al*., 2015). Such infusion temperature plays a role both in the organoleptic properties of the tea brew as well as in the amount of bioactive compounds extracted. However, as discussed in the previous section, both the consumer and the analytically trained panelists preferred the white tea infusion obtained after brewing at 98ºC for 7 min. Therefore, these extraction conditions were used to test the differences in the antioxidant capacity and total phenols of commercial white (n = 34) and green teas (n = 46) sold in Spain (**Table 17**). The effect of tea form (loose leaves Vs. bagged teas) was also evaluated. A statistically higher antioxidant capacity ($p < 0.05$) was found in green teas compared to white teas, whichever the physical form of tea and antioxidant method. These results are in line with those reported by other authors (Unachukwu, Ahmed, Kavalier, Lyles & Kennelly 2010; Carloni *et al*., 2013) who described a slightly higher antioxidant capacity of green tea compared with white tea. The same differences were observed for total phenols, which could be attributed to a higher content of catechins in green tea leaves compared to those of white teas, related with the maturity state (Zhang, Li, Ma & Tu, 2011; Zhao, Chen, Lin, Harnly, Yu & Li, 2011). As expected, bagged teas showed a higher antioxidant capacity that loose leaves teas, both for white and green teas (**Table 17**). The phenomenon has been explained by other authors (Komes *et al*., 2010; Sharpe, Hua, Schuckers & Andrescu, 2016) taking into account that loose leaves are usually hand-rolled into tiny pellets, so the extraction time is most likely insufficient to extract a higher antioxidant content. On the contrary, bagged teas have lower quality but they are ground in fine particles so the extraction efficiency of bioactive compounds is higher than in loose leaves teas.

Table 17. Antioxidant capacity and total phenols of commercial white and green teas.

Tea type	$TEAC_{ABTS}$ (mmol Trolox/L)	$TEAC_{FRAP}$ (mmol Trolox/L)	Total phenols (mg gallic acid/L)
WTL	3.7 ± 0.3^{a}	2.8 ± 0.1^{a}	885 ± 64^{a}
WTB	5.2 ± 0.3^{b}	5.4 ± 0.3^{b}	906 ± 77^{a}
GTL	6.0 ± 0.2^{c}	3.8 ± 0.2^{c}	1043 ± 5^{b}
GTB	10.8 ± 0.3^{d}	7.9 ± 0.2^{d}	1131 ± 96^{b}

Different letters indicate statistically significant differences $p < 0.05$.
WTL: White tea leaves; WTB: White tea bag; GTL: Green tea leaves; GTB: Green tea bag.
$TEAC_{ABTS}$: Trolox Equivalent Antioxidant Capacity measured with the ABTS method.
$TEAC_{FRAP}$: Trolox Equivalent Antioxidant Capacity measured with the FRAP method (Ferric Reducing Capacity of Plasma).

Every food consumed has an impact on the overall antioxidant capacity, with the corresponding effect on human health. Thus, the contribution of white and green tea consumption on the daily intake of antioxidant compounds and polyphenols was calculated. The mean antioxidant capacity intake in Spain range from 6014 to 3549 μmol Trolox equivalents/day for the FRAP and ABTS methods, respectively (Saura-Calixto & Goñi, 2006). The consumption of tea in Spain in 2016 was 36.6 g/inhabitant/ year, corresponding to 0.10 g/inhabitant/day (Mercasa, 2017). Thus, an intake of 7-89 μmol Trolox equivalents/day could be expected (**Table 18**), which means a contribution of 0.9-2.5% of the daily antioxidant activity intake for the ABTS method and 0.1-0.3% for the FRAP method. Although such contribution is calculated taking into account the mean intake in Spain, a realistic approach could be the calculation based on the intake of antioxidant capacity per serving (150 mL). For the ABTS method, the contribution increased up to 16-46% of the daily intake and 2-5% for the FRAP method (**Table 18**). Thus, one serving provides a high amount of antioxidant capacity as far as 1620 μmol Trolox equivalents. The differences found depending on the type of tea and physical presentation should be highlighted. Therefore, green tea provides the highest antioxidant capacity, although a serving of white tea also contains up to 780 μmol Trolox equivalents (with probably superior sensory properties). Another point to take into

account is that bagged teas contributed almost doubled the value of loose leaves to the daily intake of antioxidant capacity.

In the case of polyphenols, the daily intake of polyphenols in Spain (Saura-Calixto & Goñi, 2006) is 1171 mg gallic acid equivalents. Thus, tea intake contributes only to 0.6-0.8% of the daily polyphenols intake. However, when a serving (150 mL) is used for calculations, the contribution of teas to the daily intake of polyphenols reaches 11-14%, since each serving provides around 150 mg of polyphenols. In this case, no large differences were observed between white-green teas of bagged-loose leaves teas.

Table 18. Contribution of tea consumption to the daily antioxidant activity (AOX) and polyphenols intake in the Spanish diet.

Tea type	**Analytical assay**	**AOX/daily intake**[1] (μmol trolox/day)	**Contribution to daily antioxidant capacity intake** (%)	**AOX/serving intake**[2] (μmol trolox/serving)	**Contribution to daily antioxidant capacity intake** (%)
WTL	$TEAC_{ABTS}$	31	0.9	555	16
	$TEAC_{FRAP}$	7	0.1	135	2
WTB	$TEAC_{ABTS}$	43	1.2	780	22
	$TEAC_{FRAP}$	8	0.1	150	2
GTL	$TEAC_{ABTS}$	50	1.4	900	25
	$TEAC_{FRAP}$	12	0.2	225	4
GTB	$TEAC_{ABTS}$	89	2.5	1620	46
	$TEAC_{FRAP}$	17	0.3	300	5
Tea type	**Analytical assay**	**Polyphenols/daily intake**[1] (mg/day)	**Contribution to daily polyphenols intake** (%)	**Polyphenols/serving intake**[2] (mg/serving)	**Contribution to daily polyphenols intake** (%)
WTL	*Folin-Ciocalteu*	7	0.6	133	11
WTB	*Folin-Ciocalteu*	7	0.6	136	12
GTL	*Folin-Ciocalteu*	9	0.7	156	13
GTB	*Folin-Ciocalteu*	9	0.8	170	14

[1]Considering tea consumption for a whole year.

[2]Consideing the complete serving ingested a particular day.

WTL: White tea leaves; WTB: White tea bag; GTL: Green tea leaves; GTB: Green tea bag.

$TEAC_{ABTS}$: Trolox Equivalent Antioxidant Capacity measured with the ABTS method.

$TEAC_{FRAP}$: Trolox Equivalent Antioxidant Capacity measured with the FRAP method (Ferric Reducing Capacity of Plasma).

4. Conclusions

After chemical and sensory analysis, optimal infusion conditions for white tea were set at a water temperature of 98ºC and a brewing time of 7 min. Under such conditions, a large amount of bioactive compounds and antioxidant capacity can be extracted into the tea brew while obtaining a pleasant mildly bitter-astringent brew with flowers and citrus notes. In addition, although Spanish commercial green teas have a higher antioxidant capacity, the daily contribution of a white tea cup should not be underestimated since the amount of polyphenols provided to the Spanish diet is noteworthy. Therefore, this research stresses the importance of linking sensory and chemical data to obtain the best sensorial quality and the optimal healthy properties in white tea infusions.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Furosine and 5-hydroxymethylfurfural as chemical markers of tea processing and storage

En este trabajo se describe cómo durante el tratamiento térmico del té (las etapas de secado) así como durante el almacenamiento de las hojas de té, se desarrolla la reacción de Maillard, con las consiguientes modificaciones en la composición química del té. Así, se demuestra que se ve afectado el contenido en lisina (aminoácido esencial) y en catequinas (compuestos antioxidantes). Estudiar la influencia del tratamiento térmico y, por consiguiente, del pardeamiento químico en alimentos es uno de los objetivos principales de la presente tesis doctoral.

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Furosine and 5-hydroxymethylfurfural as chemical markers of tea processing and storage

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ABSTRACT

One of the most consumed beverages around the world is tea, but little is known about how tea quality can be monitored during storage and processing. Thus, the objective of this research was to study the usefulness of furosine and 5-hydroxymethylfurfural (HMF) content -two Maillard reaction markers- as quantitative parameters that control tea processing and storage. Two different experiments were carried out: one using green and black teas (processed at laboratory scale) and another one using commercial green, white, black and red teas that were under storage for one year (shelf life) and under accelerated conditions (55ºC at 40% of humidity for 3 months). The samples were analyzed for the content of furosine, HMF, and free and total lysine. The combination of HMF and furosine was useful as an indicator to control tea processing and storage regardless the type of tea. HMF and furosine are useful chemical indicators (when combined) to monitor tea quality during processing and storage. Furosine analysis is especially sensitive to control the first drying steps, while HMF reflects those changes occurred in the last tea processing steps.

KEYWORDS: Tea, heat processing, storage, Maillard reaction, furosine, HMF.

ABBREVIATIONS: HMF: 5-hydroxymethyl-furfural.

1. Introduction

Tea is consumed as infusion of dried tea leaves after several steps of processing, where oxidation of the polyphenols can occur. Several types of teas are obtained depending on the intensity of the drying process and whether they suffer or not an ulterior fermentation (Hilal & Engelhardt, 2007). Fermentation is actually an oxidation carried out by the enzymes polyphenol oxidase and peroxidase released in broken leaves. Oxidation converts catechins into theaflavins and thearubigins, and chlorophylls into other pigments (pheophytins and pheophorbides) modifying all of the flavor and color. Green, red and black teas suffer a more harmful treatment than white tea. After harvesting, they all are withered. Green tea is firstly steam-dried, rolled up and finally, dried with hot air (Alcázar et al., 2007). However, red and black teas, after harvesting and withering, are rolled up to break up cell structure allowing to undergo a partial oxidation process (in the case of red tea) or a complete oxidation process (in black tea) (Vuong, 2014). This process oxidizes catechins into more complex polyphenols.

Tea processing could affect its beneficial properties due to the loss or modification of its bioactive compounds during the treatments (Gorjanović et al., 2012). In addition, a decrease in bioactive compounds and antioxidant capacity has been reported to occur during the storage of tea and other plants commonly used for infusion (Jiménez-Zamora, Delgado-Andrade, & Rufián-Henares, 2016). The chemistry of tea leaves is also modified through processing and storage by the development of the Maillard reaction, which has been previously described in tea beverages (Zhang et al., 2012). The reaction starts with condensation among the carbonyl group of a reducing sugar and a free amino group of an amino acid or a protein. Along the different steps involved in the Maillard reaction, different molecules can be used as chemical markers

to evaluate the extension of the Maillard reaction as well as heat damage (Rufián-Henares & Pastoriza, 2016). Furosine (ε-N-2-furoylmethyl-L-lysine) is an amino acid derivative formed during acid hydrolysis of Amadori compounds, which are formed in the early stage of the Maillard reaction from the interaction of the ε-amino groups of lysine with glucose, lactose and maltose (Resmini & Pellegrino, 1999). Maillard reaction medium stages have been widely monitored through the use of 5-hydroxymethylfurfural (HMF) (Berg & Boekel, 1994). However, HMF also has a potential toxicological effect since it can exert genotoxic and mutagenic activity through metabolic activation to 5-sulfooxymethylfurfural, giving rise to the generation of DNA adducts in human beings (Pastoriza de la Cueva et al., 2017).

In order to guarantee tea quality, the tea industry consequently applies different quality control measures such as sensory analysis as well as the presence of contaminants like pesticides or heavy metals (Salvador, Lopes, Filho, & Zucchi, 2002). Thus, the novelty of this study resides on the use of Maillard reaction products to monitor tea quality along processing and storage. Therefore, the main objective proposed for the present study was to investigate whether some Maillard reaction products (i.e. Furosine and HMF) can be monitored along the processing-storage line of tea leaves. Thus, these chemical species can be proposed as useful indicators for tea quality control, being useful to diminish nutritional losses.

2. Materials and methods

2.1 Reagents

FMOC-Cl (9-Fluorenylmethoxycarbonyl chloride), boric acid, lysine, and HMF were obtained from Sigma-Aldrich (St. Louis, USA). Furosine standard was obtained from NeoMPS (Strasbourg, France). Bidistilled deionized water was obtained from a Milli-Q system (Millipore, Milford, MA).

2.2 Samples

Tea plants (*Camellia sinensis*) were acquired from a garden center (Viveros Moreira, A Coruña, Spain). Fresh leaves were processed in triplicate in the laboratory in order to obtain green and black tea according to procedure of McKinley & Jamieson, (2009) **(Table 19)**:

- *Black tea:* the first step undertaken was withering, while tea leaves were subjected to a temperature of 20-22ºC for 12-18 hours until they reached a moisture content of 55-58%. Next step was rolling up of the tea leaves by hand at room temperature for 15-24 min. The leaves were then fermented/oxidized for 1-2 hours at 22-30ºC and at 90-95% of humidity. Next, there was a progressive drying in four stages: The first one was carried out for 40 min at 110ºC until reaching a 50% of moisture; the second drying step lasted 30 min at 50ºC, until tea leaves attained 30% of moisture; the third drying process was 40 min long at 90ºC, reaching a 13% of moisture. Finally, the last drying took 25-30 min at 70-90ºC until the tea leaves attained 3-5% of moisture content.

- *Green tea:* Fresh leaves were subjected to a pre-drying process for 2 h at room temperature. The next step consisted on a more effective drying process at 95-100ºC for 45-60 seconds until tea leaves reached 75% of moisture content. They were then rolled

up, which was followed by another drying phase at 110ºC for 40 min until the tea leaves attained 50% of moisture. Thereafter, a three-step progressive drying process was undertaken under the same conditions described for black tea, without rolling up. At each step (1-6; **Table 19**), tea leaves were freeze-dried before each analysis in order to express data in dry weight.

Moreover, in order to study the effect of storage on tea, eight commercial tea samples (packaged in tea bags) of each type (green, white, black and red (oolong) tea) were purchased in specialized tea-shops in Granada (Spain). These commercial samples were analyzed in triplicate at three different times as previously proposed:

- right after acquiring (time 0).
- after one year of storage at room temperature under controlled humidity (40%; product shelf life).
- after three months of storage at 55ºC under controlled humidity (40%; accelerated storage).

The corresponding teas obtained after laboratory processing or storage were frozen at -80ºC until analysis.

Table 19. Green and black tea processing carried out in the laboratory.

Processing steps		Black Tea (BT)		Green Tea (GT)
Withering	1BT	12-18 hours 20-22 ºC 55-58% moisture content		
Rolling up		15-24 minutes Room temperature Hand-made		
Fermentation/ oxidation	2BT	1-2 hours 22-30 ºC 90-95% humidity		
Previous drying			1GT	2 hours Room temperature
Drying			2GT	45-60 seconds 95-100 ºC 75% Moisture content
Rolling up and drying	3BT	Rolling up 40 minutes 110 ºC 50% Moisture content	3GT	Rolling up 40 minutes 110 ºC 50% Moisture content
		Rolling up		30 minutes

		30 minutes		50 ºC
Rolling up and drying	4BT	50 ºC	4GT	30% Moisture content
		30% Moisture content		
		Rolling up		40 minutes
		40 minutes		90 ºC
Rolling up and drying	5BT	90 ºC	5GT	13% Moisture content
		13% Moisture content		
		Rolling up		No rolling up
		25-30 minutes		25-30 minutes
Final drying	6BT	70-90 ºC	6GT	70-90 ºC
		3-5% moisture content		3-5% Moisture content

2.3 Chemical indicators of non-enzymatic browning

Furosine assay

Furosine determination was performed following the method described by (Rufián-Henares, García-Villanova, & Guerra-Hernández, 2008). 0.250 g of tea samples were hydrolyzed with 4 mL of 7.95 M HCl at 110°C for 23 h in a Pyrex screw-cap vial with PTFE-faced septa. High-purity N_2 gas was bubbled through the solution for 2 min. The hydrolysate was filtered with a medium-grade filter paper. A 0.5 mL portion of the filtrate was applied to a Sep-pack C_{18} cartridge (Millipore, MA) pre-wetted with 5 mL of methanol and 10 mL of deionized water and was then eluted with 3 mL of 3 M HCl. Fifty microliters of the solution was analyzed by ion-pair RP-HPLC,

which consisted of a HPLC system (Thermo Fisher Accela 600) equipped with a quaternary pump, an autosampler, a column oven and a variable wavelength UV–vis detector (PDA) set at 280 nm. The analytical column was a C_{18} column Tracer Extrasil ODS2 5 µm 250 x0.4 from Teknokroma thermostated at 32°C. The mobile phase was water:acetonitrile (80:20), 1.022 g of sodium heptanosulphonate and 0.1% of formic acid. Running time was 15 minutes. Quantification was performed by the external standard method, using a commercial standard of pure furosine. Furosine was quantified by the external standard method within the range 0.01–1,000 mg/L. LOQ and LOD were respectively 0.1 µg/L and 0.05 µg/L. The R2 of the calibration curve was 0.9987. The recovery study was carried out by adding incresing concentrations of furosine to a tea beverage of know furosine content. The range of furosine concentrations added were 0.25-1 mg/mL. The recovery obtained was 98.4%. Repeatability was tested intraday and interday for which we obtained variation coefficients (CV) of 1.23 and 3.25% respectively. The analysis was performed in triplicate and the data are the mean values expressed as micrograms per g of dried tea leaves.

HMF

HMF determination was performed according to the method described by (Rufián-Henares et al., 2008) in each tea beverage obtained, previously filtered (0.45 µm). No previous extraction was requiered. The HPLC system, and the analytical column were the same as that of the furosine analysis. The mobile phase was composed of a mixture of acetonitrile in water (5% v/v) delivered at the flow rate of 1 mL/min under isocratic conditions for a 15 minutes run. The UV detector was set at 284 nm and 20µL were injected. HMF was quantified by the external standard method within the range 0.01–5.00 mg/L. LOQ and LOD were respectively 0.6 µg/L and 0.1 µg/L. The

R2 of the calibration curve was 0.9996. The recovery study was carried out by adding incresing concentrations of HMF to a tea beverage of know HMF content. The range of HMF concentrations added were 0.25-1 μg/mL. The recovery obtained was 97.97%. Repeatability was tested intraday and interday for which we obtained variation coefficients (CV) of 1.76 and 2.95% respectively. The analysis was performed in triplicate and the data are the mean values expressed as micrograms per g of dried tea leaves.

2.4 Lysine content in tea sample

The analysis of lysine content in each type of tea (both processed and commercial teas) was performed by means of the method of Horanni & Engelhardt, (2013). The derivatization procedure was performed with of the hydrolizate obtained for furosine analysis (total lysine) as well as the infusion prepared for the analysis of antioxidant capacity (free lysine). Four hundred μL of hydrolizate or tea beverage were mixed with 100 μL of potassium borate buffer (0.5 M, pH 8.5) and 500 μL of FMOC-Cl (3 mM in acetonitrile), mixed on a vortex and incubated at room temperature for 10 min. The reaction was then stopped by addition of 100 μL of acetic acid (1 M). Finally, the derivatized sample was filtrated through a 0.45 mm nylon membrane filter. The analysis of lysine derivate was performed on the same HPLC system used for furosine and HMF analysis. For gradient elution, mobile phase A was 0.1 M sodium acetate buffer containing 0.05% (v/v) triethyl amine and adjusted to pH 5.8 with glacial acetic acid. Mobile phase B was a mixture of acetonitrile/water (80:20, v/v). The gradient elution was as follows: 80% A and 20% B (for 5 min), then 10 min to 30% B (for 8 min), then 3 min to 32% B, then 9 min to 38% B, then 3 min to 50% B, then 5 min to 60% B, then 5 min to 100% B (for 8 min) and finally come back in 2 min to 20% B (and the column being equilibrated for 12 min). The flow rate was 0.25 mL/min and the

injection volume was 5 µL. The detection wavelength was 262 nm. LOQ and LOD were respectively 0.91 µg/mL and 0.24 µg/mL. The R2 of the calibration curve was 0.9988. The recovery study was carried out by adding incresing concentrations of Lysine to a tea beverage of know Lysine content. The range of lysine concentrations added were 0.25-1 mg/mL. The recovery obtained was 97.27%. Repeatability was tested intraday and interday for which we obtained variation coefficients (CV) of 2.24 and 4.13% respectively. The analysis was performed in triplicate and the data are mean values expressed as mg/g of dried tea leaves.

2.5 Statistical analyses

Statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by Duncan Multiple Range test to compare the means that showed significant variation ($P < 0.05$). When the comparisons were carried out exclusively between green and black teas, a Student's t-test was applied. Evaluation of the correlation between assays was carried out by computing the relevant correlation coefficient (Person linear correlation) at $P < 0.05$ confidence level. All the statistical analyses were performed using Statgraphics Plus software (Statpoint Technologies, Inc., The plains, VA, USA), version 5.1, 2001.

3. Results and discussion

Some indicators of Maillard reaction such as furosine (marker of the first steps) and HMF (intermediate stages) have been previously demonstrated as reliable markers of thermal treatment in different vegetables (Rufián-Henares et al., 2008). Moreover, tea has substrates for Maillard reaction in its composition: lysine, reducing sugars and quinone derivatives. Total carbohydrate and total protein are 25% and 15% (dry weight), respectively (Graham, 1992). Lysine content is 9.65 mg/g and (Li & Ni, 2016), reducing sugar content is 19.7 mg/g (glucose 6.8 mg/g, fructose 7.2 mg/g, and lactose 5.7 mg/g) (Shanmugavelan et al., 2013). The concentration of these precursors is quite homogeneous regardless the type of tea (Cabrera & Giménez, 2010), so that it is not expected they could play a role in variation on the formation of Maillard reaction products. Thus, the researchers decided to analyze such chemical species in the different steps of tea processing (laboratory-made teas) along with tea storage time (commercial teas, shelf life). However, in the case of black tea, quinones are formed from the oxidation of tea catechins (Tanaka & Kouno, 2003). These chemical species are highly reactive compounds that can attack nucleophilic groups such as lysine (Akagawa, Shigemitsu, & Suyama, 2005). Therefore, it could be expected a higher development of the Maillard reaction in black tea.

3.1 Laboratory made teas

3.1.1 Furosine analysis

The furosine evolution along green and black teas processing is shown in **Figure 36A**. There was a gradual increase of furosine levels from the first stage to the last one, which is a logical result since as processing advances, more drying steps are applied and thus thermal damage increases. In the case of **green tea**, furosine levels increased up to step 4, then decreased gradually in stage 5 and 6 of green tea processing (**Figure 36A**, grey bars). The larger increase in furosine content was observed in step 4, which could be related with the high temperature used in such step (50ºC) and intermediate moisture content (from 30 to 13%) (Rufián-Henares & Pastoriza, 2016). Then, furosine levels dropped, which could be related with the decrease of initial substrates (amino groups and sugars) and intermediates (i.e. Amadori compound N-ε-fructosellysine) to proceed with the Maillard reaction to later steps (Rufián-Henares & Pastoriza, 2016). This was in line with the decrease on free (**Figure 36B**, grey bars) and total lysine (**Figure 36C**, grey bars). Free lysine decreased significantly ($P < 0.05$) in the first and second steps, with the first drying processes; then it remained unchanged in step 3 (as furosine did) and a large decrease was obtained for step 4, correlating with the highest furosine content during green tea processing (**Figures 36B** and **36A**, respectively); no further decrease of free lysine was observed for steps 5 and 6. In a complementary way, total lysine was almost stable during the first three processing steps (**Figure 36C**), which could indicate that the Maillard reaction proceeded in the first drying processes mostly through free lysine reaction. However, a statistically significant decrease ($P < 0.05$) on total lysine levels (**Figure 36C**, grey bars) was observed for the last three steps (4-6), which will support how the Maillard reaction

progressed by means of the lysine contained within tea proteins when more drastic drying processed were achieved.

In **black tea**, furosine levels increased gradually from step 1 to step 4 suffering in step 5 a massive increase (**Figure 36A**, black bars). This could be related with the delayed increase of furosine, compared to that of green tea: i) The first oxidation steps in black tea processing (stages 1-2) did not produced a statistically significant increase in furosine levels ($P > 0.05$) due to the absence of thermal treatment (**Table 19**), although some reactive quinones should be generated (Tanaka & Kouno, 2003); ii) The next two steps tripled the furosine content due to the amount of heat applied to the fermented tea leaves. As depicted in **Figures 36B** and **36C** (black bars), the transformation of substrates (mainly free lysine, since no differences were obtained for total lysine) along the fermentation process could explain why furosine levels increased less in black tea during step 4, compared to green tea; iii) Finally, the last drying steps (5-6) gave rise to a strong furosine development in black tea, while furosine decreased in green tea. This could be linked with the previous degradation of free lysine (steps 3-4) in the oxidized tea leaves; thus, the lower moisture (low water activity) and the intense heat treatment could favor the reaction of the ε-amino groups of those lysine found in tea proteins with sugars and the highly reactive quinones (Tanaka & Kouno, 2003). (**Figure 36C**, black bars). In addition, the presence of four rolling up steps (Table 1) in black tea processing (compared with green tea, in which only 1 rolling up step was used) could favor the rupture of tea cells, then releasing more sugars, which in turn could improve their reaction with the ε-amino group of lysine attached to the protein skeleton; this could allow a larger development of the Maillard reaction in black tea. Therefore, furosine could be a valuable-sensitive marker to control tea processing regardless the type of tea.

3.1.2 HMF analysis

HMF results are depicted in **Figure 36D**. HMF appeared during the third processing step of **green tea** (the first drastic drying); no changes were obtained for the fourth step (a milder drying at 50ºC). Then a statistically significant increase in HMF levels ($P < 0.05$) was obtained for stage 5 (the second drastic drying at 90ºC). Finally, HMF decreased in step 6: the decrease of furosine-HMF in the last processing step, shortage of free lysine degradation in the last three steps and slow total lysine degradation in the last drying step could indicate the progression of the Maillard reaction to advanced steps through the degradation of furosine intermediates like the Amadori compound N-ε-fructosellysine (Rufián-Henares & Pastoriza, 2016). On the contrary, in the case of **black tea**, HMF statistically increased ($P < 0.05$) in steps 3, 5 and 6, in line with the decrease of total lysine. This could indicate the progression of the Maillard reaction and a higher thermal damage of the samples so obtained (Rufián-Henares & Pastoriza, 2016). Thus, HMF analysis could improve the evaluation of tea processing through furosine analysis.

3.2 Commercial teas

The researchers then analyzed total and free lysine, furosine and HMF in commercial green, white, black and red teas, as soon as they had been acquired, after one-year storage (shelf life) and after three-months storage at high temperature (accelerated storage). In the case of total lysine, a statistically higher amount ($P < 0.01$) was found in commercial white teas (**Table 20**). Tea storage for 1 year decreased the levels of total lysine in a non-significant manner except for white tea. However, the storage under accelerated conditions gave rise to a decrease on total lysine for all the

samples, probably because it was reached enough energy to favor the Maillard reaction in the lysine contained within tea proteins. Free lysine values ranged from 0.11 to 0.26 mg/g of dry tea leave for red and white teas, respectively (**Table 20**). These values are in line with those reported for similar teas by other authors (Horanni & Engelhardt, 2013; Wang, Tsai, Lin, & Ou, 2006). The behavior of free lysine during storage was similar to that of total lysine.

Table 20. Total and free lysine and heat treatment indicators in commercial stored teas.

Tea	Storage	Total lysine[1]	Free lysine[1]	HMF[2]	Furosine[2]
Green	0	1.36 ± 0.29[a]	0.12 ± 0.02[a]	0.19 ± 0.08[a]	1233 ± 640[a]
	1 year	1.32 ± 0.12[a]	0.11 ± 0.01[a]	4.23 ± 2.92[b]	1275 ± 388[a]
	55/3	0.87 ± 0.07[b]	0.06 ± 0.01[b]	40.39 ± 16.27[c]	-
White	0	1.89 ± 0.32[a]	0.26 ± 0.05[a]	ND	1068 ± 391[a]
	1 year	1.46 ± 0.13[b]	0.20 ± 0.03[b]	5.38 ± 6.44[b]	1337 ± 435[a]
	55/3	0.98 ± 0.14[c]	0.11 ± 0.05[c]	33.35 ± 11.63[c]	-
Black	0	1.27 ± 0.30[a]	0.13 ± 0.01[a]	1.05 ± 0.03[a]	1246 ± 694[a]
	1 year	1.24 ± 0.11[a]	0.11 ± 0.02[a]	9.18 ± 8.31[b]	1432 ± 717[a]
	55/3	0.87 ± 0.12[c]	0.05 ± 0.02[b]	85.68 ± 31.07[c]	-
Red	0	1.25 ± 0.31[a]	0.11 ± 0.02[a]	ND	1121 ± 354[a]
	1 year	1.21 ± 0.10[a]	0.10 ± 0.01[a]	7.23 ± 3.10[b]	1159 ± 352[a]
	55/3	1.01 ± 0.12[b]	0.06 ± 0.02[b]	9.97 ± 8.85[c]	-

[1] Total and free lysine expressed as mg g^{-1} of tea leaves (dry weight).

[2] HMF and furosine expressed as μg g^{-1} of tea leaves (dry weight).

Different letters within the same column and type of tea indicate statistically significant differences ($P < 0.05$).

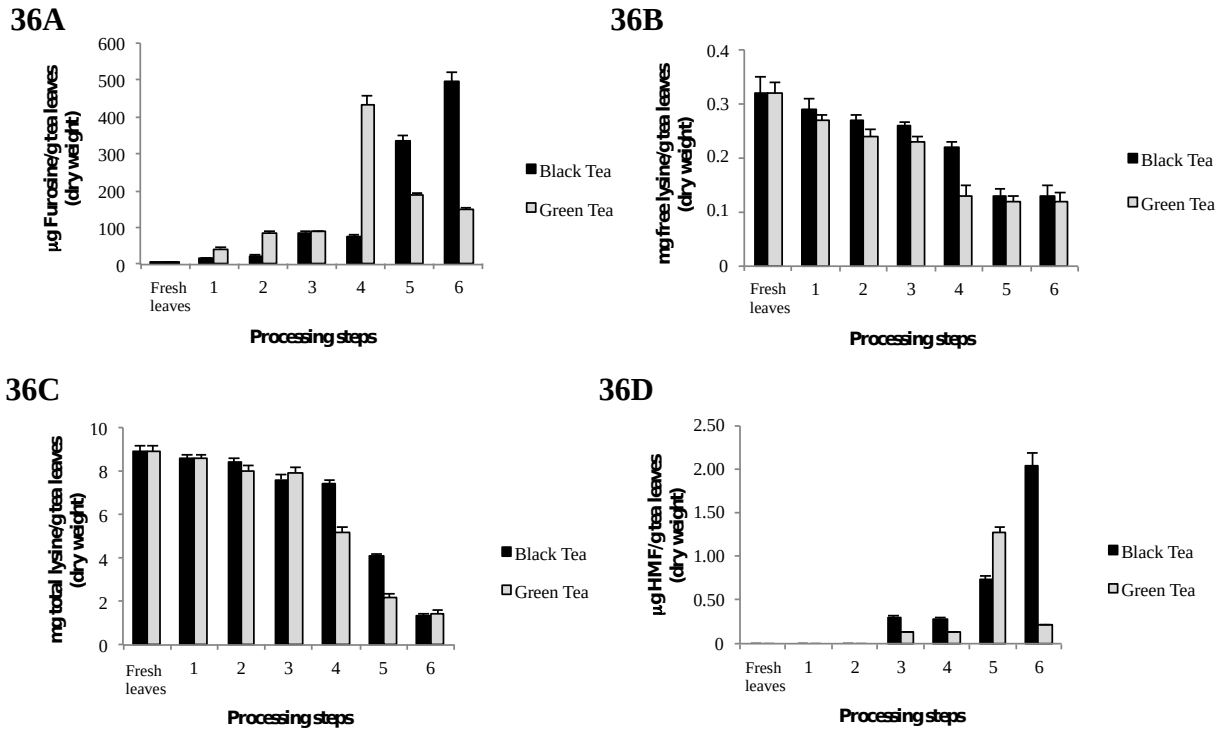
ND: not detected.

Regarding furosine, there were no significant differences ($P > 0.05$) among green, white, black and red teas (**Table 20**) right after purchasing them (furosine values ranged from 1.068 to 1246 μg/g of dry tea leave for white and black teas). The absence of statistical significance could be explained taking into account that although white tea suffers a less aggressive drying process, furosine is an indicator for the initial stages of Maillard reaction, so that the energy needed to generate furosine is quite low and enough with the treatment that such teas had undergone. Finally, tea storage gave rise to the increase of furosine levels in all types of tea, although no statistically significant differences were obtained ($P > 0.05$).

In the case of HMF, it was detected in commercial green and black teas (**Table 20**). This is in line with the results obtained for processed teas **(Figure 36D**). HMF was detected after applying high drying temperature step in black tea, which was related to the aggressive drying process combined with the fermentation process (providing more substrates like quinones and energy for the Maillard reaction). On the contrary, during green tea processing, less substrates are available for the Maillard reaction, so that the generation of HMF is lower. Moreover, after a year of storage at room temperature, there was a statistically significant increase in HMF levels ($P < 0.05$) in all types of teas (**Table 20**). However, although HMF levels were higher in the binomial black-red tea compared to white-green teas, they were not different within each pair of samples. These teas were also subjected to storage at 55ºC for 3 months. In this case, the higher values were obtained again in black tea (**Table 20**), followed by green, white and red teas ($P < 0.05$). The higher increase of HMF levels under accelerated conditions could be attributed to the higher thermal load, which in turn provided more energy to exceed the activation energy of the Maillard reaction. The initial steps (measured through furosine) led to intermediate stages, where HMF is formed. Therefore, although

furosine could be a marker of tea processing, it can be concluded that HMF could be a valuable marker of storage conditions, since room temperature *per se* is enough to proceed with the development of the Maillard reaction leading therefore to an accumulation of HMF at the usual temperature and humidity conditions of storage.

Figure 36. Effect of processing steps on furosine (36A), free lysine (36B), total lysine (36C) and HMF (36D) levels of green and black teas. Processing steps for <u>black tea</u>: fresh leaves (no treatment applied), withering (1), oxidation (2), drying: 110ºC, 50% moisture content (3), drying: 50ºC, 30% moisture content (4), drying: 90ºC, 13% moisture content (5), drying: 70-90ºC, 3-5% moisture content (6). For <u>green tea</u>: Room temperature drying (1), second drying 95-100ºC, 75% moisture content (2), third drying 110ºC, 50 % moisture content (3), drying: 50ºC, 30% moisture content (4), drying 90ºC, 13% moisture content (5), drying: 70-90ºC, 3-5% moisture content (6). HMF: 5-hydroxymethylfurfural.



Conclusions

The study of tea processing has demonstrated that the combination of furosine and HMF analysis could be a reliable method to control drying conditions for black and green teas. As HMF and furosine results indicate, the Maillard reaction advances along tea processing and storage, showing that these molecules could serve as good indicators to monitor tea quality during both processing and storage.

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Effect of in vitro digestion fermentation on green and roasted coffee bioactivity: the role of the gut microbiota

Dentro de los objetivos principales de la presente tesis doctoral se encuentra la digestión gastrointestinal in vitro y fermentación de distintos alimentos. Así, en este trabajo se evalúa la capacidad antioxidante y perfil fenólico tanto del café verde como tostado, tras ser sometidos una digestión y fermentación in vitro. Además de poder evaluar la capacidad antioxidante del café (uno de los objetivos principales) también nos permite evaluar el efecto del procesado térmico (tostado) sobre dicha actividad biológica. Por otro lado, la inclusión de la etapa de fermentación in vitro nos ha permitido cuantificar el efecto tanto del café verde como tostado en la composición de las comunidades microbianas y en su funcionalidad, probando la utilidad del modelo de digestión-fermentación desarrollado en la presente tesis doctoral.

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Effect of *in vitro* digestion fermentation on green and roasted coffee bioactivity: the role of the gut microbiota

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ABSTRACT

Coffee is one of the most consumed beverages and has been linked to health in different studies. However, green and roasted coffees have different chemical composition and therefore their health properties might differ as well. Here, we study the effect of *in vitro* digestion-fermentation on the antioxidant capacity, phenolic profile, production of short-chain fatty acids (SCFAs), and gut microbiota community structure of green and roasted coffee brews. Roasted coffees showed higher antioxidant capacity than green coffees, with the highest level achieved in fermented samples. Polyphenol profile was similar between green and roasted coffees in regular coffee brews and the digested fraction, but very different after fermentation. Production of SCFAs was higher after fermentation of green coffee brews. Fermentation of coffee brews by human gut microbiota led to different community structure between green and roasted coffees. All these data suggests that green and roasted coffees behave as different types of food.

KEYWORDS: coffee, polyphenols, Maillard reaction, antioxidant capacity, short chain fatty acids, gut microbiota.

1. Introduction

Coffee is the food product most consumed in the world after water (Farah & Ferreira dos Santos, 2015). Coffee has been related to health since ancient times and still today is studied frequently in many research projects related to chronic diseases (Galluzzi Bizzo, Farah, Ann Kemp, & Berguinins Scancetti, 2015). The protective role of coffee on health is thought to be mostly related to its high antioxidant capacity and is also linked to its high consumption (Saura-Calixto & Goñi, 2006). Coffee brews have a wide range of antioxidant compounds such as different types of phenolics (hydroxycinnamic acids such as caffeic, ferulic, coumaric and chlorogenic acids), aromatic compounds and Maillard reaction products such as melanoidins (Pastoriza & Rufián-Henares, 2014). Polyphenols have been reported to exert a protective role against several diseases such as cardiovascular or neurodegenerative diseases and cancer thanks to their ability to protect cells against oxidation (Galluzzi et a., 2015). Further, 5-caffeoylquinic acid (a chlorogenic acid) has been shown to have a protective role against neurodegenerative diseases (Jeszka-Skowron, Stanisz, & De Peña, 2016). Melanoidins which are produced during roasting process, are also potent antioxidants (Jiménez-Zamora, Pastoriza & Rufián-Henares, 2015).

During the roasting step, most of the aromatic compounds of coffee are generated while others are affected (mostly chlorogenic acids) by the heat treatment. During this process, some other compounds (like melanoidins or quinic acid) appear, which contributes to coffee's antioxidant capacity (Ludwig, Bravo, De Peña, & Cid, 2013). Generally, the main changes in composition involve loss of polysaccharides, oligosaccharides, chlorogenic acids, and trigonelline (Gniechwitz, Reichardt, Blaut, Steinhart & Bunzel, 2007). Moreover, the lactones of the chlorogenic acids are formed during roasting, but such formation depends on the degree of roasting.

Dietary polyphenols are mostly utilized by the gut microbiota, since most escape digestion and have low absorption rate in the small intestine (Selma, Espín, & Tomás-Barberán, 2009). Phenolic compounds feed the human gut microbiota, and microbial metabolites of polyphenols are absorbed in the large intestine and reach the blood stream (Russell & Duthie, 2011).. It has been reported that coffee is able to change some microbial communities and modify the end-products of community metabolism (Cowan et al., 2014). On the other hand, roasted coffee is also able to modify the gut microbiota due to the high content of melanoidins, which can behave as fiber-like compounds in the gut (Jiménez-Zamora et al., 2015).

Taking all this information into account, green and roasted coffee could be considered as two different products due to the different chemical composition they have, which in turn will shape the composition and functionality of the gut microbiota. Therefore, the aim of the present paper is to unravel the differences between green and roasted coffee after *in vitro* digestion-fermentation process. To do that, the differences in antioxidant capacity, individual polyphenols and gut microbiota structure of digested-fermented extracts of three green and roasted coffees from different locations were studied.

2. Materials and methods

2.1. Chemicals

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ), potassium persulphate, 3,5-dicaffeoylquinic acid, caffeic acid, dimethyl caffeic, chlorogenic acid, ferulic acid, coumaric acid, gallic acid, tyrosol, p-hydroxybenzoic acid, m-hydroxyphenylacetic acid, acetic acid, propionic acid, butyric acid, Folin-Ciocalteu reagent, sodium hydroxide, hydrochloric acid, iron (III) chloride hexahydrate, sodium acetate, potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide, resazurin, salivary alpha-amylase, pepsin from porcine, bile acids (porcine bile extract) and ethanol were from Sigma-Aldrich (Darmstadt, Germany). Pancreatin from porcine pancreas was purchased from Alpha Aesar (United Kingdom).

2.2. Samples

Coffee beans, green and roasted, were supplied by the local company Cafes Cumbal (Granada, Spain). Green coffee beans from three different locations (Brazil, Colombia and Vietnam) and their respective commercial roasted coffees were coded as follows: Brazil Arabica green coffee (BA), Brazil Arabica roasted coffee (BAR), Colombia Arabica green coffee (CA), Colombia Arabica roasted coffee (CAR), Vietnam Robusta green coffee (VR) and Vietnam Robusta roasted coffee (VRR). Coffee beans were ground using a coffee mill and coffee infusions were prepared using a mocha coffeemaker. Right after milling, 40g of ground coffee were extracted with 500

mL of distilled water using a mocha coffee machine. Aliquots were taken for individual phenolics, *in vitro* digestion-fermentation, and antioxidant capacity assays. For antioxidant capacity and individual phenolics assays three different fractions for each sample were collected: one coming from the coffee brew prior to digestion-fermentation, another one coming from the *in vitro* digestion and the last one coming from the *in vitro* fermentation. These fractions will be called from now on "coffee brew", "digested coffee brew" and "fermented coffee brew", respectively.

2.3. In vitro gastrointestinal digestion

All samples were subjected to an *in vitro* digestion process followed by an *in vitro* fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out according to the protocol described by Pérez-Burillo, Rufián-Henares, & Pastoriza (2018). The gastrointestinal *in vitro* digestion was composed of an oral phase (5 minutes at 37ºC with alpha-amylase 75 U/mL, pH 7.0), a gastric phase (2 hours at 37ºC with pepsin 2000U/mL at pH 3.0) and an intestinal phase (2 hours at 37ºC with pancreatin 13.37 mg/mL at pH 7.0).

2.4. In vitro fermentation

The *in vitro* fermentation was carried out according to the protocol described by Pérez-Burillo et al. (2018). *In vitro* fermentation was carried out using faecal samples from three healthy donors (not taking antibiotics, people with body mass index within the "normal weight range", mean Body Mass Index = 21.3). The solid residue obtained after *in vitro* gastrointestinal digestion plus 10% of the digestion supernatant was fermented (500 mg).

After *in vitro* gastrointestinal digestion and *in vitro* fermentation three different fractions were obtained: digestion supernatant (fraction available for absorption at the small intestine), fermentation supernatant (fraction available for absorption at the large intestine) and fermentation solid residue (fraction not available for absorption and excreted with feces).

2.5. Antioxidant assays

The antioxidant capacity of the undigested, digested, and fermented (supernatant) coffee brews was measured with three different methods:

- *$TEAC_{ABTS}$ assay:* The antioxidant capacity was estimated in terms of radical scavenging activity, following the procedure described by Re et al. (1999). Briefly, ABTS was produced by reacting ABTS stock solution (7 mM) with potassium persulphate (2.45 mM) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS solution was diluted with an ethanol and water mixture (50:50) to an absorbance of 0.70 ± 0.02 at 730 nm. After placing 20 µL of sample or Trolox standard and 280 µL of diluted ABTS solution on a transparent 96-well polystyrene microplate (Biogen Científica, Spain), absorbance readings were taken every 60 seconds for 20 minutes on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37ºC). Calibration was performed with a Trolox stock solution ranging from 0.01 to 1.00 mg/mL. The results obtained are expressed as µmol Trolox equivalents per mL of sample.

- *$TEAC_{FRAP}$ assay:* The ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie & Strain (1996) and adapted to a microplate reader. Briefly, 280 µL of FRAP reagent, prepared freshly and warmed at 37ºC, were mixed in each well of a transparent 96-well polystyrene microplate (Biogen

Científica, Spain) with 20 µL of sample or water to provide appropriate blank reagent. The FRAP reagent contained 2.5 mL of a TPTZ solution (10 mM) in HCl (40 mM), plus 2.5 mL of $FeCl_3 \cdot 6H_20$ (20 mM), and 25 mL of acetate buffer (0.3 M; pH 3.6). Readings of maximum absorbance (595 nm) were taken every 60 seconds using a FLUOStar Omega microplate reader (BMG Labtech, Germany). The temperature was maintained at 37ºC and the reaction was monitored for 30 min. Trolox stock solutions ranging from 0.01 to 1.00 mg/mL were used to perform the calibration curves. The results obtained are expressed as µmol Trolox equivalents per mL of sample.

- *Folin-Ciocalteu (total phenolics assay):* The Folin-Ciocalteu method is widely used to measure the amount of total polyphenols in foods, although it also measures the antioxidant capacity of foods due to the similar mechanism involved in the chemical reaction (as in the case of the FRAP and ABTS methods). The procedure described by Singleton & Rossi (1965) was used with few modifications, and adapted to a microplate reader. Briefly, sodium carbonate 10% was prepared and then the needed volume was diluted to 2.35%. 30 µL of sample were placed into the wells of a transparent 96-well polystyrene microplate (Biogen Científica, Spain), followed by 255 µL of the sodium carbonate and finally 15 µL of the Folin-Ciocalteu reagent. All tests were run in triplicate. Absorbance measurements at 725 nm were taken for 60 minutes at 37ºC. The equipment used was a FLUOStar Omega microplate reader (BMG Labtech, Germany). Calibration curve was performed using Gallic acid as the standard with concentrations ranging from 0.01 to 1.00 mg/mL. The results obtained are expressed as µmol Gallic acid equivalents per mL of sample.

2.6. *Short chain fatty acids determination*

The production of short chain fatty acids (SCFAs) as a measure of the gut microbiota functionality was assessed according to the procedure described in by Delgado-Andrade et al. (2017) with few modifications. The analysis of SCFAs was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 210 nm; the mobile phase used was 0.1 M phosphate buffer (pH 2.8)/acetonitrile 99:1 v/v delivered at a 1.25 mL/min flow rate; the column used was an Aquasil C18 reverse phase (Thermo Scientific) (150 × 4.6 mm, 5 μm), with a total run-time of 30 min. The sample did not require any pretreatment before injecting. Briefly, the SCFAs standards were prepared in the mobile phase at concentrations ranging from 5 to 10000 ppm. After the fermentation process, 1 mL of fermentation supernatant was centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter and finally transferred to a vial for HPLC analysis.

2.7. Individual phenolic analysis

The profile of individual polyphenols was obtained by means of UPLC-MS/MS-QTOF equipment (Esteban-Muñoz, Barea-Álvarez, Oliveras-López, Giménez-Martínez, Rufián-Henares & Olalla-Herrera, 2018).

- *Extraction procedure.* 1 mL of sample was mixed with 1 mL of diethyl ether in a 2 mL tube and was kept with agitation at 5ºC wrapped in foil for 24 hours. Then the supernatant was recovered and poured into a 10 mL tube. Right after, 1 mL of diethyl ether was added again to the 2 mL tube, vortexed and the supernatant was recovered and poured into a 10 mL tube. This step was repeated once. Sodium sulphate anhydrous was added to remove any humidity and the 3 mL aliquot was vacuum-evaporated at 30ºC. Finally, the solid residue was resuspended in 1 mL of a water-methanol mix (50:50 v/v)

and the sample was filtered through a 0.20 μm filter, making it ready for UPLC analysis.

- *UPLC-MS/MS-QTOF equipment and operating conditions.* The ESI-MS2 experiments were performed on a liquid chromatography system hybrid mass spectrometer UPLC-MS/MS-QTOF Synapt G2 HDMS (Waters, Mildford, USA). The UPLC separation was performed using in an ACQUITY UPLC™ system, equipped with a chromatographic column HSS T3 2.1 x 100 mm, 1.8 mm. The elution program was set with a binary gradient consisting of (A) water with 0.5% acetic acid and (B) acetonitrile, as follows: 0.0–15.0 min, 5% (B); 15.0–15.1 min, from 5 to 95% (B); and 15.1–18.0 min, from 95% to 5% (B). Ten microliters of sample were injected and the flow rate was set at 0.4 mL/min. The TOF conditions consisted of a full MS, and data-dependent scanning was performed in negative mode with electrospray ionisation (ESI).

- *Identification and quantification.* Individual phenolic compounds were identified by comparing the negative masses recorded in previous research, using the MassLynx V4 software (Waters Laboratory Informatics, Mildford, USA) for instrument control, data acquisition and data analysis. Individual phenolic compounds were quantified by obtaining a series of solutions, with a concentration of 0.1-40ppm of standard with different retention times. For each phenolic compound selected, a five-point calibration curve was performed (with $R^2 \geq 0.9$) to ensure the linearity of the method. The standards were analysed under the same working conditions as the samples. Identification was performed by means of the retention times and mass spectrum of the corresponding standards.

2.8. High-throughput amplicon sequencing.

Bacterial genomic DNA was isolated from each fermented sample using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, USA) as previously stated (Rigsbee, Agans, Foy, & Paliy, 2011). The V4 region of the 16S rRNA gene was amplified using primers complementary to flanking conserved sequences (forward primer 16S gene complementary sequence GCCAGCMGCCGCGG and reverse primer 16S gene complementary sequence GGACTACHVGGGTWTCTAAT). Forward primers also contained Ion Torrent P1 adapter sequence and 6-nucleotide barcode. PCR amplification was performed with 25ng of starting DNA material and included 10 cycles of linear elongation with only the forward primers used, followed by 25 cycles of traditional exponential PCR (Paliy & Foy, 2011). Inclusion of linear PCR step decreased the stochasticity of first few PCR reaction steps (Rigsbee et al., 2011) and allowed the use of a single PCR amplification reaction per sample. Purified amplicons were pooled equimolarly and sequencing libraries were prepared with the Ion PGM Template OT2 400 kit (Life Technologies, Inc.) according to the manufacturer's protocol. High throughput sequencing was performed on Ion Torrent PGM using Ion PGM Sequencing 400 kit and Ion 316 chip. An average of 16,954 sequence reads per sample were obtained and were processed in QIIME as previously described (Shankar *et al.*, 2017). Sequence read counts for each operational taxonomic unit (OTU) were adjusted by dividing them by known or predicted number of 16S rRNA gene copies in that organism's genome following a previously described approach (Rigsbee et al., 2011). Thus derived cell counts were sub-sampled (rarefied) to the lowest value among all samples.

2.9. Statistical analysis

Statistical significance of the data and differences among samples were tested by Student's t-test at $p < 0.05$ significance level. Evaluation of the relationship among different assays was carried out by computing the Pearson correlation coefficient. These statistical analyses were performed using Statgraphics Plus software (Statpoint Technologies, Inc., The plains, USA), version 5.1, 2001 and R version 3.4.0.

Multivariate statistical analyses were performed on the genus-level microbial abundance dataset generally following the approaches previously described (Paliy & Shankar, 2016). These included unconstrained principal coordinates analysis (PCoA) utilizing phylogenetic weighted UniFrac distance as a measure of sample dissimilarity, and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The statistical significance of the separation of green and roasted coffee samples in PCoA space was determined by a permutation test of the Davies-Bouldin index measure ran with 1,000 iterations. DB index compares the intra-cluster distances among samples to the distance between cluster centroids.

3. Results and discussion

3.1. Antioxidant capacity

ABTS assay. The antiradical antioxidant capacity of coffees measured with the ABTS assay showed values in the same range for the regular coffee brew, digested and fermented coffee brews (**Figure 37A**). Regular coffee brews had values ranging from 18.1 to 30.1 mmol Trolox equivalents/L for BA and VRR, respectively. In general, undigested coffee brews obtained from roasted coffees were statistically more antioxidant ($p < 0.05$) than their respective green coffees, with an exception of Brazilian coffee. The effect of roasting over the antioxidant capacity of coffee has been previously studied, though there are some discrepancies in the matter. For example, some reports demonstrated an increase on antioxidant capacity with roasting (Liang, Xue, Kennepohl, & Kitts, 2016), while others described a decrease in antioxidant activity (Perrone, Farah, & Donangelo, 2012). During roasting some compounds, mainly phenolic acids like chlorogenic acids, are either incorporated into melanoidins or degraded, which could result in a loss of antioxidant capacity (Perrone et al., 2012). However, the high temperatures used during coffee roasting allow the generation of other compounds (like melanoidins) via the Maillard reaction; these compounds can also contribute to antioxidant capacity of the coffee brew, compensating for the degradation of polyphenols (Ludwig et al., 2013).

When coffee brews were subjected to *in vitro* digestion, the antioxidant capacity ranged from 13.8 to 36.4 mmol Trolox equivalents/L for BA and VRR, respectively. In the case of fermentation, the antioxidant activity was between 21.3 and 36.4 mmol Trolox equivalents/L for VR and BA, respectively **(Figure 37A)**. Digestion of roasted coffees resulted in higher antioxidant capacity ($p < 0.05$) compared to green coffees except for Colombian coffees (as in the case of regular coffee brews). Surprisingly, no

differences were found for fermented coffee brews except for Brazilian green coffee, which was more antioxidant ($p < 0.05$) than its roasted counterpart. Altogether, these results point out to a low effect of the digestion process on the antioxidant capacity of coffee brews, while the activity of microorganisms during fermentation could release more antioxidant compounds (or modify their structure allowing then a higher antioxidant activity) from green coffees. This could be related to the microbial metabolism of coffee polysaccharides present in the green coffee (Gniechwitz et al., 2007) or polyphenolic compounds that are not present in roasted coffee (Selma et al., 2009; Russell & Duthie, 2011; Liang et al., 2016).

FRAP assay. The analysis of the reducing capacity of samples with the FRAP assay showed higher values ($p < 0.05$) for digested and fermented coffee brews than in the case of the ABTS assay (**Figure 37B**). For regular coffee brews the reducing capacity was significantly higher ($p < 0.05$) in roasted coffees, which is in accordance with the results obtained from the ABTS method. In this case the values ranged from 14.0 to 24.8 mmol Trolox equivalents/L for BA and VRR coffees, respectively. When the samples were subjected to digestion, only the Vietnamese coffee showed significant differences between green and roasted varieties, being again roasted coffee higher than green coffee ($p < 0.05$). The antioxidant capacity values of digested samples were noticeably higher and ranged from 41.6 to 76.8 mmol Trolox equivalents/L for VR and CAR, respectively. The results obtained with the FRAP method about the higher antioxidant capacity of roasted coffees could reinforce the idea that some compounds (like polyphenols) are lost during roasting, while others appear (Liang et al., 2016). It is noteworthy to mention that the reducing capacity of fermented samples was very high, ranging from 99.7-120 mmol Trolox equivalents/L (**Figure 37B**). However, no significant differences were found among the assessed coffees. Such large values of

antioxidant capacity could be related to the generation of new molecules arising from the microbial fermentation of melanoidins (Jiménez-Zamora et al., 2015), polysaccharides (Asano, Ikeda, Fujii, & Iino, 2007) and phenolic compounds (Selma et al., 2009).

Folin-Ciocalteu assay. The measurements of total phenolic content showed a similar tendency to that revealed with the FRAP assay. The content of polyphenols was higher in the digested and fermented coffee brews ($p < 0.05$) than in regular coffee brews (**Figure 37C)**. In this sense, values ranging from 1802 to 2587 mg of gallic acid/ L were obtained for regular coffees, from 3153 to 4693 mg of gallic acid/L for digested coffees and from 4692 to 7034 mg of gallic acid/L in the case of fermented ones. No statistically significant differences ($p < 0.05$) were found between green and roasted coffees except for digested BAR and fermented VRR (see **Figure 37C**). These results suggest that roasted brews have somewhat higher content in phenolic compounds, which could be explained by the degradation of chlorogenic acids to their corresponding hydroxycinnamates such caffeic, ferulic, and quinic acids (Liang et al., 2016). In addition, it has been shown that the content of benzoic acids is higher in roasted coffee than in green coffee (Köseoğlu-Yılmaz & Kolak, 2017). Even more, the analysis of individual polyphenols (**Table 21; Figure 38**) reflects a higher content of m-hydroxybenzoic and p-hydroxybenzoic acids in roasted coffees than in green ones, which could lead to a higher value in the Folin-Ciocalteu assay.

Figure 37. Antioxidant capacity (**37A**, **37B**), total polyphenols (**37C**) and SCFAs (**37D**) of regular, digested and fermented green and roasted coffee brews. Abbreviations: BA (green coffee beans from Brassica arabica), CA (green coffee beans from Colombia arabica), VR (green coffee beans from Vietnam robusta), BAR (roasted coffee beans from Brassica arabica), CAR (roasted coffee beans from Colombia arabica), and VRR (roasted coffee beans from Vietnam robusta). Ns - not significant, * - $p \leq 0.05$), ** - $p \leq 0.01$.

Figure 37A

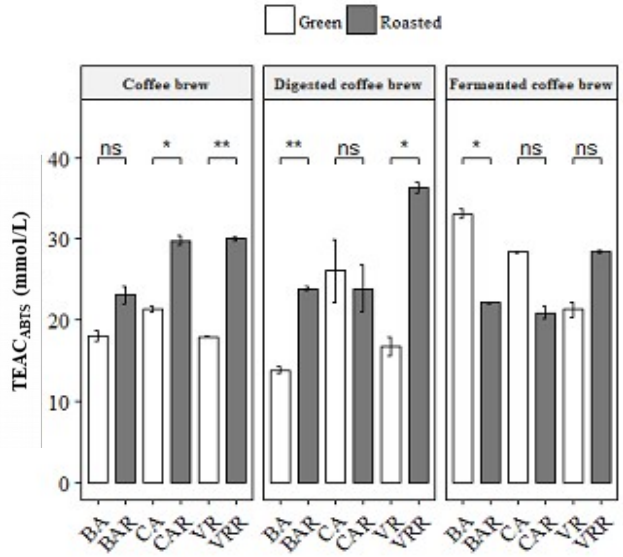


Figure 37B

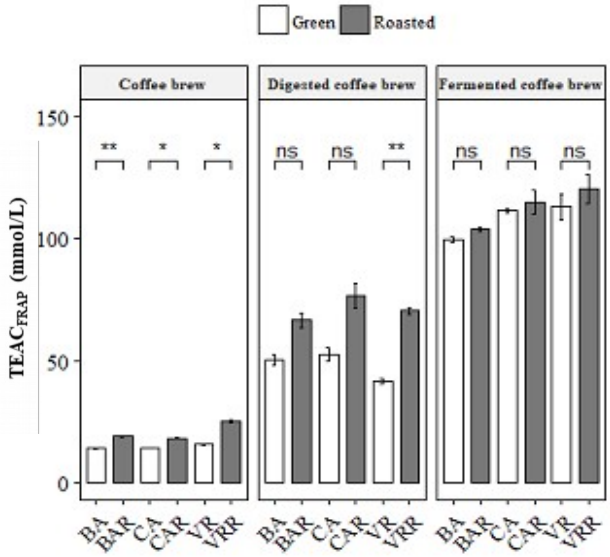


Figure 37C

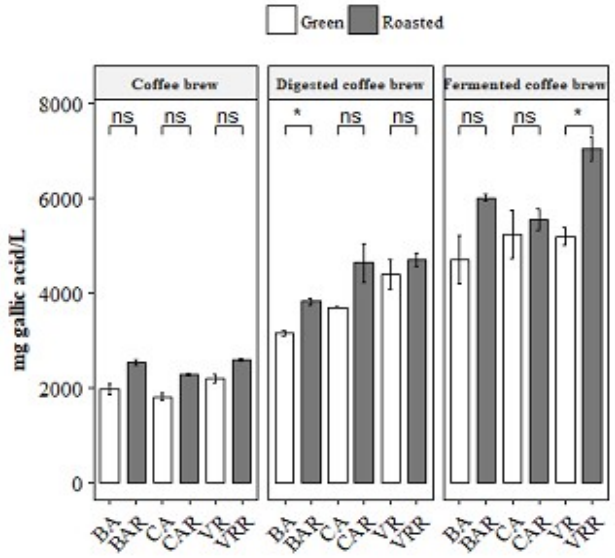
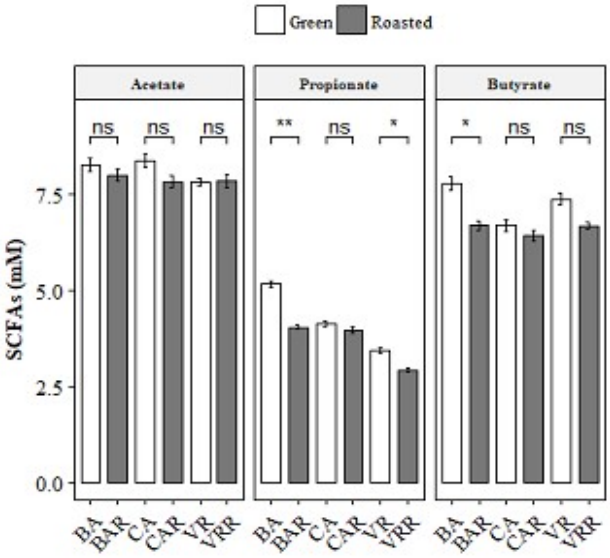


Figure 37D



3.2. Individual phenolic compounds

Nine individual phenolic compounds were quantified in the analyzed coffee brews: 3,4-di-O-Caffeoylquinic acid, caffeic acid, dimethyl caffeic acid, chlorogenic acid, ferulic acid, coumaric acid, tyrosol, m-hydroxybenzoic acid and p-hydroxyphenylacetic acid. The polyphenolic profile of regular and digested coffee brews was quite similar (**Table 21; Figure 38**) although the amounts of all the studied phenols in regular coffee brews was slightly higher (not statistically significant) than those found in digested coffee brews. The concentration of individual polyphenols ranged from 0.81 to 11315 mg/L for tyrosol and p-Hydroxyphenyl acetic acid, respectively. In addition, the levels of the chlorogenic acids fraction were much higher in green coffees than in their respective roasted ones (**Table 21**). This is in agreement with the findings reported by other authors (Clifford, 2000; Perrone et al., 2012; Ludwig et al., 2013; Liang et al., 2016) and is explained by the degradation of such compounds during roasting. On the other hand, higher amounts of caffeic and ferulic acid were found in roasted coffees, which could be related to the degradation of chlorogenic acids during roasting, yielding their corresponding cinnamates (Clifford, 2000). Moreover, m-hydroxybenzoic and p-hydroxyphenylacetic acids were found only in roasted coffees. These phenolic compounds could be products of the degradation of more complex polyphenols during roasting, since m-hydroxybenzoic acid was not detected in green coffee and the levels of p-hydroxyphenylacetic acid were 100-fold lower in green coffee samples compared to roasted coffees.

The phenolic profile found in fermented coffee brews was different from either digested or regular coffees (**Table 21; Figure 38**). Caffeic and ferulic acids were detected, but in much lesser amounts than in regular or digested coffee brews. Hydroxycinnamates are extensively metabolized by the gut microbiota, which could

explain the decrease (even disappearance) of these polyphenols in fermented samples (Clifford, 2000; Selma et al., 2009; Russell & Duthie, 2011). The main products are usually hydroxybenzoic acids such as m-hydroxybenzoic and p- hydroxyphenylacetic acids (Selma et al., 2009). Thus, the higher amount of these metabolites found in fermented samples could be related to the microbial metabolism of other phenolic compounds.

Figure 38. Heatmap representation of polyphenols amounts in different coffee brews. Amounts are represented by a color gradient as shown in the legend; note the log2-scale of the gradient.

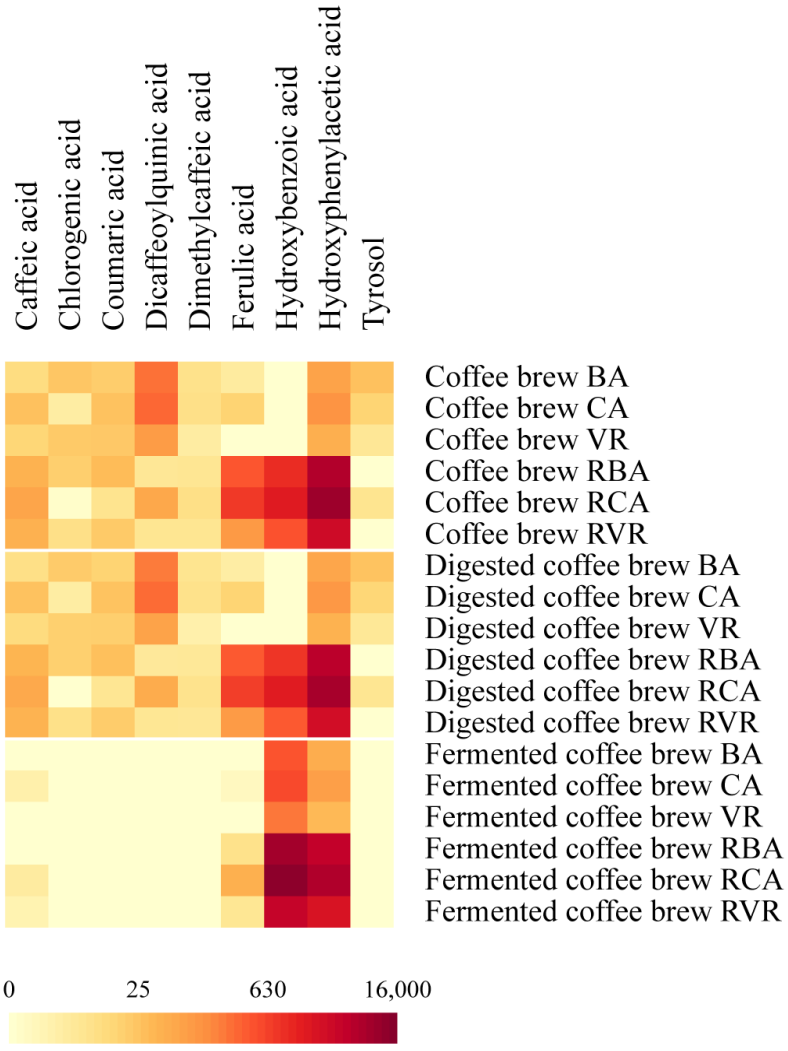


Table 21. Individual phenolic compounds (mg/L) in coffee brews and digestion-fermentation extracts.

Samples	3,4-Di-O-Caffeoylquinic acid	Caffeic acid	Dimethyl Caffeic acid	Chlorogenic acid	Ferulic acid	Coumaric acid	Tyrosol	m-Hydroxybenzoic acid	p-Hydroxyphenyl acetic acid
BA brew	268 ± 6.32^{a}	9.91 ± 0.24^{a}	7.67 ± 0.18^{a}	23.3 ± 0.47^{a}	4.09 ± 0.09^{a}	18.3 ± 0.51^{a}	28.0 ± 0.57^{a}	ND	78.6 ± 2.37^{a}
CA brew	317 ± 3.90^{b}	28.4 ± 0.36^{b}	8.19 ± 0.10^{a}	3.63 ± 0.04^{b}	15.1 ± 0.17^{b}	27.0 ± 0.39^{b}	14.6 ± 0.15^{b}	ND	126 ± 1.98^{b}
VR brew	99.1 ± 4.19^{c}	13.5 ± 0.59^{c}	3.76 ± 0.16^{b}	20.7 ± 0.74^{c}	1.01 ± 0.04^{c}	21.9 ± 1.09^{a}	5.31 ± 0.19^{c}	ND	51.0 ± 2.75^{c}
BAR brew	5.43 ± 0.34^{d}	45.3 ± 2.91^{d}	5.80 ± 0.36^{c}	18.1 ± 0.96^{c}	463 ± 25.7^{d}	32.9 ± 2.41^{c}	0.87 ± 0.05^{d}	1161 ± 61.5^{a}	7383 ± 321^{d}
CAR brew	65.3 ± 2.69^{e}	72.9 ± 3.09^{e}	8.41 ± 0.35^{a}	1.15 ± 0.04^{d}	870 ± 32.0^{e}	6.72 ± 0.33^{d}	6.30 ± 0.22^{c}	1910 ± 66.9^{b}	11315 ± 594^{e}
VRR brew	6.01 ± 0.15^{d}	46.9 ± 1.23^{d}	6.00 ± 0.15^{c}	8.11 ± 0.17^{e}	106 ± 2.41^{f}	20.7 ± 0.62^{a}	ND	499 ± 10.8^{c}	3609 ± 116^{f}
BA Digested	222 ± 3.02^{a}	8.42 ± 0.12^{a}	6.21 ± 0.08^{a}	20.3 ± 0.23^{a}	3.52 ± 0.04^{a}	15.4 ± 0.25^{a}	26.7 ± 0.31^{a}	ND	67.6 ± 1.17^{a}
CA Digested	301 ± 7.10^{b}	27.6 ± 0.67^{b}	7.62 ± 0.18^{b}	3.59 ± 0.07^{b}	14.8 ± 0.31^{b}	25.9 ± 0.72^{b}	13.7 ± 0.28^{b}	ND	109 ± 3.30^{b}
VR Digested	78.2 ± 4.20^{c}	10.9 ± 0.60^{a}	2.90 ± 0.16^{c}	17.1 ± 0.78^{c}	0.83 ± 0.04^{c}	17.6 ± 1.11^{a}	5.10 ± 0.23^{c}	ND	45.4 ± 3.10^{c}
BAR Digested	4.83 ± 0.16^{d}	41.3 ± 1.38^{c}	5.05 ± 0.16^{d}	16.8 ± 0.46^{c}	426 ± 12.3^{d}	29.6 ± 1.13^{b}	0.81 ± 0.02^{d}	952 ± 26.3^{a}	6054 ± 251^{d}
CAR Digested	56.2 ± 2.36^{e}	64.1 ± 2.78^{d}	7.06 ± 0.30^{b}	1.04 ± 0.04^{d}	774 ± 29.1^{e}	5.85 ± 0.29^{c}	5.92 ± 0.21^{c}	1814 ± 65.0^{b}	916 ± 492^{e}
VRR Digested	5.53 ± 0.06^{d}	44.1 ± 0.48^{c}	5.40 ± 0.06^{a}	7.79 ± 0.07^{e}	101 ± 0.95^{f}	19.2 ± 0.24^{d}	ND	419 ± 3.74^{c}	3139 ± 42.1^{f}
BA Fermented	ND	0.52 ± 0.02^{a}	ND	ND	0.22 ± 0.01^{a}	ND	ND	481 ± 5.23^{a}	54.8 ± 0.56^{a}
CA Fermented	ND	2.90 ± 0.16^{b}	ND	ND	1.55 ± 0.07^{b}	ND	ND	593 ± 54^{c}	88.4 ± 1.89^{c}
VR Fermented	ND	0.02 ± 0.00^{c}	ND	ND	0.00 ± 0.00^{c}	ND	ND	237 ± 10.22^{e}	36.1 ± 1.06^{e}
BAR Fermented	ND	0.74 ± 0.01^{a}	ND	ND	7.64 ± 0.07^{d}	ND	ND	10026 ± 432^{d}	5038 ± 289^{d}
CAR Fermented	ND	3.96 ± 0.16^{d}	ND	ND	47.8 ± 1.72^{e}	ND	ND	15680 ± 475^{d}	7680 ± 412^{d}
VRR Fermented	ND	2.42 ± 0.06^{b}	ND	ND	5.55 ± 0.13^{f}	ND	ND	4732 ± 247^{f}	2530 ± 111^{f}

Different letters within the same column and type of food indicate statistically significant differences ($p < 0.05$).

3.3. Short chain fatty acids production

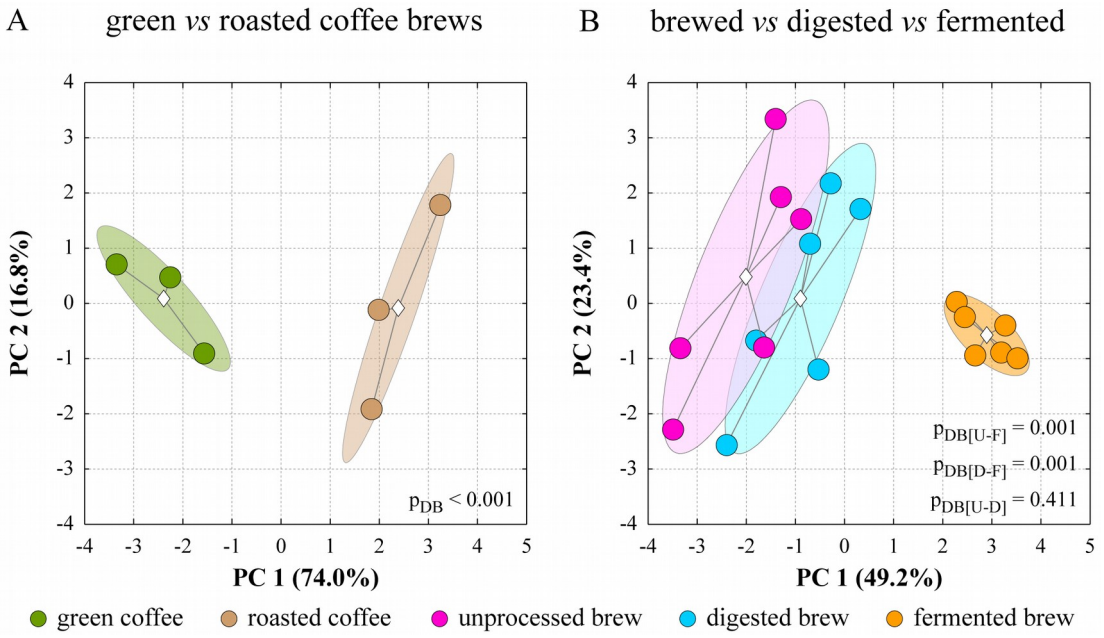
Coffee brews contain polysaccharides that can be fermented by the gut microbiota (Gniechwitz et al., 2007). This polysaccharide fraction is mainly composed of arabinogalactans, galactomannans, and manooligosaccharides (Asano et al., 2007). However, during roasting some of these carbohydrates are degraded, taking part in the Maillard reaction and sugar caramelization. This is the reason for the almost 2-fold higher carbohydrate content of green coffee compared to roasted coffee (Gniechwitz et al., 2007). In addition, different Maillard reaction products are readily metabolized by the gut microbiota (behaving as prebiotics), leading to the formation of SCFAs (Delgado-Andrade et al., 2017).

We have measured the levels of three main schort-chain fatty acids (acetate, butyrate, propionate) in all fermented samples (see **Figure 37D)**. Overall, the SCFAs levels are in line with those reported by other authors (Gniechwitz et al., 2007; Delgado-Andrade et al., 2017). The results obtained for acetic acid ranged from 7.81 to 8.37 mM for regular coffee brews. In addition, no statistically significant differences were found among green or roasted coffees ($p > 0.05$); this could probably be due to the presence of melanoidins in roasted coffees, which generate SCFAs upon fermentation (Jiménez-Zamora et al., 2015), compensating for the breakdown of polysaccharides during roasting process. Secondly, the levels of propionic acid were comprised between 2.92 and 5.15 mM. For this SCFA, statistically higher ($p > 0.05$) levels were found in Brazilian and Vietnamese green coffee fermented beverages, compared to their roasted coffees brews (**Figure 37D**). In the case of butyrate the values ranged from 6.42 to 7.79 mM, being higher ($p > 0.05$) only for the Brazilian green coffee. According to these results, green coffee has a tendency to produce higher amounts of SCFAs, though the differences are relatively small (up to 20%). Fermentation of coffee melanoidins by

bacterial species able to produce SCFAs (Jiménez-Zamora et al., 2015) could explain the observed differences between green and roasted coffee SCFAs production.

In order to unravel the overall effect of processing (green *vs.* roasted coffee) or *in vitro* metabolization (regular *vs.* digested *vs.* fermented brew) on SCFAs production, antioxidant capacity and polyphenolic content, a principal component analysis was carried out. As depicted in **Figure 39A**, a clear separation between green and roasted coffee samples was observed, since only two components were able to explain 90.8% of the variability. Regular coffee and digested coffee brews were very similar, while fermented coffee samples were separated in the PCA space, likely because of the significant effect of gut microbiota metabolismn **(Figure 39B)**. This makes sense since the properties of the later depends on the gut microbiota metabolism, which in turn change the original profile of bioactive compounds. In this case, a 69.2% of the differences were explained with two principal components.

Figure 39. PCA ordination analysis of the dataset of measured metabolites and antioxidant capacities. Panel A shows the separation between green and roasted coffee. Panel B shows the separation between regular coffee brew, digested coffee brew and fermented coffee brew. Different groups are denoted by colors as shown in the legend. Group clouds represent areas of three standard errors around the group centroid (diamond). P_{DB} values denote the statistical significance of the separation of green and roasted coffee samples in PCoA space based on the permutation analysis of Davies-Bouldin index as described (Shankar et al., 2015). The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles.



3.4. Fermentation of green and roasted coffee by human gut microbiota promotes different community structures.

The effect of the different coffee brews on the microbiota community structure was determined after the fecal microbiota fermented coffee samples for 20 hours. Overall, the community composition was comparable among all samples, with *Escherichia*/*Shigella*, *Bifidobacterium* and *Bacteroides* being the most abundant genera. Among the top nine genera, only *Bacteroides* was statistically differentially abundant between green and roasted coffee samples (**Figure 40A**). However, the differences among other genera were sufficient to separate these two types of samples in PCoA space with statistical significance (**Figure 40B**). OPLS discriminant analysis of microbiome dataset (**Figure 40C**) similarly separated the sample groups well (R^2 = 0.998), though the predictive power of the generated model was modest (Q^2 = 0.528). The top discriminating genera included *Escherichia*/*Shigella*, *Lactobacillus*, several *Clostridium* clusters, *Providencia*, *Enterobacter*, and *Bacteroides* (**Figure 40C**). Many of the discriminating genera play a role on human health. The beneficial-detrimental effects of different bacteria are described in **Table 22**.

Figure 40. Fermentation of green and roasted coffee by human fecal microbiota promotes different community structures. Panel **A** displays relative abundances of the top nine most abundant microbial genera. Each column represents a community derived from an independent fermentation of green or roasted coffee sample. Abundances of other genera were summed and are represented cumulatively as "other genera". Star represents the statistically significant difference (at $p < 0.05$ level) in the abundance of *Bacteroides* genus between sample types as determined by paired samples T-test. Panel **B** shows the output of the unconstrained PCoA ordination analysis of genus abundance dataset among all profiled samples. Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix. Different groups are denoted by colors as shown in the legend. Group clouds represent areas of three standard errors around the group centroid (diamond). P_{DB} value denotes the statistical significance of the separation of green and roasted coffee samples in PCoA space based on the permutation analysis of Davies-Bouldin index as described (Shankar et al., 2015). The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles. Panel **C** displays the T-vs-$T_{orthogonal}$ scatter plot of the OPLS discriminant analysis of the genus abundance dataset. R^2Y denotes the amount of group-related variation in the data explained by the model. The genera with the largest discriminating weights are listed in the tables. IS – incertae sedis.

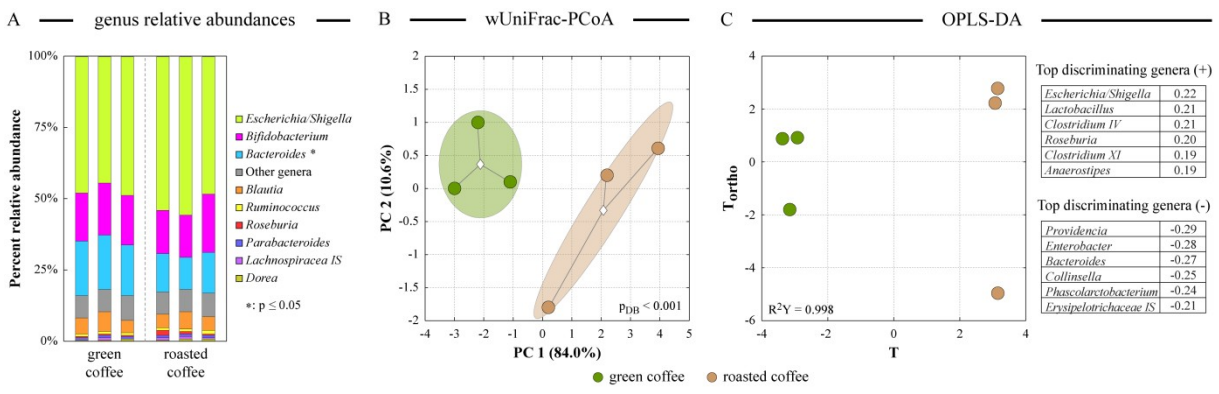


Table 22. Beneficial or detrimental effects of bacteria in human health.

Bacteria		Health effect	References
Anaerostipes	+	Produce acetic, lactic and butyric acid	Ríos-Covián et al. (2016)
Bacteroides	-/+	*B. fragilis* is involved in inflammatory processes that could lead to colorectal cancer; associated with western diets and low fiber intake	Simpson & Campbell (2015)
Bifidobacterium spp.	+	Reduced in colorectal cancer and in type_I diabetes	Murri et al. (2013)
Clostridium IV	-/+	Reduced in dysbiosis in IBD, seems to be increased in children with food sensitization;	Chen, Chen, Kong, Chang, & Huang (2016)
Clostridium XI	+	Decreased in Budd-Chiari symdrom and increases after treatment along with *Bacteroides*	Sun et al. (2017)
Collinsella	+	Reduced in irritable bowel syndrome patients with more severe symptoms	Malinen et al. (2010)
Enterobacter	-	Member of the ESKAPE pathogens	Pendleton, Gorman & Gilmore (2013)
Escherichia/shigella	-	Responsible for gastrointestinal disorders such as constipation, diarrheic symptoms.	Verbeke, Boesmans, & Boets (2014)
Lactobacillus	+	Represents the largest and most diverse genera of all lactic acid bacteria with uses in biotechnology, medical field, probiotic, etc.	Stefanovic, Fitzgerald, & McAuliffe (2017)
Phascolarctobacterium	+	SCFA producer. It has been found increased in rats fed with inulin	Zhang et al. (2018)
Providencia	-/+	Participates in communication between bacteria and host	Hughes & Sperandio (2008)
Roseburia	+	Associated with weight loss and decreased glucose intolerance in mice, reduced in ulcerative colitis patients, differ in abundance between type II diabetes patients and non-diabetic people	Ryan et al. (2014)

4. Conclusions

This manuscript reports the effect of *in vitro* digestion-fermentation of green and roasted coffees on the antioxidant capacity, phenolic profile, SCFAs production and microbial community structure. In many cases, roasted coffee brew showed higher antioxidant values than green coffees. Moreover, in FRAP and Folin-Ciocalteu assays, digested coffee brews displayed significantly higher values than regular coffee brews, and even higher values were obtained for fermented coffees. On the other hand, the phenolic profile of regular and digested coffee brews were very similar, while the profile of fermented coffees differed greatly from the others, probably due to microbial metabolism. SCFA production was higher for microbial communities fermenting green coffee brews. All these data allowed the separation of green and roasted coffees through PCA ordination analysis. In addition, the metabolite profiling also allowed the differentiation of regular-digested coffee brews from fermented ones with PCA. Finally, the microbial community structure also differed between fermented green and roasted coffee samples. Therefore, green and roasted coffees behave in many ways as different types of food and therefore they should probably be considered as such, especially taking into account the effect of the gut microbiota on human health. More studies to link specific compounds from green or roasted coffee with individual microbial taxa are needed.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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High antioxidant action and prebiotic activity of hydrolyzed spent coffee grounds (HSCG) in a simulated digestion-fermentation model: toward the development of a novel food supplement

El café es uno de los alimentos más consumidos en la dieta española. Durante su preparación se obtienen los posos de café, que son el residuo alimentario más producido a nivel mundial, siendo además muy contaminantes para el medio ambiente. Por ello, en este trabajo y en el siguiente se estudian la bioactividad de los posos de café para poder ser usados como ingredientes funcionales, bien como tales o tras un tratamiento adecuado. Así, en este trabajo se evalúa la capacidad antioxidante de los posos de café en líneas celulares, tras someter los posos al proceso *in vitro* de digestión-fermentación. Además, se evalúa su capacidad prebiótica mediante el proceso de fermentación *in vitro*. De esta manera, el contenido del presente trabajo se encuentra estrechamente relacionado con la presente tesis doctoral, al estudiar su posible uso como ingrediente funcional tras estudiar su capacidad antioxidante y capacidad prebiótica tras digestión-fermentación *in vitro*.

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High antioxidant action and prebiotic activity of hydrolyzed spent coffee grounds (HSCG) in a simulated digestion-fermentation model: toward the development of a novel food supplement

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Abstract

Spent coffee grounds are a by-product with a large production all over the world. The aim of this study was to explore the effects of a simulated digestion-fermentation treatment on hydrolyzed spent coffee grounds (HSCG) and to investigate the antioxidant properties of the digestion and fermentation products in human hepatocellular carcinoma HepG2 cell line. The potentially bioaccessible (soluble) fractions exhibited high chemoprotective activity in HepG2 cells against oxidative stress. Structural analysis of both the insoluble and soluble material revealed partial hydrolysis and release of the lignin components in the potentially bioaccessible fraction following simulated digestion-fermentation. A high prebiotic activity as determined from the increase in *Lactobacillus spp.* and *Bifidobacterium spp.* as well as the production of short chain fatty acids (SCFAs) following microbial fermentation of HSCG was also observed. These results pave the way toward the use of HSCG as a food supplement.

Keywords: spent coffee grounds; antioxidant; reactive oxygen species; simulated digestion-fermentation; HepG2 cells; short chain fatty acids

Introduction

Coffee is by far one of the most consumed beverages in the world and the resulting spent coffee grounds (SCG), because of the high content in caffeine, tannins, and polyphenols, can have negative effects on the environment, requiring proper management and disposal.[1] Proposed use of SCG includes production of biofuel, removal of pollutants from water and as a source of natural phenolic antioxidants for use as nutritional supplements, foods, or cosmetic additives.[1-9]

SCG contain mainly carbohydrates (38–42%), proteins (8%), and chlorogenic acids (3–4%).[10,11] As a major outcome of the roasting process, SCG contain also melanoidins, which are usually quantified to account for up to around 25% w/w of the dry weight of roasted coffee beans.[12,13]

We recently developed an expedient chemical procedure to convert SCG into an all-natural biocompatible material, termed hydrolyzed spent coffee grounds (HSCG), involving an hydrolytic protocol with 6 M HCl, at 100 °C, overnight. The black powder thus obtained displayed potent antioxidant properties for diverse applications, including e.g. cell protection, food lipid preservation and thermal and photo-oxidative stabilization of polymers.[14]

Yet, simple chemical assays are inadequate to evaluate the actual antioxidant activity of food, and the importance of *in vitro* digestion combined with cellular assays to determine the antioxidant activity has been recently emphasized.[15-20]

SCG have been found to be good sources of prebiotic compounds following *in vitro* digestion.[12] The chemical characterization of potentially prebiotic oligosaccharides in SCG have been also recently reported.[21]

The anti-inflammatory potential of the metabolites produced by colonic fermentation of SCG has also been recently described, supporting the use of SGC in the food industry as dietary fiber source with health benefits.[12,22] However, the physiological potential and health benefits of HSCG have not yet been adequately investigated.

We report herein the modifications induced by *in vitro* gastrointestinal digestion followed by a fermentation step on the antioxidant activity of HSCG. The potential antioxidant and oxidative stress protection of both the potentially bioaccessible digestion and fermentation products and the residual, undigested fraction of HSCG were investigated by validated cellular assays using human liver HepG2 cell line. This is generally held as a sensitive model for the determination of the chemoprotective potential of antioxidant compounds.[23] Preliminary structural investigation on both the soluble, potentially bioaccesible, fractions and the residual, indigestible, solid fraction was also carried out. The prebiotic activity of HSCG was also determined.

Materials and Methods

Chemicals. HSCG were prepared from espresso SCG collected from a local coffee shop as previously described.[14] Salivary -amylase, pepsin from porcine, bile acids (bile extract porcine), tryptone, cysteine, sodium sulphide, resazurin, inulin, *o*-phthaldialdehyde (OPT), glutathione (GSH), and 2,7-dichlorofluorescin diacetate (DCFH-DA) were from Sigma-Aldrich. Pancreatine from porcine pancreas was purchased from Alpha Aesar, *tert*-butylhydroperoxide (t-BOOH) from Panreac, Bradford reagent from BioRad.

General Experimental Methods. FTIR analysis was performed using a Perkin Elmer Spectrum 100 spectrometer in attenuated total reflectance (ATR) mode, with an

average of 32 scans and resolution of 4 cm^{-1}, in the range 4000-400 cm^{-1}. UV-vis spectra were recorded on a Agilent/HP 8453 spectrophotometer. NMR spectra were recorded in D_2O or CD_3OD on a Bruker 400 MHz NMR spectrometer. HPLC analysis was performed with an Agilent instrument equipped with a UV-vis detector (G1314A); the chromatographic separation was performed on a Phenomenex Sphereclone ODS column (250 × 4.60 mm, 5 μm), at a flow rate of 0.7 mL/min; the mobile phase was a 1% formic acid (solvent A)/methanol (solvent B) gradient as follows: from 5 to 90% B, 0-45 min; the detection wavelength was 254 nm. Short chain fatty acid (SCFAs) determination was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 210 nm; the mobile phase used was 0.1 M phosphate buffer (pH 2.8)/acetonitrile 99:1 v/v delivered at a 1 mL/min flow rate; the column used was an Aquasil C18 reverse phase (Thermo Scientific) (150 × 4.6 mm, 5 μm), with a total run-time of 20 min.

***In vitro* Digestion.** The *in vitro* digestion method followed was an adaptation to the method previously described,[17,24] composed of an oral phase, a gastric phase and an intestinal one. Briefly, for the oral phase, 5 mL of simulated salivary fluid containing α-amylase were added to 0.5 g of grinded HSCG. Such mix was incubated at 37 ºC for 2 min. Then, 10 mL of simulated gastric fluid containing pepsin were added and the pH lowered to 3.0 by adding 1 N HCl. The mixture was incubated at 37 ºC for 2 h, after that 20 mL of simulated intestinal fluid containing pancreatin and bile salts were added and the pH increased to 7.0 with 1 N NaOH. The mixture was then incubated at 37 ºC for 2 h. In order to stop the enzymatic reactions, the tube was buried in iced water, centrifuged at 14000 g for 10 min at 4 ºC and the supernatant stored at -80 ºC until further analysis. A 10% of the liquid fraction was added to the solid residue in order to mimic the fraction that is not readily absorbed after digestion.[25] Then, the mixed

fractions were frozen for further lyophilization. When required, the supernatant from the digestion mixture was lyophilized, too, and then subjected to chemical extraction: in brief, 300 mg of material were dissolved in 120 mL of water, then the solution was taken to pH 1 with 3 M HCl and extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried over Na_2SO_4 and taken to dryness to give a dark yellow oily residue (ca. 30 mg). For comparison ethyl acetate extraction was performed also on the supernatant form a control mixture containing enzymes and other ingredients but no HSCG.

In vitro **Fermentation.** The method used was adapted from a previously described protocol.[26] In brief, to 300 mg of lyophilized digestion solid residue, 200 μL of distilled water were added into a screw-cap tube to make up the volume up to 0.5 mL. Then, 7.5 mL of fermentation final solution (peptone water + resazurine) was added. Finally, 2 mL of inoculum was added being the final volume 10 mL. The inoculum consisted of a solution of 32% feces in phosphate buffer 100 mM, pH 7.0 (fecal content composed of a mixture of equal weight of fresh morning feces of three healthy adult human donors, mean body mass index = 21.3). Nitrogen was bubbled in order to reach an anaerobic atmosphere and the mixture was incubated at 37 ^{o}C for 20 h under oscillation. Right after, the samples were buried in ice to stop microbial activity and centrifuged. Supernatant were collected and stored at -80^{o}C. The solid residue was also stored for direct antioxidant activity measure. Both digestion and fermentation were performed in triplicate. When required, the supernatant was lyophilized and subjected to chemical extraction: in brief, 100 mg of material were dissolved in 40 mL of water, then the solution was taken to pH 1 with 3 M HCl and extracted with ethyl acetate (3 × 30 mL). The combined organic layers were dried over Na_2SO_4 and taken to dryness to give a dark yellow oily residue (ca. 4 mg). For comparison ethyl acetate extraction was

performed also on the supernatant of a control mixture containing all the ingredients but no HSCG.

SCFAs Assay. SCFAs determination (acetic, propionic and butyric acids) was carried out by HPLC as described in the General Experimental Methods. The sample did not require any pretreatment before injecting. Briefly, the SCFA standards were prepared in the mobile phase at concentrations ranging from 5 to 10000 ppm. After the fermentation process, 1 mL of supernatant was centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter and finally transferred to a vial for HPLC analysis. A probiotic commercial milk beverage was also analyzed for comparison.

Prebiotic activity. The ability of bacteria to utilize HSCG as carbon source was performed as previously described.[12] After the digestion-fermentation step, the growth of different bacterial strains was assessed by qRTi-PCR as reported previously.[27] For DNA extraction, the QIAamp DNA Stool Mini Kit (Qiagen) was used according to the manufacturer's instructions after diluting the stool contents 1:10 (w/v) in PBS. DNA was eluted in buffer AE (provided in the kit), and then purified DNA extracts were stored at −20°C. A series of genus-specific primer pairs were used.[27] PCR amplification and detection was performed in an Eco Illumina thermocycler as follows. Each reaction mixture (10 μL) was composed of 5 μL of KAPA SYBR Fast Master Mix (Kapa Biosystems), 0.25 μL of each specific primer (at a concentration of 10 μM) and 2 μL of template DNA. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from pure cultures with a bacterial population ranging from 2 to 9 log10 CFUs, as determined by plate counts. One strain belonging to each of the bacterial genera or groups targeted in this study was used to construct the standard curve. More specifically, the strains from which the DNA was extracted were the following: *Bifidobacterium longum* CECT 4551, *Clostridium coccoides* DSMZ 935, *Bacteroides*

fragilis DSMZ 2151, *Lactobacillus salivarius* CECT 2197. All of them were obtained from the Spanish Collection of Type Cultures (CECT) or the German Collection of Microorganisms and Cell Cultures (DSMZ).

Cellular Assays. Human hepatoma HepG2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 ºC. They were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F-12) from Biowhitaker, supplemented with 2.5% Biowhitaker foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin.

To assay direct effect, cells were incubated with doses ranging from 1 to 100 μg/mL (depending of the assay) of HSCG, digested HSCG, fermented HSCG or residual fraction from fermented HSCG for 20 h. To assay for a protective effect, cells were pre-treated with the noted doses (see Table 1 and Figures 1 and 2) of the four samples for 20 h, then the medium was discarded, the cells were washed with phosphate buffer saline (PBS) and medium containing 400 μM t-BOOH was added for 2 h, after which the cell cultures were processed as detailed below for each assay.

Cell viability was determined by using the crystal violet assay.[28] Briefly, HepG2 cells were seeded at low density (10000 cells per well) in 96-well plates, grown for 20 h under the different conditions and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with water, allowed to dry, and 1% sodium dodecylsulfate added. The absorbance of each well was measured using a microplate reader at 570 nm.

Cellular reactive oxygen species (ROS) were quantified by the dichlorofluorescein assay using a microplate reader to screen the antioxidant effect of the different quantities of samples.[15,29] Briefly, the cells were seeded in 24-well plates (200000 cells per well) in medium containing FBS and replaced with the FBS-free

medium the following day. After 20 h, 5 μM DCFH-DA was added to the wells at 37 ºC for 30 min, cells were then washed with PBS, placed in fresh FBS-free medium with the different concentrations of the four HSCG samples and ROS production was monitored for 120 min. For the protection assay, cells were seeded and left overnight before treating them with the HSCG samples for 20 h. Then DCFH-DA was added for 30 min and cells were washed with PBS prior to the addition of 400 μM t-BOOH to every well but controls with further incubation for 2 h. Control cells without t-BOOH treatment were used as negative control. Multiwell plates were measured in a fluorescent microplate reader at excitation wavelength of 485 nm and emission wavelength of 528 nm. Results are expressed as percent of fluorescence units.

GSH content was quantitated by a fluorometric assay.[15] The method takes advantage of the reaction of GSH with OPT at pH 8.0. HepG2 cells were plated in 60 mm diameter plates at a concentration of 1.5×10^6 cells/plate. Cells were treated with the different quantities of the samples for 20 h, collected by scraping in 1.5 mL of PBS and centrifuged (1500 rpm, 4 ºC, 5 min). Cells were lysed by adding 110 μL of 5 % w/v trichloroacetic acid containing 2 mM EDTA and protein was measured by the Bradford reagent. Following centrifugation of cells at 7500 rpm and 4 ºC for 30 min, 50 μL of the clear supernatant were transferred to wells in a 96-well plate. Then, 15 μL of 1 M NaOH were added to neutralize the sample followed by 175 μL of 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA and finally 10 μL per well of a stock solution of OPT (10 mg/mL) in methanol were added. After 15 min at room temperature in the dark, fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the treatments were referred to those of a standard calibration curve of GSH (5–1000 ng) and expressed as percent related to untreated cells.

Statistical Analysis. Statistical significance of the data from the antioxidant assays was tested by one-way analysis of variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($p < 0.05$); all the statistical analyses were performed using Statgraphics Plus software, version 5.1. For the cellular assays, statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$. A SPSS version 23.0 program was used.

Results and Discussion

Determination of Antioxidant Properties of HSCG Following Simulated Digestion and Fermentation in HepG2 cells. Evaluation of the actual bioavailability of polyphenols in food and food supplements based on data concerning their absorption, metabolism, tissue and organ distribution is crucial to establish their effects on human body.[18,30,31] Drug bioavailability depends on several factors such as administration route, phenotype, age, gender, and food interaction,[31] however studies carried out on animals or human subjects are necessarily complex, expensive, and lengthy. This is the reason why different *in vitro* procedures that mimic the physiochemical and biochemical conditions encountered in the gastrointestinal tract have been actively developed and tested, providing preliminary data on the potential bioavailability of different components of the food under evaluation.[32-37] The importance of *in vitro* digestion combined with cellular assays to determine the antioxidant activity has been recently emphasized,[15-20] and the HepG2 cell line is widely used to study the biotransformation and the chemopreventive potential of different drugs and chemicals as a model system of the human liver.[23,38] Although we are aware that phase-II metabolism of the phenolic

compounds produced after the simulated gastrointestinal digestion should be duly considered since it can limit their bioavailability and reduce their biological activity,[39] and that expression of drug-metabolizing enzymes and drug transporters in transformed cell lines is often low and variable, genotyping of phase I and phase II enzymes, and drug transporter polymorphisms in these cells confirmed HepG2 as a suitable model for metabolic studies, as the low levels of sulfotransferase and *N*-acetyltransferase reported in this cell line are still high enough to allow metabolic assays by these enzymes.[38]

To test the biocompatibility and cytoprotective properties of both the liquid (potentially bioaccessible) fractions from digestion (HSCG-dig) and fermentation (HSCG-ferm) treatment and the solid residue after fermentation (HSCG-res), together with raw HSCG for comparison, doses of 1-20 μg/mL were considered physiological and realistic,[14] but higher concentrations were also tested in order to ensure that no cytotoxic effect induced by the samples was observed in the cell culture. Thus, crystal violet assay (**Table 23**) shows that doses up to 100 μg/mL of any of the four products did not affect HepG2 cell viability after 20 h.

Despite a slight increase in ROS observed for HSCG-dig at 1 and 5 μg/mL, no physiologically relevant changes in ROS production (**Figure 41A**) and GSH concentration (**Figure 42A**) were observed after plain treatment of cells with all four samples, indicating no harmful alteration of the redox status. Thus, direct treatment with the HSCG products evoked no cellular stress or oxidative damage which could influence the functional conditions of cells to face a potential stressful injury. In order to test the cytoprotective effect of the products in a stressful condition, a model of oxidative stress induced by a potent pro-oxidant, *tert*-butylhydroperoxide (t-BOOH) was established.[15] In agreement with this model, 400 μM t-BOOH evoked a condition of oxidative stress exhibited by decreased cell viability (**Table 23**) and GSH (**Figure 42B**),

Table 23. Direct and Protective Effect of HSCG, HSCG-dig, HSCG-ferm, and HSCG-res on Cell viability (Mean ± SD, *n*=6-10)[a, b]

Sample	Dose	Direct effect[c]	Protective effect[c]
control		100.0 ± 5.1 a	100.0 ± 4.6 b
t-BOOH (400 μM)		-	83.3 ± 4.4 a
HSCG+ t-BOOH	1 μg/mL	102.6 ± 5.9 a	86.6 ± 7.5 ab
	5 μg/mL	94.8 ± 6.7 a	92.3 ± 6.6 b
	10 μg/mL	101.3 ± 4.3 a	91.7 ± 8.6 b
	50 μg/mL	92.9 ± 6.6 a	-
	100 μg/mL	86.8 ± 7.8 a	-
HSCG-dig+ t-BOOH	1 μg/mL	102.6 ± 10.9 a	94.5 ± 3.1 b
	5 μg/mL	92.7 ± 7.3 a	100.8 ± 9.6 bc
	10 μg/mL	105.4 ± 11.5 a	96.0 ± 8.5 bc
	50 μg/mL	108.9 ± 7.0 a	-
	100 μg/mL	118.0 ± 12.0 a	-
HSCG-ferm+ t-BOOH	1 μg/mL	103.4 ± 7.1 a	92.1 ± 6.6 b
	5 μg/mL	107.6 ± 14.3 a	99.1 ± 5.8 bc
	10 μg/mL	103.7 ± 7.8 a	106.3 ± 5.5 c
	50 μg/mL	116.6 ± 5.8 a	-
	100 μg/mL	116.4 ± 16.1 a	-
HSCG-res+ t-BOOH	1 μg/mL	112.1 ± 13.2 a	80.3 ± 5.5 a
	5 μg/mL	108.9 ± 5.7 a	83.8 ± 2.5 a
	10 μg/mL	108.9 ± 9.8 a	83.6 ± 3.3 a
	50 μg/mL	103.4 ± 7.9 a	-
	100 μg/mL	99.7 ± 8.6 a	-

[a]HepG2 were treated with the noted concentrations of mentioned compounds during 20 h (direct effect, without t-BOOH, left column) or for 20 h, then the cultures were washed and 400 μM t-BOOH was added for 2 h to all cultures except controls (protective effect, with t-BOOH, right column).

[b] Means in a column without a common letter differ, $P< 0.05$.

[c] Results are expressed as percentage of viable cells.

as well as overproduction of ROS (**Figure 41B**). Interestingly, data in **Table 23** show that pre-treatment with 1-10 μg/mL of HSCG-dig and HSCG-ferm and 5-10 μg/mL HSCG completely protected HepG2 cell viability from stress-induced death, indicating a clear defense of cell integrity by the spent coffee products against a stressful challenge. The antioxidant activity observed for HSCG-ferm would indicate that the gut microbiota has a strong metabolic activity against HSCG, releasing antioxidant compounds that could be absorbed through the colonic intestinal tract.

The ROS-scavenging effect of HSCG has been recently reported.[14] In the present study, **Figure 41B** shows that 1-20 μg/mL of HSCG-dig, HSCG-ferm and raw HSCG significantly reduced ROS overproduction evoked by t-BOOH, suggesting a quenching ability of reactive species by the products in a cellular environment. Thus, the high levels of ROS generated by the pro-oxidant were significantly quenched by the HSCG products in cells pre-treated for 20 h. We have previously reported a similar ROS reducing effect in this same cell line treated with coffee melanoidin[15] and with a green coffee bean extract or pure chlorogenic acid.[40] This ROS-quenching effect by digested, fermented and raw HSCG could be a first explanation for the reduced oxidative stress and subsequent cell protection.

Comparable to what previously reported with HSCG,[14] a dose-dependent recovery of the depleted GSH was observed with 5-10 μg/mL of HSCG-dig and a complete rescue was confirmed with 5-10 μg/mL HSCG, whereas the other conditions tested showed no significant recovery of the decreased GSH (**Figure 42B**). GSH is the main non-enzymatic antioxidant defense as a substrate in glutathione peroxidase-catalyzed detoxification of organic peroxides, by reacting with free radicals and by repairing free radical induced damage through electron-transfer reactions. Therefore, maintaining GSH concentration above a critical threshold while facing a stressful

situation represents a crucial advantage for cell survival. The present GSH data agree with previous results obtained in the same cell line with other coffee constituents,[15,40] and clearly indicates that cells exposed to HSCG and HSCG-dig showed a protected redox status in a situation of oxidative stress. Thus, the protective mechanism of HepG2 cell integrity and functionality by HSCG products can be illustrated in terms of regulation of the cellular redox status, i.e. scavenging of oxygen radicals by HSCG favors the recovery of GSH levels and decreases oxidative damage and subsequent cell death.

Notably, in all the cellular assays performed no significant protective effect was observed for the HSCG-res sample, suggesting that most part of the beneficial antioxidant activity exhibited by HSCG is transferred to the soluble, potentially bioaccessible fraction. Although much of the evidence for bioactivity of polyphenols evaluated on human cell lines may have little significance *in vivo*,[41] a more significant biological relevance could be attributed to HSCG-ferm as containing human gut microbiota derived metabolites.

Figure 41. Direct (A) and protective (B) effect of the different fractions from simulated digestion-fermentation of HSCG on ROS generation. (A) HepG2 cells were treated with the noted quantities for 2 h and ROS production was determined and expressed as percent of fluorescence units relative to control. (B) HepG2 cells were treated with the noted quantities for 20 h, and then submitted to 400 μM t-BOOH for 2 h and ROS production determined and expressed as above. C= control cells; t= t-BOOH only treated cells. Data are means ± SD (n=6-8). Different letters indicate statistically significant differences ($P<0.05$) among different groups.

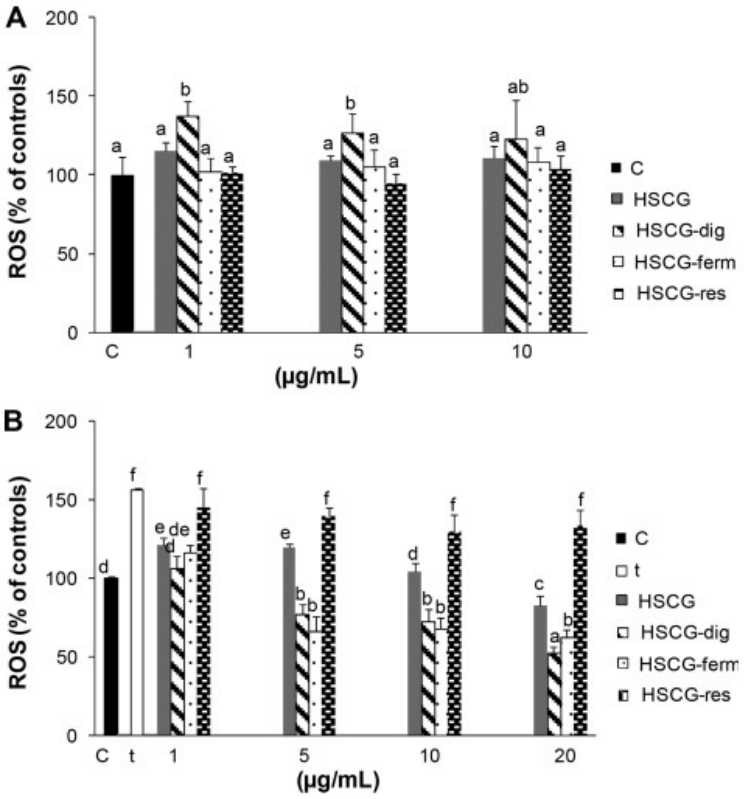
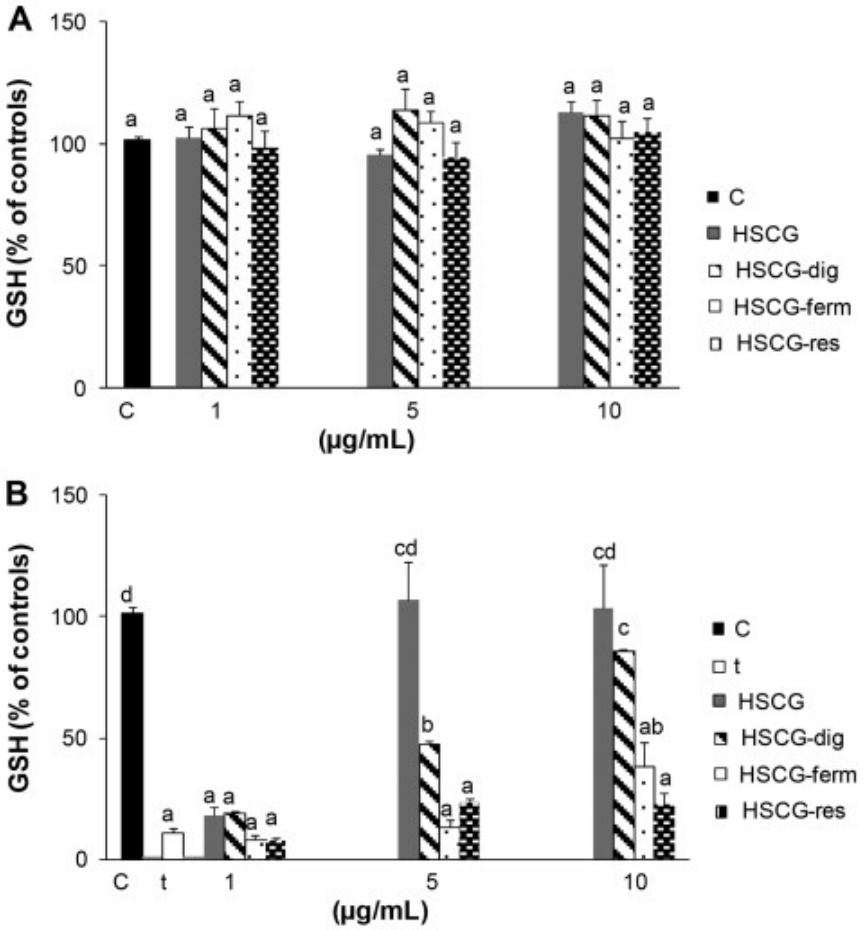
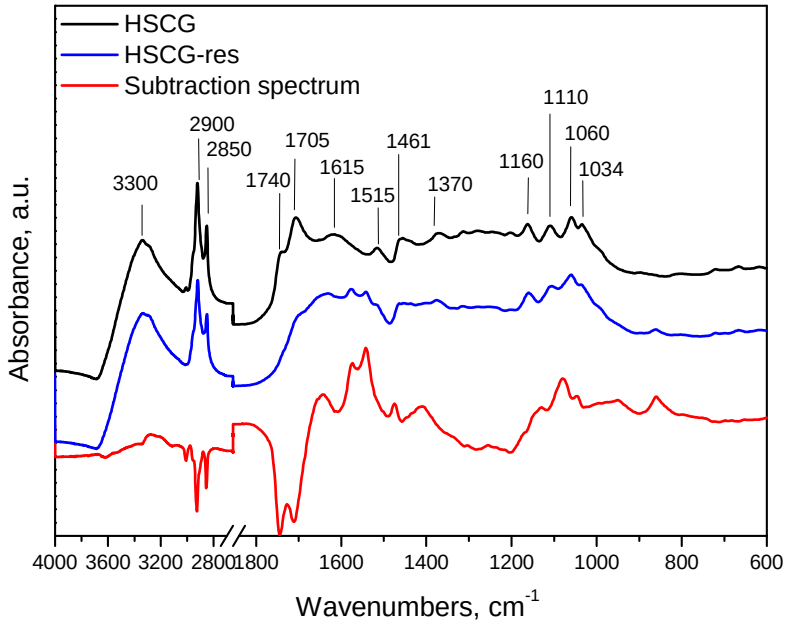


Figure 42. Direct (A) and protective (B) effect of the different fractions from simulated digestion-fermentation of HSCG on GSH. (A) HepG2 cells were treated with the noted quantities for 20 h and GSH levels were determined and expressed as percent of fluorescence units relative to control. (B) HepG2 cells were treated with the noted quantities for 20 h, and then submitted to 400 μM t-BOOH for 2 h and GSH levels determined and expressed as above. C= control cells; t= t-BOOH only treated cells. Data are means ± SD (n=6-8). Different letters indicate statistically significant differences ($P<0.05$) among different groups.



Structural Transformations of HSCG Following Simulated Digestion and Fermentation. To gain an insight into the structural transformations that occur following simulated digestion-fermentation of HSCG, the ATR-FTIR spectra of HSCG and HSCG-res were recorded and are shown in **Figure 43**, together with the subtracted spectrum (HSCGS-res – HSCG). In the high frequency region, HSCG and HSCG-res showed similar spectral profiles, with a broad band located at 3300 cm^{-1} and a pattern of signals in the 2900-2800 cm^{-1} range. These features are due to hydroxyl groups of cellulose and hemicellulose[10] and to the hydrocarbon moieties of lignins,[42] respectively. A decrease in these latter signals is observed in the subtracted spectrum, indicating partial hydrolysis of lignins following the simulated digestion-fermentation, which could in part account for the higher antioxidant activity observed for the soluble, potentially bioaccessible fractions compared to HSCG-res. At lower wavenumbers, HSCG shows two absorption bands in the carbonyl stretching region, located at 1740 and 1705 cm^{-1}, likely due to acetyl groups of hemicellulose and carboxylic acids, respectively.[43] The subtracted spectrum shows a remarkable reduction of these signals in HSCG-res, likely due to hydrolysis and removal of hemicellulose and carboxylic acid constituting HSCG following the enzymatic treatment. No other significant differences could be observed among the two samples, either in the cellulose/hemicellulose alcohol C-O stretching region (bands at 1160, 1060 and 1034 cm^{-1})[44] or in the aromatic skeleton vibrations (1615 ant 1515 cm^{-1}) and C-H bending of aromatic methoxy groups (1461 and 1110 cm^{-1}).[45] The increase in absorption in the 1600-1500 cm^{-1} range in the HSCG-res spectrum can be attributed to the amide groups of some residual enzymes used for the simulated digestion-fermentation treatment.

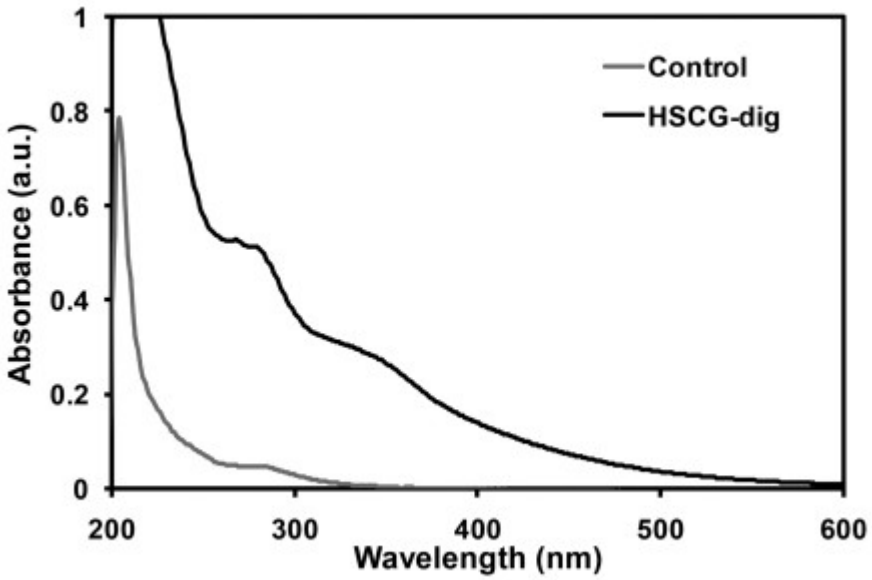
Figure 43. ATR-FTIR spectra of HSCG and HSCG-res.



To gain information about the nature of the components released from HSCG following simulated digestion and fermentation, the soluble fractions HSCG-dig and HSCG-ferm were preliminarily analyzed by both HPLC and ^{1}H-NMR, but no significant differences were observed compared to control mixtures containing enzymes and other ingredients but no HSCG. Accordingly, to avoid interferences from these components, in other experiments the HSCG-dig and the HSCG-ferm samples were taken to pH 1 and repeatedly extracted with ethyl acetate. HPLC analysis with UV detection at 254 nm of the organic extractable fraction from HSCG-dig showed the presence of minute amounts of chromatographically defined compounds but significant quantities of organic polymeric material eluted as a broad peak at the end of the gradient program. The presence of conjugated, aromatic polymer was also well apparent from the UV-vis spectrum of the organic extract of HSCG-dig reported in **Figure 44**, featuring a broadband absorption in the range 200-600 nm, with maxima at around 280

and 310 nm, typical of lignin moieties with hydroxycinnamic acid type structures;[46-48] for comparison the UV-vis spectrum of the organic extractable fraction from the control, simulated digestion mixture not containing HSCG is also shown at the same concentration, displaying no significant absorption above 300 nm. Unfortunately, all attempts to further characterize the main extractable components of HSCG-dig by NMR failed, likely due to the great chemical heterogeneity of lignin polymers.

Figure 44. UV-vis spectra of the ethyl acetate extractable fraction of HSCG-dig and of a control digestion mixture containing enzymes and other ingredients but no HSCG (both at 0.15 mg/mL in methanol).



Very little information could be obtained on the ethyl acetate extractable fraction of HSCG-ferm: apparently no polymeric material was present in this case, as highlighed by both HPLC and UV-vis analysis, suggesting efficient metabolization of the lignin moieties by the colonic microflora; however, not very significant differences could be observed either in the elutoghraphic profiles or in the UV-vis or NMR spectra

when the extractable fraction of HSCG-ferm was compared with that obtained form a control fermentation mixture not containing HSCG.

Prebiotic Activity of HSCG. Taking into consideration the nutritional composition of SCG,[12] which is especially enriched in insoluble components, these by-products should have an interesting activity on the gut microbiota. Since it was found that SCG increase the levels of *Lactobacillus spp.* and *Bifidobacterium spp.* after *in vitro* fermentation,[12] we decided to analyze the effect of microbial fermentation over HSCG by determination of changes in microbial composition and production of SCFAs, which are known to have healthy properties such as immunomodulation through their attachment to the GPR43 receptor.[49] **Table 24** shows the changes in microbial composition after fermentation of HSCG, compared to those produced by a commercial milk beverage enriched with a probiotic strain known to produce large amount of SCFAs after fermentation, a positive control made of inulin (a commercial prebiotic) and a control sample (fermentative medium without any nutrient). HSCG showed a clear prebiotic activity since its fermentation increased the population of *Bifidobacterium spp.* and *Lactobacillum spp.* (compared with the control), which are known microbial species with healthy properties. At the same time, a decrease (compared with the control) was observed on *Clostridium spp.* and *Bacteroides spp.*, which are microorganisms related with different pathologies. The same effect was also obtained for the milk beverage and inulin (probiotic and prebiotic controls, respectively). However, the amount of *Bifidobacterium spp.* and *Lactobacillum spp.* was statistically higher ($P < 0.05$) in the case of the milk beverage. In the case of inulin, although the concentration of both healthy bacterial strains was higher than those found after fermentation of HSCG, such differences were not statistically significant.

Table 24. Prebiotic Activity of HSCG.[a]

Sample	*Clostridium spp.*	*Bacteroides spp.*	*Bifidobacterium spp.*	*Lactobacillum spp.*
HSCG	(5.21 ± 0.47)E+07a	(8.02 ± 0.16)E+08a	(8.52 ± 0.44)E+07a	(2.41 ± 0.35)E+10a
Milk beverage	(4.43 ± 0.26)E+07a	(7.16 ± 0.17)E+08a	(1.12 ± 0.04)E+08b	(7.16 ± 0.16)E+10b
Inulin	(6.17 ± 0.19)E+07a	(8.78 ± 0.32)E+08a	(9.31 ± 0.47)E+07a	(3.27 ± 0.07)E+10a
Control	(9.31 ± 0.13)E+07b	(1.23 ± 0.09)E+09b	(4.41 ± 0.39)E+06c	(3.72 ± 0.46)E+09b

[a]Different letters within the same column indicate statistically significant differences ($P < 0.05$)

Table 25. Fermentative Activity Measured as Release of SCFAs (μmol/g dry matter) of HSCG.[a]

Sample	acetic acid	propionic acid	butyric acid
HSCG	2534 ± 37 a	663 ± 7 a	23 ± 3 a
Milk beverage	478 ± 27 b	89 ± 6 b	83 ± 4 b
Inulin	1561 ± 32 c	428 ± 16 c	48 ± 7 c
Control	49 ± 6 d	26 ± 4 d	4 ± 1 d

[a]Different letters within the same column indicate statistically significant differences ($P < 0.05$)

Table 25 shows the levels of acetic, propionic and butyric acids released after fermentation of the same samples depicted in **Table 24**. It can be observed that the fermentation of HSCG released very high amounts of acetic and propionic acid. This is in accordance with the results obtained for the antioxidant activity, since an extensive fermentation of HSCG means a high release of antioxidant compounds due to a stronger metabolic activity of the gut microbiota, which in turn release more SCFAs as by-products of their metabolism. When the HSCG were compared with the milk derivative, statistically higher ($P < 0.05$) concentrations of acetic and propionic acids (up to 5-times higher) were obtained. However, the amount of butyric acid obtained for HSCG was lower as expected considering that the lactic bacterial strains used as probiotics in milk derivatives are specialized in the production of butyric acid. This is also in accordance with the higher proportion of *Bifidobacterium spp.* and *Lactobacillum spp.* in the milk derivative (**Table 24**), since these microorganisms are butyrate producers. The comparison with inulin showed also statistically significant differences ($P < 0.05$) for the three SCFAs. HSCG produced more acetic and propionic acid than inulin, while the amount of butyric acid produced by HSCG was half of that produced by inulin. This could be related, as in the case of the milk derivative, with a higher microbial load of *Bifidobacterium spp.* and *Lactobacillum spp.* in the inulin-fermented samples (**Table 24**). Therefore, all these results indicate that the gut microbiota effectively ferments HSCG, releasing both SCFAs and antioxidant compounds, behaving as dietetic fiber as known prebiotics like inulin. It is known that SCG are a rich source of galactomannans and arabinogalactans, whose beneficial effects on large bowel function, including promotion of growth of bifidobacteria as well as production of SCFAs, have been amply documented.[21,50-52] Notwithstanding, the role of other constituents, e.g. phenolic compounds, in the observed prebiotic activity can not be ruled out since it should be

taken into account that one of the main effect of the treatment performed for preparation of HSCG is removal of the hydrolyzable components such as carbohydrates.[14] Oligomeric and polymeric polyphenols such as condensed and hydrolyzable tannins, which are not absorbed in the small intestine and are able to reach the colon, are known to have effects on gut microbes, modulating the colon microbial composition and function.[53] Grapeseed, wine, cocoa, green tea, blueberry and pomegranate extracts have been shown to enhance the growth of lactobacilli and bifidobacteria while reducing numbers of potential pathogens, both in *in vitro* and *in vivo* assays.[53,54] In this context, the present findings hopen the way toward a systematic evaluation of HSCG as a cheap and an easily accessible source of prebiotic carbohydrates and phenolic compounds for application in human health as food supplements.

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Notes

The authors declare no competing financial interest.

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Spent coffee grounds extract rich in manooligosaccharides promote a healthier gut microbial community in a dose dependent manner

Dados los buenos resultados obtenidos en el anterior trabajo, en el que se demostraron los posibles efectos beneficiosos de los posos de café, en el presente paper se ha ido más allá y se trató de elaborar un extracto rico en manooligosacaridos y polifenoles, de forma que se incrementaran la capacidad antioxidante y prebiótica de los posos de café. Así, en este trabajo se evaluó la capacidad prebiótica de este extracto así como su contenido en polifenoles totales mediante el método Folin-Ciocalteu, método que permite evaluar a la vez el contenido en polifenoles y capacidad antioxidante. Además, se cuantificó el efecto de compuestos furánicos procedentes del procesado térmico sobre la microbiota intestinal.

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Spent coffee grounds extract rich in manooligosaccharides promote a healthier gut microbial community in a dose dependent manner

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ABSTRACT

Coffee is one of the most consumed beverages around the world and as a consequence, spent coffee grounds are a massively produced residue that is causing environmental problems. Reusing them is a major focus of interest nowadays. We extracted mannoligosaccharides (MOS) from spent coffee grounds and submitted them to an *in vitro* fermentation with human feces. Results obtained suggest that MOS are able to exert a prebiotic effect on gut microbiota by stimulating the growth of some beneficial genera such as *Barnesiella, Odoribacter, Coprococcus, Butyricicoccus, Intestinimonas, Pseudoflavonifractor,* or *Veillonella.* Moreover, SCFA production also increased in a dose dependent manner. However, we observed that 5-hydroxymethylfurfural, furfural and polyphenols (which are either produced or released from spent coffee grounds matrix during hydrolysis) could have an inhibitory effect on some beneficial genera such as *Faecalibacterium, Ruminococcus, Blautia, Butyricimonas, Dialister, Collinsella,* or *Anaerostipes* which could affect negatively to the prebiotic activity of MOS.

KEY WORDS: Mannooligosaccharides, mannan, MOS, short chain fatty acids, gut microbiota, prebiotic activity, 5-hydroxymethylfurfural, polyphenols.

Introduction

Coffee brew is consumed all over the world and, in fact, is the food product most consumed in the world, after water.[1] Coffee has been related to health since ancient times and still today is the main object of study in many research projects related to chronic diseases.[2] Coffee is thought to have a protective role on health, mostly due to its phytochemical compounds such as chlorogenic acids and other polyphenolic substances.[3] However, carbohydrates also account for an important part of the coffee bean. In green coffee, carbohydrates account for up to 60% of the dry weight, with sucrose as the main monosaccharide. Among polysaccharides, which can range from 37 to 55% of the dry weight, cellulose, hemicellulose, galactomannans and arabinogalactans are found. Although galactomannans are the main polysaccharide present in beans, in coffee infusion they are not the main ones due to a low solubility.[4] On the other hand, roasting has an important influence on carbohydrate content. Sucrose and low molecular weight oligosaccharides are almost completely degraded whereas polysaccharide content is reduced to around 24-39% of the dry weight. Roasting also partially hydrolyses polysaccharides to a lower degree of polymerization making them more soluble.[5]

Due to its high consumption, coffee by-products are massively produced specially spent coffee grounds. The main problem is that they are not properly disposed of, generating a growing environmental concern.[5,6] Therefore, spent coffee grounds have become a major focus of interest regarding their reutilization as a source of bioactive compounds.[7-12]

Since hot water is not able to extract the whole carbohydrate fraction during coffee brewing, spent coffee grounds are still rich in polysaccharides, with galactomannans representing around 50% of them and arabinogalactans and cellulose

accounting for the other 50%.[4] Therefore, mannanoligosaccharides (MOS) are one of the bioactive compounds that can be obtained from spent coffee grounds. MOS are short chain carbohydrates produced from coffee galactomannans usually through acid, alkaline or enzymatic hydrolysis.[13] Their physiological effects arise from different functional properties: they are able to enhance the growth of beneficial bacteria, decrease blood pressure, reduce fat absorption, or decrease attachment of pathogenic bacteria to the intestinal mucosa.[14-15]

Finding ingredients that can promote the growth of beneficial gut bacteria is becoming a major focus of interest in order to produce functional foods. That is because now it is very clear that the gut microbiota has important implications in different diseases and is therefore closely related to health status.[16] In this sense, the gut microbiota has been linked to inflammatory bowel disease,[17,18] obesity,[19, 20] autism spectrum disorders,[21] and immune system disorders,[22] among others. It has been reported that around 40 g of polysaccharides reach the colon every day, being an important source of nutrients for gut microbes and acting as prebiotic agents that enhance the growth of some of them.[23] The metabolization of fiber takes places specially in the proximal colon resulting in the production of several metabolites such as short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, which have an important role in host health, not only in the gut.[24] Dietary fiber is the main nutrient for gut microbes and it has been proven to modulate gut microbiota *in vitro* and *in vivo* with beneficial effects for host health.[23]

Taking all this information into account, the objective of this study was to evaluate the effects of MOS produced from spent coffee grounds on gut microbial communities and also on SCFAs production, after *in vitro* fermentation with human fecal samples.

Materials and methods

Reagents

Acetic, formic and propionic acids, 1-phenyl-3-methyl-5-pyrazolone (PMP), chloroform, mannose, manobiose, manotriose, manotetraose, mannopentaose and mannohexaose standards, sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide, resazurin, Folin-Ciocalteu reagent, sodium carbonate, gallic acid, 5-(Hydroxymethyl)furfural (HMF), furfural and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Darmstadt, Germany).

Samples

A mix of spent coffee grounds and water (10% w/w) was hydrolyzed in a reactor (270 mL of distilled water and 30 g of spent coffee grounds) by applying high temperatures and high pressure for 60 minutes and taking samples after 10 minutes, 30 minutes and 60 minutes. Four experiments were carried out varying only the temperature: experiment A was carried out at 220 ^{o}C, experiment B was carried out at 185 ^{o}C, experiment C was carried out at 170 ^{o}C and experiment D was carried out at 180 ^{o}C. Each experiment was composed of the three samples taken after 10 minutes, 30 minutes and 60 minutes. Aliquots were taken and stored at -80 ^{o}C for MOS quantification, HMF, furfural and total polyphenols quantification and for *in vitro* fermentation.

In vitro fermentation

The *in vitro* fermentation method was carried out according to the protocol described by Pérez-Burillo et al.[25] A Fecal sample from four healthy donors (not taking antibiotics, people with body mass index within the “normal weight range”: mean Body

Mass Index = 21.3), was obtained in the morning, placed in sterile containers and stored at 4 ºC until the inoculum was prepared (as an equal mixture of feces from all four donors). Briefly, 0.5 mL of MOS mixture obtained from the reactor was placed in a screw-cap tube. Then, 7.5 mL of fermentation medium (948.75 mL of peptone water (15 g/L), 50 mL of reductive solution (312 mg of cysteine and 312 mg of sodium sulphide in 48 mL of water and 2 mL of NaOH 1M), and 1.25 mL of resazurin (0.1% w/ v)) and 2 mL of inoculum (consisting of a solution of 32% faeces in phosphate buffer 100 mM, pH 7.0) were added, to reach a final volume of 10 mL. Nitrogen was bubbled through the mix to produce an anaerobic atmosphere and the mix was then incubated at 37 ºC for 24 hours under oscillation. Immediately afterwards, the samples were immersed in ice, to stop microbial activity. Aliquots were taken for DNA isolation and SCFAs analysis and stored at -80 ºC until analysis.

Short chain fatty acids determination

Short chain fatty acid (SCFAs) determination was performed on the supernatant obtained after *in vitro* fermentation. The analysis was carried out according to the procedure described by Panzella et al.[11] with few modifications. SCFAs determination was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 210 nm; the mobile phase used was 0.1 M phosphate buffer (pH 2.8)/acetonitrile 99:1 v/v delivered at a 1.25 mL/ min flow rate; the column used was an Aquasil C18 reverse phase (Thermo Scientific) (150 × 4.6 mm, 5 µm), with a total run-time of 30 min.

The sample did not require any pretreatment before injecting. Briefly, the SCFA standards were prepared in the mobile phase at concentrations ranging from 5 to 10000 ppm. After the fermentation process, 1 mL of MOS fermentation supernatant was

centrifuged to remove solid particles, filtered through a 0.22 μm nylon filter and finally transferred to a vial for HPLC analysis. Results are expressed as μmol/g of spent coffee grounds. Chromatograms are depicted in **Figures 45A-45C**.

MOS HPLC determination

Pre-column derivatization: MOS mixtures obtained from the reactor were labeled with PMP following the procedure described by Zang et al.[26] with few modifications. Briefly, 0.1 mL of sample was mixed in a screw-cap pyrex tube with 0.1 mL of NaOH 0.3 M. Right after, 0.12 mL of PMP 0.5 M was added and the mixture was vortexed for 10 seconds and placed at 70 ºC for 60 minutes. Thereafter, 0.1 mL of HCl 0.3 M was added to neutralize the NaOH. The mixture was then mixed with 0.5 mL of chloroform, vortexed, and the aqueous layer collected, discarding the organic one. This step was done two more times. Samples were filtered through a 0.22 μm filter and injected in the HPLC.

HPLC analysis: Determination of MOS was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 245 nm; the mobile phase used was A) Milli-Q water with 0.1% of formic acid, and B) acetonitrile with 0.1% of formic acid running with the following gradient: 2% of B to 20% of B in 25 minutes (0-25 minute), 20% of B for 15 minutes (25-40 minute), 20% of B to 2% of B in 5 minutes (40-45 minute), and 2% of B for 15 minutes (45-60 minute). Standard curves were performed for each sugar in the range of 5-0.15 ppm. The column used was an Accucore C18 reversed phase 150 mm x 2.1 mm and 2.6 μm of particle size. Results are expressed as mg/g of spent coffee grounds. Chromatograms are depicted in **Figures 45D-45F**.

Folin-Ciocalteu total phenolics assay

Total phenolic content was estimated following the procedure described by Singleton and Rossi[27] with few modifications and adapted to a microplate reader. The assay was performed on the samples obtained from the reactor. Briefly, sodium carbonate 10% was prepared and then the needed volume was diluted to 2.35%. 30 µL of sample were placed by triplicate in the wells of a transparent 96-well polystyrene microplate (Biogen Científica, Spain), followed by 255 µL of the sodium carbonate and finally 15 µL of the Folin-Ciocalteu reagent. Absorbance lectures at 725 nm were taken for 60 minutes at 37 ºC. The equipment used was a FLUOStar Omega microplate reader (BMG Labtech, Germany). A calibration curve was performed using gallic acid as the standard with concentrations ranging from 0.01 to 1.00 mg/mL. The results obtained are expressed as µmol gallic acid equivalents (GAE) per g of spent coffee grounds.

HMF and furfural assay

HMF and furfural determination was performed according to the method described by Rufián-Henares et al.[28] One mL of the MOS mixture taken from the reactor was centrifuged at 10000 rpm for 5 minutes. Then, it was filtered through a 0.22 µm filter. The sample was ready for HPLC injection. Determination was carried out on Accela 600 HPLC (Thermo Scientific) equipped with a pump, an autosampler and a UV-VIS PDA detector set at 284 nm. The mobile phase was made up of a mixture of acetonitrile in water (5% v/v) delivered at the flow rate of 1 mL/min under isocratic conditions through C18 reverse phase column. 20 µL of sample were injected. HMF and furfural was quantified by the external standard method within the range 0.01–50.00 mg/L. Results are expressed as mg/g of spent coffee grounds.

DNA extraction and sequencing

DNA extraction was performed using a NucliSENSEasyMAG platform (Biomérieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μL in 10 mMTris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al.[29] Primer sequences are: Forward 5'TCGTCGGCAGCGTCAGATG-TGTATAAGAGACAGCCTACGGGNGGCWGCA-G3' and Reverse 5'GTCT-CGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3'. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification, amplicons were multiplexed and 1 mL of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001).

Bioinformatic analysis

Quality assessment of sequencing reads was performed with the prinseq-lite program applying the following parameters:[30] a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3'-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing where joined using fastq-join from the ea-tools suite.[31] Taxonomic affiliations were assigned using the RDP_classifier from the Ribosomal Database Project (RDP).[32] Reads that had an RDP

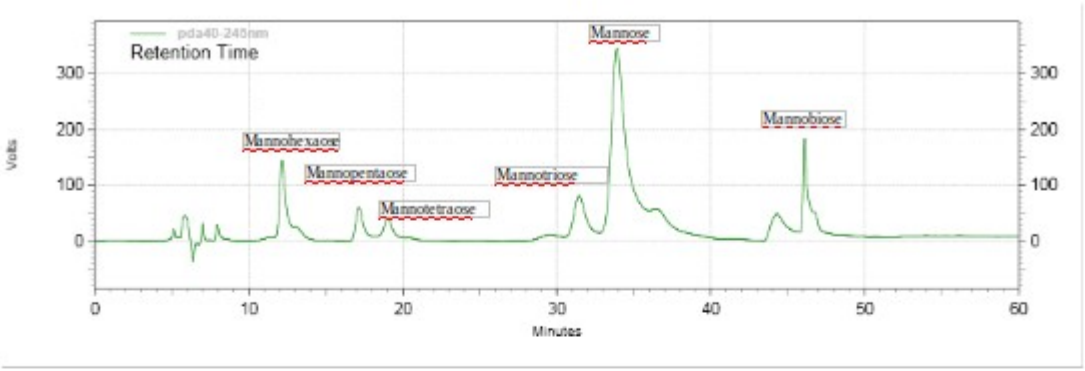
score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank as unidentified. We assigned 6 taxonomic levels, which were kingdom, phylum, class, order, family and genus.

Statistical analysis

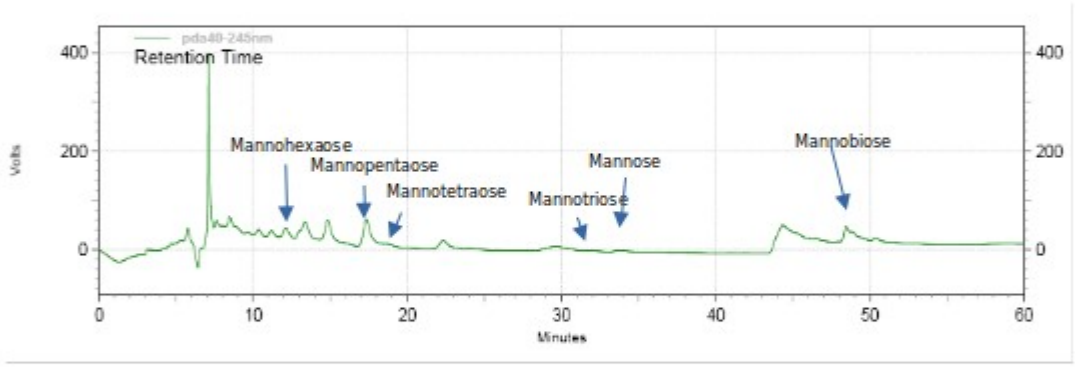
Pearson correlations were carried out through multivariate analysis with Statgraphics Centurion XVI.I. Bacterial abundances between groups, SCFA, HMF and polyphenols were compared by analysis of variance (ANOVA) at $p < 0.05$ confidence level with Statgraphics Centurion XVI.I and R software.

Figure 45. Chromatograms from SCFA (A-C) and MOS (D-F).

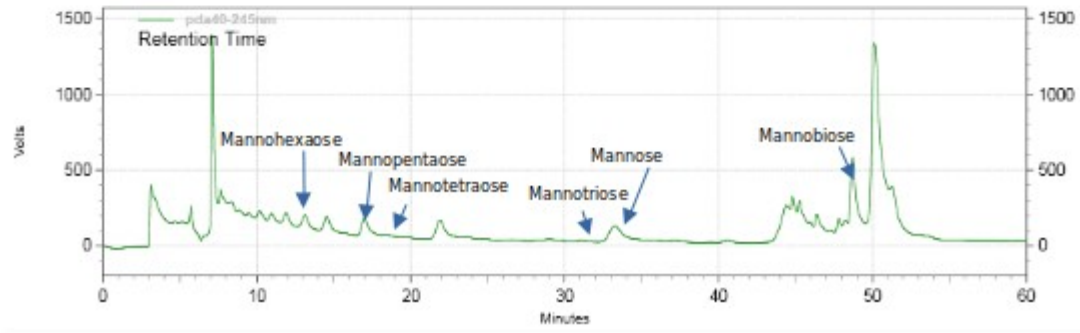
A



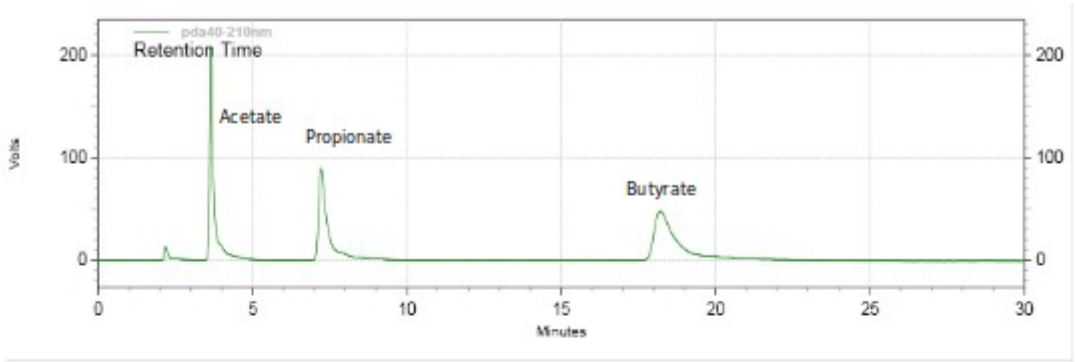
B



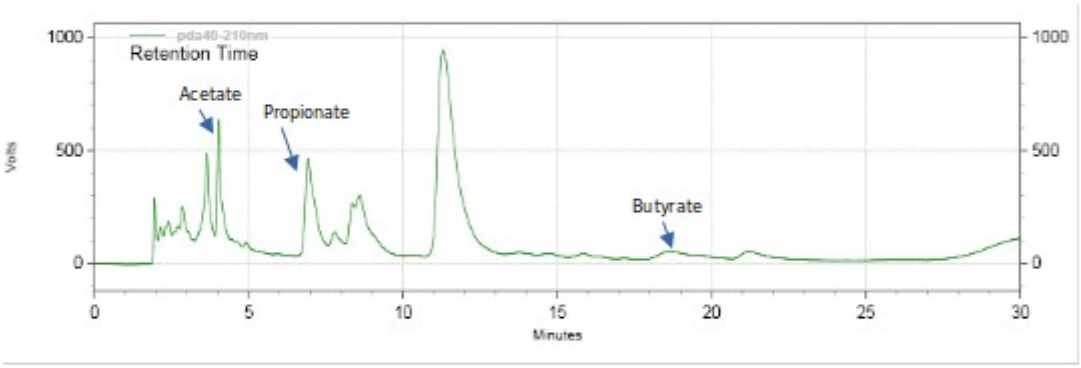
C



D


pda40-210nm
Retention Time
Acetate
Propionate
Butyrate
Volts
Minutes
0
100
200
0
5
10
15
20
25
30

E


pda40-210nm
Retention Time
Acetate
Propionate
Butyrate
Volts
Minutes
0
500
1000
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5
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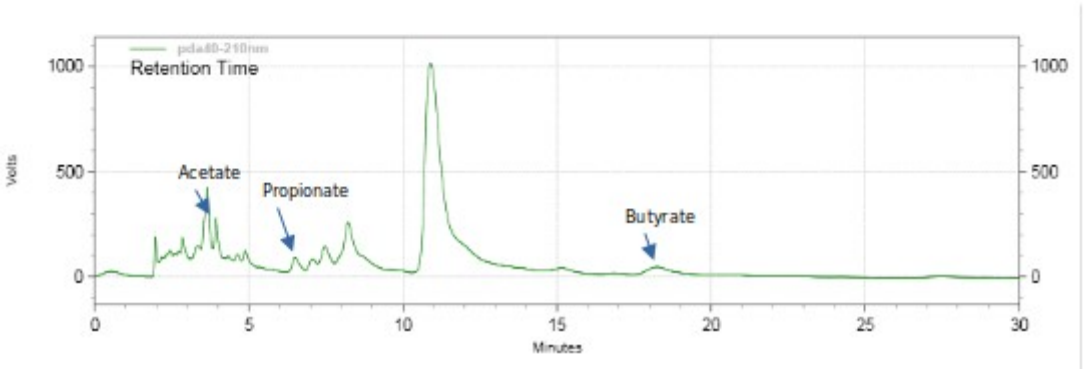
25
30

F


pda40-210nm
Retention Time
Acetate
Propionate
Butyrate
Volts
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Results and discussion

MOS quantification

MOS quantification was carried out after 10, 30 and 60 minutes of hydrolysis in every experiment. For each sample, mannose, mannobiose, mannotriose, mannotetraose, mannopentaose and mannohexaose were determined. **Table 26** shows the MOS content in each sample. All carbohydrates were detected except mannotetraose. After 10 minutes, temperature was not stable yet and MOS values are similar among the different experiments. However, experiment B had significantly lower ($p < 0.05$) amounts of total MOS than the other experiments. The highest levels of mannose, mannobiose and mannopentaose were obtained in experiment A, whereas mannotriose and manohexaose were significantly higher in experiment C.

After 30 minutes of hydrolysis, experiment A showed the highest MOS content with statistical significance ($p < 0.05$), followed by experiments B and D, with no significant differences among the two of them. Finally, experiment C showed significantly lower ($p < 0.05$) amounts than the other three experiments. This makes sense since experiment A was carried out at a higher temperature, whereas in experiment C the lowest temperature was used. On the other hand, experiments B and D showed very similar amounts of MOS, probably due to the very close working temperature. Individually, each sugar followed the tendency described above. Finally, at 60 minutes experiment A decreased in all six sugars, probably due to a thermal degradation related with the high temperatures applied; experiment B had significantly higher values than the other three experiments, whereas experiment C had significantly lower values. Individually, each sugar followed the tendency described above.

As a result of these trends, the overall highest amounts of individual and total MOS were obtained after 60 minutes in experiment B, as at this point MOS

concentrations reached values beyond those that had been obtained at 30 minutes in experiment A.

Table 26. Mannooligosaccharides Content (mg/g of Spent Coffee Ground).

	Time, minutes	Mannose	Mannobiose	Mannotriose	Mannotetraose	Mannopentaose	Mannohexaose	Total MOS
Experiment A	10	0.11 ± 0.00^{a}	0.69 ± 0.04^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.34 ± 0.07^{a}	0.45 ± 0.02^{a}	2.59 ± 0.11^{a}
(220 ºC)	30	4.16 ± 0.26^{b}	3.52 ± 0.16^{b}	0.72 ± 0.04^{b}	0.00 ± 0.00	10.02 ± 0.41^{b}	3.33 ± 0.23^{b}	21.75 ± 0.76^{b}
	60	1.11 ± 0.04^{c}	1.81 ± 0.06^{c}	0.15 ± 0.01^{c}	0.00 ± 0.00	2.61 ± 0.07^{c}	1.21 ± 0.05^{c}	6.89 ± 0.14^{c}
	Mean	1.79 ± 0.04	2.01 ± 0.03	0.29 ± 0.01	0.00 ± 0.00	4.66 ± 0.05	1.66 ± 0.04	10.41 ± 0.08
Experiment B	10	0.04 ± 0.00^{d}	1.11 ± 0.05^{c}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.42 ± 0.05^{a}	0.99 ± 0.05^{c}	3.55 ± 0.12^{d}
(185 ºC)	30	0.15 ± 0.00^{a}	2.70 ± 0.22^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.52 ± 0.11^{a}	1.94 ± 0.04^{d}	6.31 ± 0.40^{c}
	60	6.47 ± 0.53^{e}	10.28 ± 0.79^{d}	1.14 ± 0.08^{e}	0.00 ± 0.00	16.89 ± 1.15^{d}	6.57 ± 0.60^{e}	41.35 ± 2.49^{e}
	Mean	2.22 ± 0.16	4.69 ± 0.20	0.38 ± 0.03	0.00 ± 0.00	6.61 ± 0.25	3.17 ± 0.25	17.07 ± 0.48
Experiment C	10	0.04 ± 0.00^{d}	0.83 ± 0.03^{a}	0.07 ± 0.01^{c}	0.00 ± 0.00	2.09 ± 0.06^{c}	1.01 ± 0.06^{c}	4.03 ± 0.09^{f}
(170 ºC)	30	0.02 ± 0.00^{d}	1.32 ± 0.00^{c}	0.05 ± 0.00^{c}	0.00 ± 0.00	1.23 ± 0.00^{a}	0.86 ± 0.04^{c}	3.48 ± 0.08^{d}
	60	0.31 ± 0.02^{a}	2.43 ± 0.03^{b}	0.48 ± 0.02^{f}	0.00 ± 0.00	2.60 ± 0.03^{c}	1.82 ± 0.12^{d}	7.64 ± 0.04^{c}
	Mean	0.12 ± 0.00	1.53 ± 0.12	0.20 ± 0.01	0.00 ± 0.00	1.97 ± 0.13	1.23 ± 0.04	5.05 ± 0.32
Experiment D	10	0.01 ± 0.00^{d}	0.74 ± 0.06^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00	1.06 ± 0.08^{a}	0.77 ± 0.04^{c}	2.58 ± 0.17^{a}
(180 ºC)	30	0.30 ± 0.01^{a}	2.61 ± 0.24^{b}	0.02 ± 0.00^{a}	0.00 ± 0.00	3.14 ± 0.26^{e}	2.52 ± 0.11^{e}	8.59 ± 0.66^{c}
	60	2.38 ± 0.03^{f}	4.33 ± 0.05^{e}	0.34 ± 0.02^{d}	0.00 ± 0.00	8.31 ± 0.09^{f}	4.26 ± 0.06^{f}	19.63 ± 0.05^{b}
	Mean	0.90 ± 0.05	2.56 ± 0.12	0.12 ± 0.01	0.00 ± 0.00	4.17 ± 0.17	2.52 ± 0.15	10.27 ± 0.34

Microbial composition

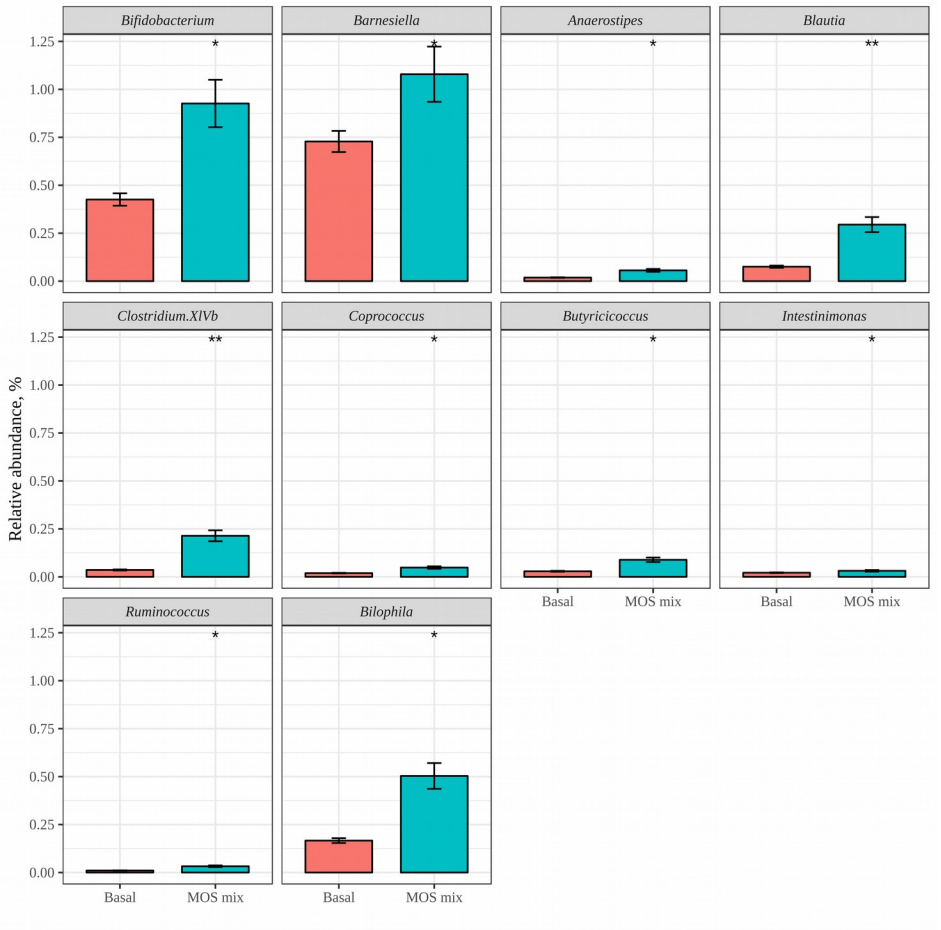
As the objective of this project was to study the possible use of spent coffee grounds rich in MOS (after hydrolysis) as potential prebiotic, an initial *in vitro* fermentation of a pure MOS mix before starting with spent coffee grounds hydrolysis was performed. Accordingly, 30 mg of MOS/mL (5 mg/mL of each compound) were fermented. Significant stimulation in the growth of some genera, in comparison with basal situation of the microbial community, were found. Therefore, *Bifidobacterium, Barnesiella, Anaerostipes, Blautia, Clostridium XVIb, Coprococcus, Butyricicoccus, Intestinimonas, Ruminococcus and Bilophila* showed significantly higher abundances when MOS where used (**Figure 46A**).

Once the prebiotic effect of pure MOS could have on some bacteria was confirmed, and since MOS have been described as prebiotic compounds, the next step was to unravel whether higher amounts of MOS would lead to a more beneficial microbial community structure. For this purpose, the samples corresponding to 30 minutes were used for several reasons: a) after 10 minutes the temperature was not stable and thus, MOS production was very variable. In this sense, MOS production was not sufficiently different among studies as to be able to make comparisons and therefore to establish clear links to certain microorganisms. b) After 60 minutes, experiment A suffered a decrease in oligosaccharide content (probably due to a thermal degradation) which would yield new compounds with potential effect on microbial growth, making this experiment useless for comparisons. c) Related to the previous reason, during carbohydrate heating there is a production of furanic compounds (such as HMF and furfural) from non-enzymatic browning.[28] In this sense, we observed an increase in the levels of furanic compounds with hydrolysis time and temperature (HMF and furfural, **Table 27**), which have been reported to inhibit some bacterial growth.[33] Therefore we

decided to avoid samples obtained after 60 minutes due to the excessive accumulation of furanic compounds; in fact, we detected that some bacteria stopped growing or even decreased in abundance after 30 minutes (described in section 3.2.1.). In addition, comparisons were made between experiments and not between samples from the same experiment, using only the sample obtained after 30 minutes of hydrolysis.

Figure 46. A) Bar plot of the relative abundance at genus level for those bacteria that were significantly different between the basal situation and after addition of a mixture of MOS (M1−M6) in equal proportions; B) Effect of adding HMF and furfural at low (1 mg/mL) and high (20 mg/mL) concentrations on gut microbiota growth.

A)



B)

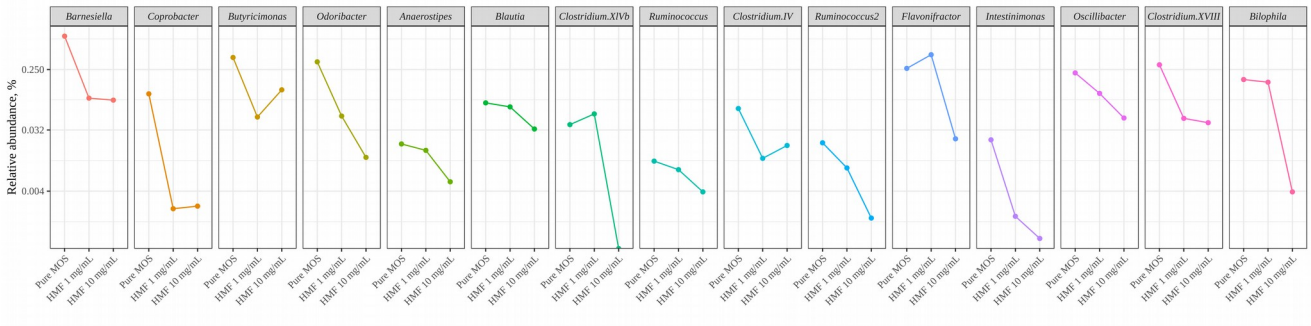

Barnesiella
Coprobacter
Butyricimonas
Odoribacter
Anaerostipes
Blautia
Clostridium.XIVb
Ruminococcus
Clostridium.IV
Ruminococcus2
Flavonifractor
Intestinimonas
Oscillibacter
Clostridium.XVIII
Bilophila
Relative abundance, %
0.250
0.032
0.004
Pure MOS
HMF 1 mg/mL
HMF 10 mg/mL

Table 27. SCFAs Content (μmol/g of spent coffee grounds), HMF and Furfural Content (mg/g of spent coffee grounds) and Total Polyphenols Content (mg GAE/g of spent coffee grounds)

	Hydrolysis time, minutes	Acetate	Propionate	Butyrate	Total SCFAs	HMF	Furfural	Polyphenols
Experiment A	10	68.32 ± 4.97^{a}	32.95 ± 1.92^{a}	24.67 ± 1.83^{a}	125.94 ± 7.09^{a}	0.29 ± 0.03^{a}	0.01 ± 0.00^{a}	16.89 ± 0.40^{a}
	30	80.21 ± 4.48^{b}	44.28 ± 2.02^{b}	43.84 ± 2.04^{b}	168.33 ± 13.85^{b}	7.99 ± 0.45^{b}	0.42 ± 0.02^{b}	20.66 ± 1.31^{b}
	60	76.61 ± 3.52^{b}	41.36 ± 1.46^{b}	35.84 ± 2.31^{c}	153.80 ± 1.89^{c}	16.84 ± 1.27^{c}	0.94 ± 0.08^{c}	26.14 ± 1.37^{c}
	Mean	75.05 ± 1.43	39.53 ± 0.56	34.78 ± 1.09	149.36 ± 6.32	8.37 ± 0.31	0.46 ± 0.03	21.23 ± 0.90
Experiment B	10	97.31 ± 4.92^{c}	53.87 ± 2.25^{c}	32.50 ± 1.20^{c}	183.67 ± 11.66^{d}	0.60 ± 0.03^{a}	0.03 ± 0.00^{a}	30.65 ± 1.95^{d}
	30	126.23 ± 12.66^{d}	66.21 ± 5.45^{d}	58.71 ± 4.73^{d}	251.15 ± 20.67^{e}	2.56 ± 0.25^{d}	0.10 ± 0.01^{d}	45.38 ± 3.88^{e}
	60	119.03 ± 10.79^{d}	69.21 ± 5.21^{d}	49.71 ± 3.15^{b}	237.95 ± 17.73^{e}	9.42 ± 0.74^{b}	0.47 ± 0.01^{b}	42.55 ± 1.94^{e}
	Mean	114.19 ± 6.68	63.10 ± 2.86	46.97 ± 3.58	224.26 ± 20.99	4.19 ± 0.38	0.20 ± 0.01	39.53 ± 2.86
Experiment C	10	105.66 ± 5.58^{c}	35.25 ± 1.40^{a}	15.79 ± 1.31^{e}	156.70 ± 8.20^{c}	0.32 ± 0.03^{a}	0.01 ± 0.00^{a}	33.01 ± 1.77^{d}
	30	134.02 ± 2.23^{d}	43.84 ± 0.38^{b}	49.73 ± 3.45^{b}	227.59 ± 0.57^{e}	2.28 ± 0.18^{d}	0.12 ± 0.00^{d}	35.37 ± 3.31^{d}
	60	109.31 ± 2.46^{c}	43.49 ± 0.68^{b}	39.49 ± 1.94^{c}	192.29 ± 2.79^{d}	6.09 ± 0.34^{b}	0.34 ± 0.02^{b}	47.81 ± 2.56^{e}
	Mean	116.33 ± 9.75	40.86 ± 2.93	35.00 ± 1.19	192.20 ± 12.49	2.90 ± 0.13	0.16 ± 0.01	38.73 ± 1.64
Experiment D	10	108.21 ± 10.62^{c}	64.59 ± 5.24^{d}	35.67 ± 2.53^{c}	208.47 ± 6.78^{d}	0.68 ± 0.06^{a}	0.03 ± 0.00^{a}	31.07 ± 2.56^{d}
	30	135.44 ± 14.27^{d}	37.40 ± 3.35^{a}	63.32 ± 3.15^{d}	236.15 ± 19.44^{e}	2.73 ± 0.18^{d}	0.17 ± 0.02^{d}	34.57 ± 1.58^{d}
	60	148.71 ± 3.94^{e}	44.92 ± 0.78^{b}	48.21 ± 3.34^{b}	241.83 ± 22.06^{e}	11.90 ± 0.94^{e}	0.48 ± 0.02^{b}	54.32 ± 4.11^{f}
	Mean	130.79 ± 7.70	48.97 ± 2.30	49.07 ± 3.02	228.82 ± 16.59	5.10 ± 0.38	0.23 ± 0.01	39.98 ± 2.49

At phylum level, nearly 100% of the bacterial populations belonged to the phyla *Actinobacteria, Firmicutes, Bacteroidetes* or *Proteobacteria.* Experiment A (with the highest MOS content) had a significant ($p < 0.05$) enrichment in phylum *Firmicutes* compared with the other three experiments. Moreover, *Bacteroidetes* showed a tendency to become more abundant in experiments with higher amounts of MOS. In addition, *Bacteroidetes* abundance was significantly higher ($p < 0.05$) in experiments A, B and D compared with experiment C (the one with the lowest amounts of MOS). These results are in accordance with previously published literature stating that fiber-rich diets increase *Firmicutes* and *Bacteroidetes* phyla.[34] The same authors described that an increase in *Firmicutes* and *Bacteroidetes* is accompanied by a decrease in *Proteobacteria.* Our results also show how *Proteobacteria* abundance is significantly higher ($p < 0.05$) in the experiment with the lowest amounts of MOS (**Figure 47A)**. On the other hand, no significant differences in the *Firmicutes:Bacteroidetes* ratio were observed.

The observed increase in *Firmicutes* could be explained by the higher abundance of some specific genera: *Clostridium XIVb, Coprococcus, Butyricicoccus, Intestinimonas, Pseudoflavonifractor* and *Veillonella*. In addition, statistically significant differences related with MOS content were found in the Bacteroidetes genera *Barnesiella* and *Odoribacter* and in the Proteobacteria *ud-Burkholderiales* and *Bilophila.* Experiment A (with the highest MOS levels) showed significantly higher ($p < 0.05$) abundances of the above mentioned taxa than the other three experiments. On the other hand, experiment C (with the lowest MOS amounts) generally showed significantly lower abundances of such bacteria than the other experiments (**Figure 47B**). These results indicate that a higher supply of MOS translates into a higher abundance of these bacteria. Moreover, these assumptions are supported by significantly

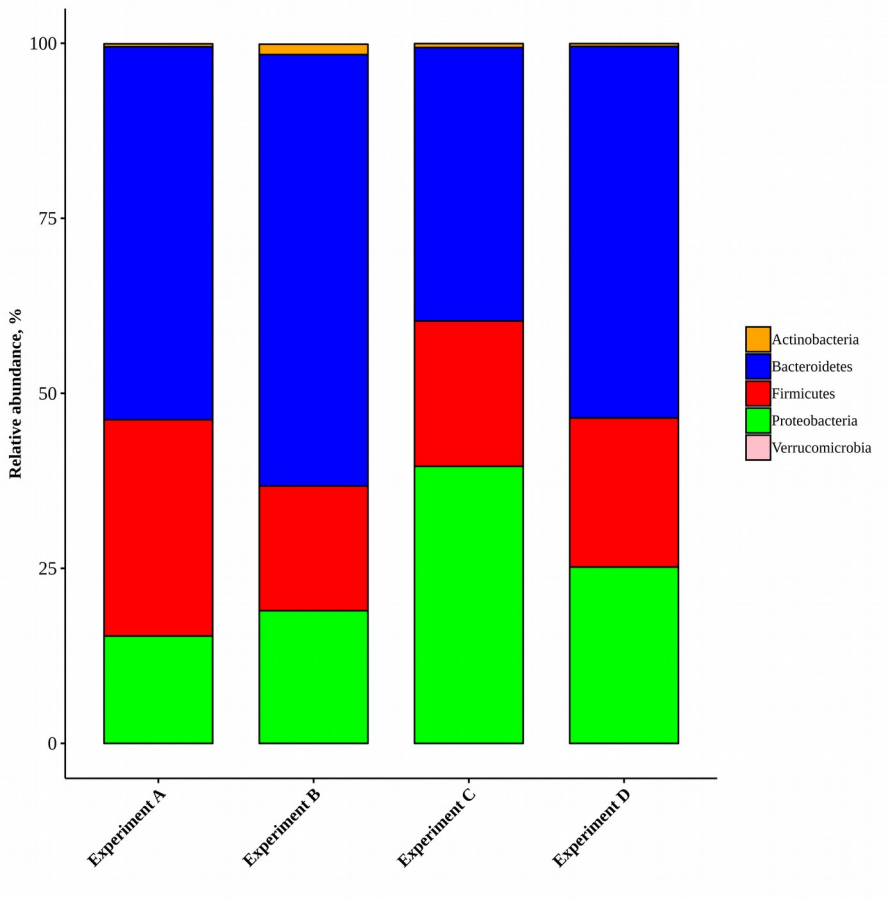
positive correlations between each bacterial abundance and every MOS analyzed (**Figure 48**). Except for *Bilophila*, all of the bacterial taxa associated with MOS have been linked mainly to positive effects on health, summarized in **Table 28.** According to these results, MOS could have a prebiotic effect over gut microbial communities, promoting a healthier microbial structure.

Table 28. Positive and Negative Effects of Microbial Genera Found to Be Significantly Different Depending on MOS Content.

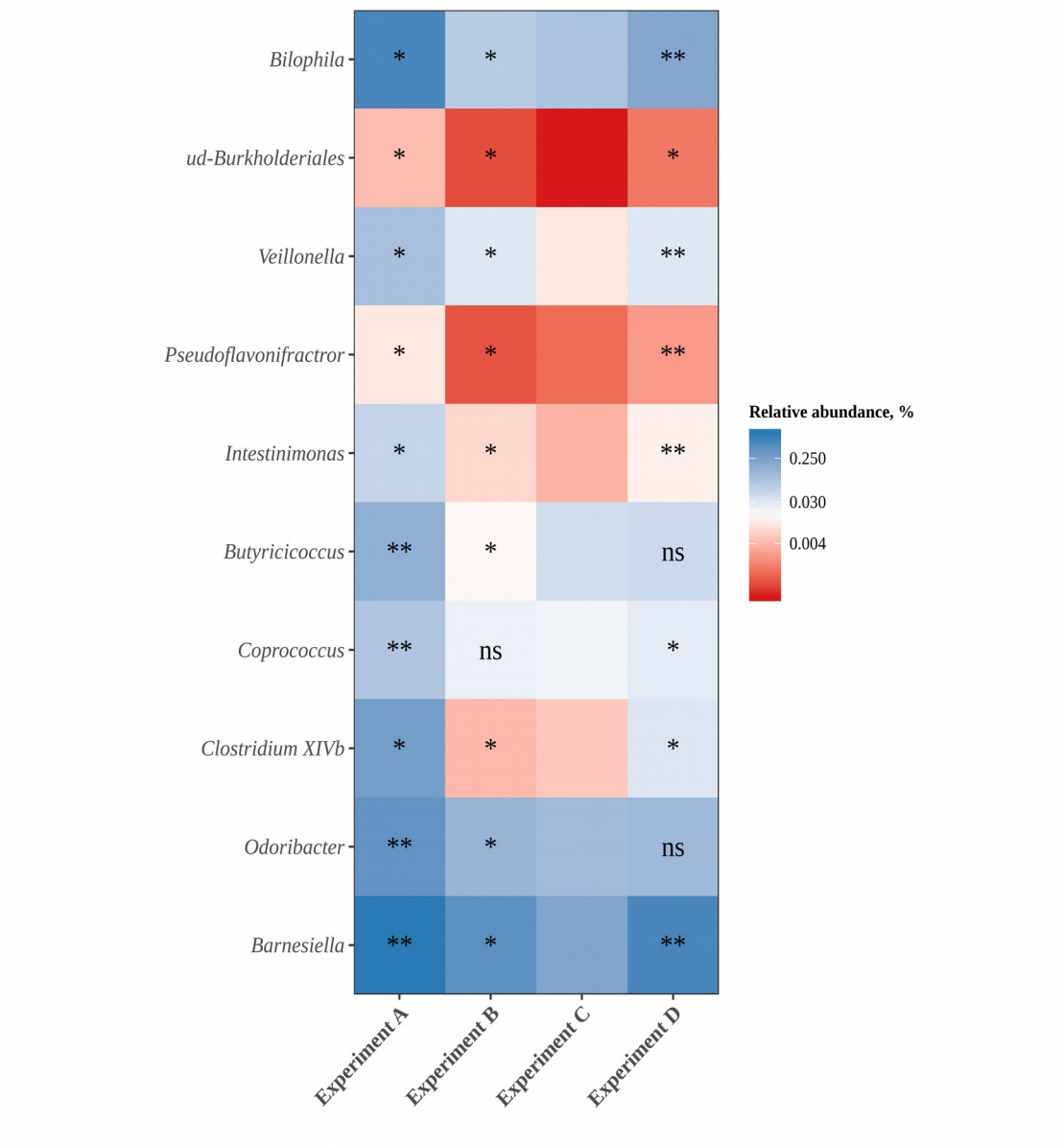
Bacteria		**Health effect**
Barnesiella	+	It could prevent or treat infections by antibiotic resistant bacteria.[42]
Odoribacter	+	Butyrate producer, lower abundance has been related to higher blood pressure in pregnant women and regulation of blood sugar in the host.[43,44]
Clostridium XIVb	+	Correlated with systemic inflammatory cytokines in patients with HIV-1.[45]
Coprococcus	+	Acetic and butyric acid producer, and lower amounts of propionic or formic acid.[16,24]
Butyricicoccus	+	Butyrate producer. Its abundance is reduced in ulcerative colitis patients and patients with inflammatory disease in general.[16,24 46,47]
Intestinimonas	+	Butyrate producer.[16,24]
Pseudoflavonifractor	+	It is related to weight loss along with *Alistipes.*[48]
Veillonella	+	Propionate and acetate producer.[29]
Bilophila	+/-	Its abundance is higher in dysbiosis with diets high in sugars or fats being therefore related to obesity and inflammation. It is also reduced in autism spectrum disorders subjects.[21,49]

Figure 47. A) Bacterial relative abundances at phylum level. **B)** Heatmap of relative abundance at genus level for those bacteria that were significantly different among experiments. Statistical labels: *: p < 0.05, **: p < 0.01, ***: p < 0.001, ns: not significant. Comparisons are made taking as the reference group Experiment C. Note the log2 scale.

47A



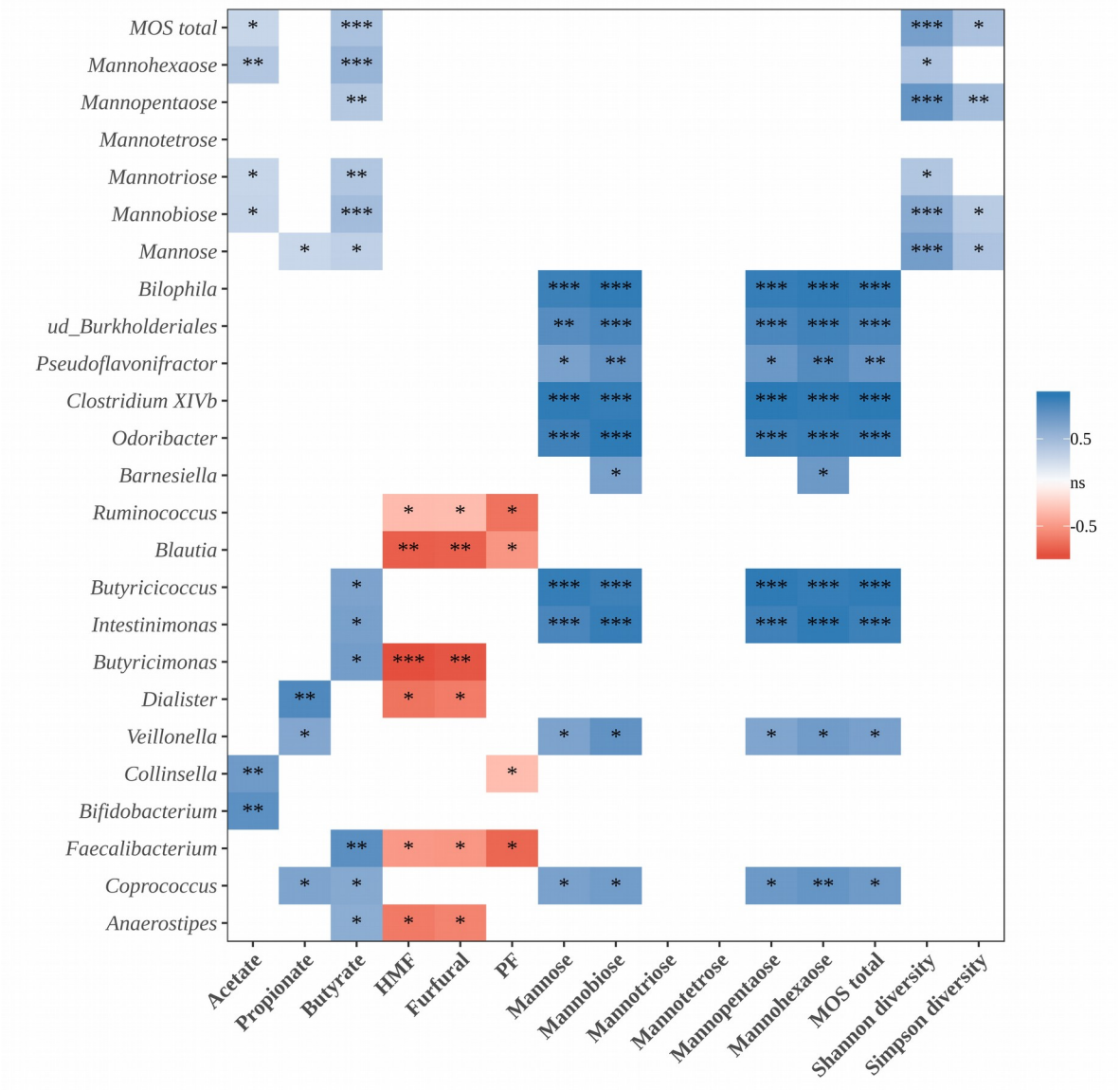
47B



The analysis of microbial α-diversity in terms of the Shannon and Simpson diversity indexes was also performed. In experiment A, the Shannon diversity index was significantly higher than in the other experiments. Moreover, the Simpson diversity index followed the same behavior, being significantly higher in A than in the other three experiments (where no significant differences were found). These results are in accordance with published literature about the effect of fiber-rich diets on the increase

of gut microbial diversity.[34] Additionally, statistically significant correlations between Shannon diversity index and mannose, mannobiose, mannotriose, mannopentaose, mannohexaose, and total MOS were found (**Figure 48)**. Finally, the Simpson diversity index was also positively correlated with mannose, mannobiose, mannopentaose and total MOS (**Figure 48)**. According to these results, we observed that a higher MOS concentration led to a richer microbial community in terms of diversity.

Figure 48. Heatmap of correlation coefficients (R). Statistical labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.



Microbial growth inhibitors

The appearance of inhibitors of microbial growth during biotransformation processes is a main issue that has been widely reported.[33] Such potential inhibitors include furanic compounds (like HMF and furfural) and some polyphenols. HMF and furfural are metabolites produced during non-enzymatic browning and therefore are already present in roasted coffee beans[35] and spent coffee grounds.[36] The inhibition caused by furanic compounds has been widely reported, mostly in relation to the fermentation of lignocellulosic residues.[33] In the case of polyphenols, it is widely known that they are strong antioxidants and therefore play a protective role in health. Some polyphenols, mostly complex ones such as flavonoids, chlorogenic acids, tannins, ellagitannins, etc., can be metabolized by some gut microbial communities, yielding simpler phenolics. These metabolites can be absorbed and exert a beneficial effect either systemically or locally in the colon.[37] However, some other polyphenols have also been described as possible growth inhibitors for some microorganisms.[33,38] In this sense, it has been reported that ferulic acid and quercetin can inhibit or reduce the growth of some fiber degrading bacterial communities in rats, leading to a lower SCFA production and an increased excretion of oligosaccharides in feces.[39]

One of the objectives of the project was to study the potential use of hydrolyzed spent coffee ground extracts without further purification, since they still have some beneficial compounds, such as polyphenols. However, it was hypothesized that along hydrolysis (due to high temperatures) some Maillard compounds (such as HMF and furfural) would appear. Therefore, the possible inhibitory effect of HMF and furfural on microbial growth we tested, by *in vitro* fermentation of a MOS mixture with and without addition of HMF-furfural mix at two concentrations (10 and 1 mg/mL). Growth inhibition of *Barnesiella, Coprobacter, Butyricimonas, Odoribacter,*

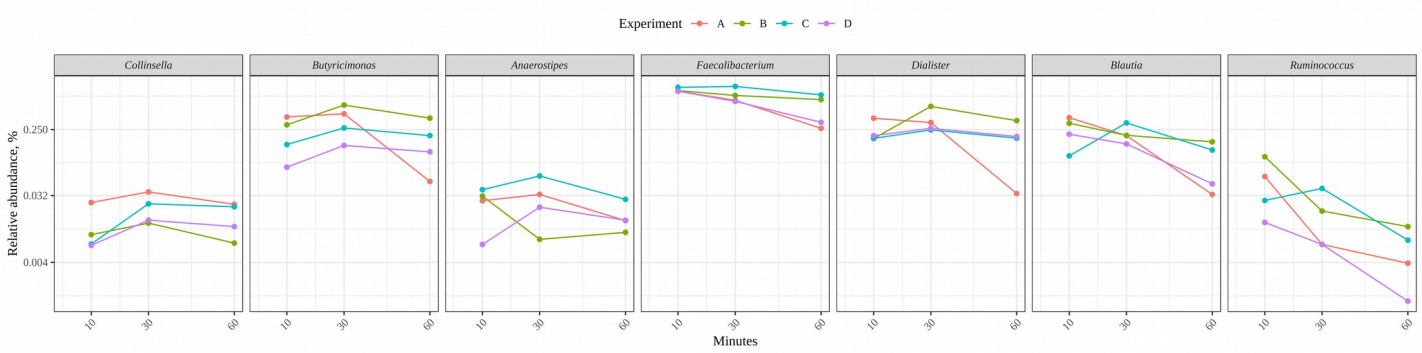
Anaerostipes, Blautia, Clostridium XVIb, Ruminococcus, Clostridium IV, Rumiococcus2, Flavonifractor, Intestinimonas, Oscillibacter, Clostridium XVIII and *Bilophila* (**Figure 46B**) were observed under this in vitro fermentation conditions. Therefore, the inhibitory activity of HMF and furfural was demonstrated.

The analysis of bacterial communities showed that the relative abundances of some genera were lower in the fermentations employing coffee grounds hydrolyzed for longer than 30 minutes (**Figure 49)**. These bacteria included some SCFA producers *Collinsella, Ruminococcus* and *Blautia* (acetate producers), *Blautia* and *Dialister* (propionate producers), and *Anaerostipes, Faecalibacterium,* and *Butyricimonas* (butyrate producers).[16] In view of this pattern, it could be hypothesized that some microbial inhibitors that accumulate during hydrolysis, such as furanic compounds and/ or polyphenols, can negatively affect such microbial genera. The relative abundance of several of these taxa had increased with coffee grounds hydrolysed for 30 minutes in comparison to those hydrolysed for 10, whereas the relative abundance of *Faecalibacterium, Ruminococcus* and *Blautia* already decreased with coffee grounds hydrolysed for 30 minutes, suggesting a more harmful effect of such inhibitors towards them. However, in experiment C, the relative abundance of these three genera increased between 10 minutes and 30 minutes of coffee grounds hydrolysis. This could be related with the lower HMF and furfural values found in experiment C.

Taking all this information into account, the analysis of HMF and furfural concentrations in the samples collected after 10, 30 and 60 minutes of hydrolysis was performed. As expected, HMF and furfural concentrations increased with hydrolysis time due to longer exposure to high temperature (**Table 27).** HMF and furfural production also depend on the temperature applied, and thus, in those experiments in which the applied temperature was higher, HMF and furfural concentrations were also

higher. We found statistically significant negative correlations ($p < 0.05$) between HMF and furfural and *Blautia, Dialister, Faecalibacterium, Ruminococcus, Butyricimonas, and Anaerostipes* (**Figure 48)**. Correlation coefficients ranged from -0.3325 (in the case of *Ruminococcus*) to -0.8312 (for *Butyricimonas*). Therefore, HMF and furfural seem to inhibit or decrease the growth of these microorganisms, and accordingly reduce the prebiotic effect of MOS.

Figure 49. Relative abundance of some bacteria depending on the hydrolysis time of coffee grounds. Note the log2 scale.



The levels of total polyphenols in the samples collected after 10, 30 and 60 minutes of hydrolysis were also analyzed. Experiment A showed, in average, lower amounts of polyphenols than the other three experiments. This could be related with the higher hydrolysis temperature, which could degrade some polyphenols. Experiments B, C and D showed similar polyphenol content in average, with no significant differences (**Table 27)**. However, polyphenol content increased along hydrolysis time, which could be related with the constant release of polyphenols as the degree of hydrolysis of the spent coffee grounds increases. As previously stated, polyphenols could also act as microbial inhibitors. In this sense, statistically significant negative correlations ($p < 0.05$) for *Blautia, Faecalibacterium, Ruminococcus* and *Collinsella* (**Figure 48)** were

found. Correlation coefficients ranged from -0.3228 (for *Collinsella*) to -0.7334 (for *Faecalibacterium*). In this sense, polyphenols released by spent coffee grounds could reduce the growth of some gut bacteria and therefore reduce fiber degradation and SCFA production. *Blautia, Faecalibacterium and Ruminococcus* showed significant negative correlations with HMF, furfural and total polyphenols. This could mean that these bacteria are negatively affected by both kinds of inhibitors. Accordingly, as depicted in **Figure 49,** these bacteria seem to be more aggressively affected and their abundances already start decreasing with coffee grounds hydrolyzed for 30 minutes.

Short chain fatty acids production

Several health effects are attributed to SCFAs. Decreasing the luminal pH is one of the most obvious, which can counter the growth of pathogenic bacteria. On the other hand, butyrate is used as substrate by the epithelial cells, being important for their functionality and, overall, the three main SCFAs (acetate, propionate and butyrate) are important for the maintenance of the gut barrier. [24] Moreover, while butyric acid is mostly metabolized by colonocytes, acetate and propionate are mostly absorbed and incorporated to different metabolic routes related to energy balance. Thus, these SCFAs have a protective role in diet-induced obesity.[40] On the other hand, butyrate and propionate have been related to the production of gut hormones and therefore in reducing food intake.[40] Moreover, it is thought that SCFAs could have an important role in colorectal cancer protection (mostly butyrate) by increasing motility, irrigation, reducing inflammation and increasing apoptosis. In fact, it has been suggested that the protective effect of dietary fiber over colorectal cancer depends upon the production of butyrate.[41] Propionate along with butyrate has also been associated with regulation of intestinal inflammation through induction of T-regulatory cell differentiation.[24]

SCFAs production is summarized in **Table 27**. SCFA production showed a similar pattern across experiments as, generally, higher amounts of MOS led to higher SCFAs production though for 60 minutes of coffee grounds hydrolysis the levels of SCFAs decreased (**Table 27**). In experiment A this makes sense since MOS production was lower at 60 minutes. However, this was found even in experiments were MOS production increased until the end. The decrease in SCFAs production with coffee grounds that have undergone more than 30 minutes of hydrolysis could be attributed to the inhibitory effect exerted by HMF, furfural and/or polyphenols. As described in **section 3.2.1,** some SCFAs producers are inversely correlated with HMF, furfural and polyphenol concentrations, which could mean that they are negatively affected by those substances. As a consequence, SCFAs production is reduced. Moreover, supporting these findings, SCFAs producers *Anaerostipes, Collinsella, Coprococcus, Faecalibacterium, Bididobacterium, Veillonella, Dialister, Butyricimonas, Butyricicoccus* and *Intestinimonas* correlated positively with the corresponding SCFAs (**Figure 48**). Therefore, this could explain, at least in part, SCFAs production.

When comparing experiments, a higher MOS production usually translated into a higher SCFAs production (**Table 27**). Mean acetate values over the three measured time points ranged from 75.05 μmol/g of spent coffee grounds for experiment A to 130.79 μmol/ g for experiment D. Experiment A showed significantly lower production of acetate ($p < 0.05$) than the other three experiments. In the case of propionate, its mean levels ranged from 39.53 μmol/g of spent coffee grounds in experiment A to 63.10 μmol/ g in experiment B. Propionate production was significantly higher ($p < 0.05$) in experiments B and D compared to A and C, both of them with similar values. Finally, mean butyrate production ranged from 34.78 μmol/g of spent coffee grounds in experiment A to 49.07 μmol/ g in experiment D. As in the case of propionate,

experiments B and D produced significantly ($p < 0.05$) higher amounts of butyrate than experiments A and C, both of them with similar production. The high amounts of MOS present in experiments B and D likely enabled the high production of SCFAs. In support of this notion, significantly positive correlations (**Figure 48**) among SCFAs production and most of the saccharides analyzed were found: acetate production was positively correlated with mannobiose, mannotriose, mannopentaose, mannohexaose and total MOS production; propionate correlated positively only with mannose production; butyrate production was positively correlated with mannose, mannobiose, mannotriose, mannopentaose, mannohexaose and total MOS production (**Figure 48**). In contrast, the low mean production of SCFAs in experiment A could be explained by 1) the decrease in MOS production at 60 minutes due to an excessive temperature, 2) the higher concentrations of HMF and furfural (**Table 28)**, and 3) the resulting significant decrease in producers of acetate (such as *Blautia*), propionate (such as *Dialister*) and butyrate (such as *Faecalibacterium* and *Butyricimonas*) in comparison with the other experiments (**Figure 49).**

As conclusions, the prebiotic activity of mannooligosaccharides obtained from spent coffee grounds was tested by studying gut microbial community structure and SCFAs production after *in vitro* fermentation with human feces. Results suggest that MOS have a prebiotic effect increasing gut microbial α-diversity and, on the other hand, promoting the growth of some beneficial bacterial genera. Moreover, a higher dose of MOS translates into a higher production of SCFAs, which reinforces the possible prebiotic effect of MOS. However, during hydrolysis some microbial inhibitors are either produced or released from the spent coffee grounds matrix, mainly HMF and furfural but also polyphenols. Accordingly, the accumulation of these compounds along the hydrolysis period seems to reduce or inhibit the growth of some beneficial bacteria.

This should be taken into account for future trials in order to clean the samples from such kind of compounds in order to improve the prebiotic effects of such oligosaccharides.

Abbreviations: HPLC: High Performance Liquid Chromatography, SCFA: Short Chain Fatty Acids, HMF: 5-hydroxymethyl-furfural, MOS: Mannoligosaccharides.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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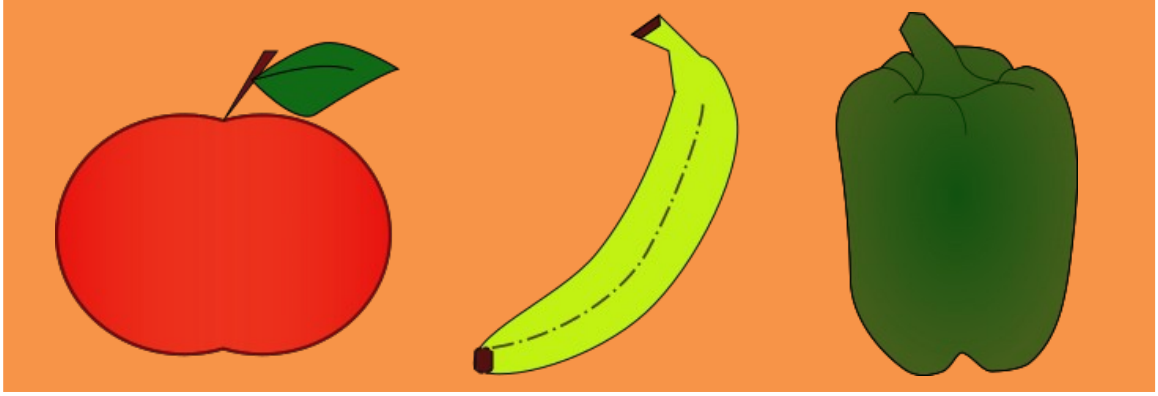
Capítulo III. Frutas y verduras.



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Characterization of commercial Spanish non-citrus juices: antioxidant and physicochemical aspects.

Este trabajo se centra en la determinación de la capacidad antioxidante de zumos de frutas no cítricas, alimentos muy consumidos en la dieta española. Además, dicha capacidad antioxidante se evaluó tras someter los zumos al proceso de digestión gastrointestinal. Por otro lado, se estudió también el posible desarrollo de pardeamiento químico durante el almacenamiento o tratamiento térmico de zumos elaborados. Todo esto hace que el presente paper esté íntimamente relacionado con la presente tesis doctoral.

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Characterization of commercial Spanish non-citrus juices: antioxidant and physicochemical aspects.

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ABSTRACT

The presence of many different antioxidant species makes fruit juices to be perceived by populations as a very healthy beverage easy to include in the daily diet. These antioxidant actions have been reported in a large number of papers, however the information correlating the antioxidant profile with the physicochemical characteristics derived from the industrial processing of fruit juices is limited. In a previous paper, our research group demonstrated that the antioxidant properties of citrus juices were underestimated when measuring by traditional methods and that our improved methodology, so-called GAR, is a better approach to analyze the global antioxidant response of juices. In this paper, we confirm that statement, establishing that the overall antioxidant capacity of non-citrus juices is 10-times higher with the GAR method (including an *in vitro* gastrointestinal digestion) that with the other methodologies. In some cases, such as pineapple juice, the antioxidant action was distributed between the soluble and non-soluble fractions almost at 50%. But, surprisingly, in some other (like tomato juice) the non-soluble fraction accounted for the higher antioxidant capacity. This fact definitively underlines the importance of the non-soluble fraction and shows the suitability of the GAR method to consider it. Physicochemical parameters, such as color, fluorescence, 5-hydroxymethylfurfural and furfural contents were correlated with antioxidant characteristics in some samples. Lastly, we unravel a mathematical model to classify non-citrus juices depending on their nature or storage conditions.

*Keywords***:** Juice; antioxidant activity; 5-hydroxymetylfurfural; Polyphenols; Color; Fluorescence.

1. Introduction

Fruit juices provide energy in the form of simple sugars, vitamins, minerals and a small amount of fiber, hydrating the body and contributing a good part of the nutritious qualities of the fruit. These beverages are fundamental in a healthy diet with the potential to positively impact metabolic outcomes mediated by the antioxidant activity (Crowe-White et al., 2017). Carotenoids, vitamin C and phenolic compounds are among the antioxidant species naturally occurring in fruit juices (Quitao-Texeira, Odriozola-Serrano, Soliva-Fortuny, Mota-Ramos & Matín-Belloso, 2009). The analysis of antioxidant capacity is usually performed with the soluble fraction resulting from *in vitro* digestion (Ryan & Prescott, 2010) or with juice extracts (Stella, Ferrarezi, dos Santos & Monteiro, 2011). However, these approaches do not measure the overall antioxidant capacity of juices, like protocols such as the GAR method (Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011), which has been successfully applied for citrus juices (Álvarez, Pastoriza, Alonso-Olalla, Delgado-Andrade & Rufián-Henares, 2014).

Thermal treatment is usually applied to fruit products aimed to ensure safety and extend their shelf life (Rattanathanalerk, Chiewchan & Srichumpoung, 2005). However, heating processes can affect the nutritional and organoleptic quality of product leading to consumer dissatisfaction. In the case of the nutritional quality it is also affected by industrial manufacture since nutrients like vitamin C, sugars and proteins take part in nonenzymatic reactions (Tulek & Yilmaz, 2006). During the Maillard reaction and caramelization, 5-hydroxymethylfurfural (HMF) and furfural are intermediate undesirable compounds which production reduces the juices' acceptance by consumers (Buedo, Elustondo, & Urbicain, 2001). HMF is an indicator of the decrease on quality since it is related with an excessive processing temperature of juices or inadequate

storage conditions (Rodrigo, Arranz, Koch, Frígola, Rodrigo, & Esteve, 2003). Furthermore, it is necessary to include the analysis of HMF in the nutritional assessment of juices, since recent finding have established that the compound is metabolized by human beings to 5-sulfoxymethylfurfural (Pastoriza, Álvarez, Végvári, Montilla-Gómez, Cruz-López, Delgado-Andrade & Rufián-Henares, 2017), a derivate with demonstrated nephrotoxicity (Nadiya, Bernhard, Heinz, Albrecht, & Hansruedi, 2009) and mutagenic activity (Glatt, & Sommer, 2006; Surh, Liem, Miller, & Tannenbaum, 1994).

In Spain, the consumption of juices along 2016 reached 457.6 million litters (Mercasa, 2017), 30% corresponding to orange juices and 70% to non-citrus juices: 19% to pineapple juices, 18.2% to peach juices, 16.8 to multifruit juices and 3.5% to apple juices. In a previous study (Álvarez et al., 2014) we investigated the antioxidant and physicochemical characteristics of citrus juices. However, due to the economic importance of non-citrus juices in Spain (spending of 428.1 million euros in 2016; Mercasa, 2017) the aim of the present study was to analyze the antioxidant and physicochemical characteristic of commercial Spanish juices with non-citric origin. In the case of the antioxidant characteristics we studied the amount of total phenols as well as the global antioxidant capacity of juices through the GAR method, specially paying attention to the insoluble fraction. For the physiochemical profile color, fluorescence and furanic compounds like HMF and furfural were measured. Then we estimated the contribution of juices consumption to the overall antioxidant capacity intake in Spain. Finally, the relationship among all the evaluated parameters was studied in order to look for an algorithm, which enables us distinguishing the kind of fruit used to manufacture these juices.

2. Materials and methods

2.1. Chemicals

The enzymes used for *in vitro* digestion (α-amylase, pepsin, pancreatin and bile salts) were from Sigma-Aldrich (St. Louis, MO, USA). For HMF-furfural assays, zinc acetate, potassium ferrocyanide and HMF-furfural standards were from Sigma-Aldrich (St. Louis, MO, USA). In the case of the antioxidant capacity methods 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2'-azobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Fluka Chemicals (Madrid, Spain). Folin-Ciocalteu reagent and gallic acid were from Panreac (Madrid, Spain).

2.2. Samples

A total amount of fifty-six juices were selected to cover the main brands consumed in Spain. They were as follows: 9 antioxidant juices (declared as "antiox" on their labels), 5 apple juices, 4 banana juices, 3 mango juices, 10 multifruit juices (declared as "multifruit" on their labels and composed of a mixture of different types of juices), 9 peach juices, 13 pineapple juices and 3 tomato juices. Samples were obtained from three different retail stores and stored under refrigeration or at room temperature (according to manufacturer's instructions) for a maximum of 3 days before analysis. Freshly squeezed juices were obtained from apple, mango and pineapple fruits. Fruits, from two different retailers, were cut and squeezed with an orange squeezing machine (Taurus TC600, Spain) and immediately frozen and stored at -80ºC until analysis. At least three determinations for each procedure were carried out in different samples.

2.3. Antioxidant capacity

The antioxidant capacity was assayed either in the whole juice or in the soluble and insoluble fractions obtained after *in vitro* gastrointestinal digestion by the GAR method (Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011). The methods used to evaluate the antioxidant activity were the standard ABTS and FRAP methods.

2.3.1. In vitro digestion

The enzymatic digestion was performed as stated in Pastoriza et al. (2011). Juice samples were digested with α-amylase, pepsin, pancreatin and bile salts simulating the oral, gastric and intestinal phases. Then, the soluble bioaccesible and the insoluble non-accessible fractions were separated and stored at -80ºC until analysis.

2.3.2. Antioxidant capacity of the soluble fraction.

The ABTS and FRAP assays were conducted as stated previously by Álvarez et al. (2014) in order to measure the antiradical and reducing capacity of the soluble fraction obtained after *in vitro* digestion of juices. Both spectrophotometric methods were performed on a Fluostar Omega microplate reader (BMG Labtech, Germany). Aqueous solutions of trolox were used for calibration and the results were expressed as mmol equivalents of trolox per litre of sample.

2.3.3. Antioxidant capacity of the insoluble fraction.

The antioxidant activity of the remaining insoluble solid fraction obtained after proper digestion of each juice was conducted as described by Álvarez et al. (2014). In brief, the lyophilized solid was mixed and vortexed with the ABTS or FRAP reagents. After a propped period of time, the samples were centrifuged and the absorbance of the

optically clear supernatant was measured by using a Fluostar Omega microplate reader (BMG Labtech, Germany). Trolox solutions were used to perform the calibration curve, being the results expressed as mmol equivalents of trolox per litre of sample.

2.3.4. Calculations of Global Antioxidant Response (GAR) of juices

The total antioxidant capacity of juices was calculated as the sum of the antioxidant capacity of each soluble fraction + the antioxidant capacity of the corresponding insoluble fractions (Pastoriza et al., 2011).

2.3.5. Total phenolic content

Total phenolics were determined according to the Folin-Ciocalteu method as described by Marfil et al. (2011) with slight modifications (Singleton, Orthofer, & Lamuela-Raventos, 1999). Measures were performed on a Fluostar Omega microplate reader (BMG Labtech, Germany). Quantification was carried out on the basis of the standard curve of gallic acid, and results were expressed as mg gallic acid equivalent per litre of sample.

2.4. Chromatographic analysis of HMF and furfural

The levels of HMF and furfural in juices were analyzed with the HPLC method described on Rufián-Henares, Delgado-Andrade & Morales (2006). In short, each juice was clarified with potassium ferrocyanide and zinc acetate solutions. After vortexing, the mixture was centrifuged and the supernatant filtered through 0.45µm acetate filters. HMF and furfural were analyzed by reversed-phase HPLC with a Perkin-Elmer series 200 HPLC (PerkinElmer, Spain). HMF and furfural were quantified by the external standard method within the range 0.1-150 µM.

2.5. Color and fluorescence analysis

The color of juices was determined with a Chroma Meter CR-400 optical sensor (Konica Minolta Sensing, Inc., Osaka, Japan) according to the CIE Lab scale (CIE Colorimeter Committee, 1974) and stated previously for citrus juices (Álvarez et al., 2014). The analysis included three values: L* (black-white component, luminosity), a* (+red to –green component) and b* (+yellow to –blue component) chromaticity coordinates (Hunter, 1942). The juices were illuminated with D65-artificial daylight (10° standard angle).

The presence of fluorescence in juices associated to Maillard compounds was evaluated using the same extract obtained for HMF and furfural analysis. Fluorescence was measured at 347 nm excitation and 415 nm emission (Delgado-Andrade, Rufián-Henares & Morales, 2006) on a fluorescence spectrophotometer (LS 55, Perkin-Elmer, Waltham, MA) with quartz glass cuvettes (QS-1.000 Suprasil, Hellma GmbH & Co, Germany). Data were expressed as arbitrary units (AU) × 10^3.

2.6. Antioxidant, polyphenols and HMF intake calculations

The dietary polyphenols and antioxidant capacity intake was calculated as the individual contribution of each juice, taking into consideration both the amount of food per serving and the daily consumption (Mercasa, 2017). Thus, polyphenols and antioxidant capacity of each juice was referred to the usual Spanish serving size (Salvador i Castells, 2000). The contribution to the daily intake of antioxidant capacity and polyphenols of each juice was referred to the results previously published by Saura-Calixto & Goñi (2006).

2.7. Statistical analysis

Statistical significance of the data was tested by one-way analysis of the variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($P < 0.05$). Evaluation of the relationship between different assays was carried out by computing the relevant correlation coefficient (person linear correlation) at the $P < 0.05$ confidence level. Multivariate analysis was performed by cluster analysis and principal component analysis. Cluster analysis is the common term for different methods used to create objective groups whose patterns of scores on variables are similar (Zheng & Lu, 2011). We used the hierarchic agglomerative cluster algorithm by adopting the squared Euclidean distance. After studying the samples clustering, those samples farther from their group that had any outlier in the parameters included in the analysis were removed. Finally, the cleaned groups were further analyzed by principal component analysis. All the statistical analyses were performed using Statgraphics Plus software, version 5.1, 2001.

3. Results and discussion

3.1. Antioxidant characteristics of juices

3.1.1. Assessment of antioxidant capacity with ABTS and FRAP methods

The content of vitamins and polyphenols of fruit juices make them one of the main sources of antioxidant compounds in the human diet (Naresh, Varakumar, Variyar, Sharma & Reddy, 2015). In Spain, the consumption of juices of non-citric origin along 2016 reached 320 million litters (Mercasa, 2017). Thus, it is assumed that these types of juices could have a high contribution to the antioxidant capacity on a daily basis. Therefore, we measured the antioxidant capacity of the main types and brands of juices marketed in Spain through the ABTS and FRAP methods. In the case of ABTS assay (**Table 29**), the activity against radicals of commercial juices ranged from 1.43 to 5.23 mmol trolox/L for tomato and mango juices, respectively. These values are in line with those reported by other authors for apple (Włodarska, Pawlak-Lemanska, Górecki & Sikorska, 2016), mango (Santhirasegaram, Razali, George & Somasundram, 2015), pineapple (Bamidele & Fasogbon, 2017) and tomato (Bamidele & Fasogbon, 2017). The antioxidant capacity of commercial mango juices was statistically higher ($p<0.05$) than that of found in apple, multifruit and tomato juices. Examinig individual juices, the highest antioxidant capacity measured with the ABTS method was for the pineapple juice J10 (15.0 mmol trolox/L) and the lowest for the multifruit juice J4 (0.48 mmol trolox/L). The behavior of freshly squeezed juices (**Table 29**) was significantly different since recently prepared apple juice had higher antioxidant capacity ($p< 0.05$) than mango juice, while pineapple juice was intermediate. In addition, excluding mango, home-made juices presented statistically higher antioxidant capacity than their respective commercial juices ($p<0.05$), which could be explained through the

destruction of antioxidant species (phenols and vitamins) due to the thermal damage occurred during manufacturing and storage (Calin, Ileana & Ionela, 2011).

The antioxidant capacity of commercial juices was also assessed as reducing power against iron (**Table 29**). In this case, the individual values ranged varied broadly from 0.14 to 94.8 mmol trolox/L for a multifruit juice (sample K5) and a banana juice (samples H11), respectively. This could be explained taking into account that some juices are artificially enriched with vitamin C (a strong reducing species), which in turn could be the explanation of why the reducing capacity was nearly 10-times higher that their antiradical activity. According to the different groups, the FRAP values were comprised from 9.00 to 35.2 mmol trolox/L for multifruit and banana, respectively. The variations obtained for the different groups were larger than those of ABTS, which could reinforce the hypothesis of vitamin C enrichment in certain juices, resulting in a lack of correlation between reducing capacity and the type of fruit used to obtain the juice. As in the case of citric juices (Álvarez et al., 2014), FRAP values were always higher than those obtained with the ABTS method, indicating a stronger reducing activity than antiradical action. Regarding freshly squeezed juices, they had 2-3 times higher antioxidant capacity than their commercial counterparts (**Table 29**). Contrary to that observed for the ABTS method, pineapple juice had higher antioxidant capacity ($p<0.05$) than apple or mango juices, in line with the results reported by Rodríguez, Gomes, Rodrigues & Fernandes (2017).

Table 29. Antioxidant characteristics of commercial and freshly squeezed juices[1]

Juice	Code	ABTS	GAR_{ABTS}			FRAP	GAR_{FRAP}			Polyphenols
			Total	*Soluble*	*Insoluble*		*Total*	*Soluble*	*Insoluble*	
Antiox	J6	5.63	59.4	43.3	17.4	36.0	372	272	98.2	307
	M1	4.72	44.6	21.8	23.7	4.28	43.3	21.2	23.2	324
	M2	2.24	21.8	12.6	9.09	5.66	57.6	33.4	23.3	216
	M3	1.06	11.8	6.71	5.87	5.80	59.9	34.2	26.4	230
	H9	2.55	23.1	16.7	7.86	11.9	124	89.5	35.5	334
	K4	3.48	34.2	24.3	10.4	2.99	30.0	21.3	9.02	116
	D13	2.59	24.7	16.0	8.43	11.5	113	73.5	38.5	640
	D14	2.79	28.1	19.1	8.67	4.51	44.5	30.2	13.4	360
	G7	4.19	42.0	31.1	11.2	4.34	43.5	32.9	12.5	237
Mean		***3.29 ± 1.52^{a}***	***32.2 ± 14.5^{a}***	***21.3 ± 10.8^{a}***	***14.4 ± 5.62^{a}***	***9.65 ± 10.4^{a}***	***98.8 ± 107^{a}***	***67.5 ± 80.1^{a}***	***31.1 ± 27.1^{a}***	***307 ± 145^{a}***
Apple	J3	2.10	24.9	18.2	7.23	22.0	225	164	58.5	161
	C4	6.39	68.5	50.7	16.4	3.54	37.7	27.9	10.1	212
	C21	0.51	5.45	3.98	1.54	3.02	32.3	23.6	8.46	436
	D12	3.11	32.4	24.3	7.89	15.1	157	118	40.3	680
	G10	2.91	30.1	22.3	8.23	7.01	68.9	51.	17.2	210
Mean		***3.00 ± 2.15^{a}***	***22.9 ± 10.6^{a}***	***23.9 ± 17.0^{a}***	***8.26 ± 5.32^{b}***	***10.1 ± 8.20^{a}***	***104.2 ± 84.0^{a}***	***76.9 ± 61.6^{a}***	***26.9 ± 21.7^{a}***	***340 ± 218^{a}***
Banana	K1	1.78	16.2	8.9	7.12	6.54	66.8	36.7	31.3	247
	H11	2.67	27.3	15.6	12.3	94.8	952	543	405	453
	C8	5.89	59.6	19.7	41.0	19.5	200	66.0	139	750
	G9	6.18	62.0	31.6	31.4	19.8	201	102	99.5	519
Mean		***4.13 ± 2.23^{ab}***	***41.3 ± 23.0^{ab}***	***19.0 ± 9.5^{a}***	***23.0 ± 15.4^{c}***	***35.2 ± 40.2^{b}***	***355 ± 403^{b}***	***187 ± 239^{b}***	***168 ± 166^{b}***	***492 ± 207^{b}***
Mango	H12	6.38	63.0	34.7	27.8	44.9	451	248	200	659
	C6	5.24	52.5	17.9	35.3	7.67	77.8	26.5	50.3	869
	CO1	4.07	42.2	22.8	20.4	4.53	46.1	24.9	22.9	398
Mean		***5.23 ± 1.15^{b}***	***52.6 ± 10.4^{b}***	***25.1 ± 8.6^{ab}***	***27.8 ± 7.42^{c}***	***21.0 ± 20.7^{b}***	***192 ± 225^{ab}***	***99.8 ± 153^{ab}***	***90.9 ± 95.0^{ab}***	***642 ± 236^{b}***
Multifruit	K5	1.43	14.0	8.2	5.54	0.14	1.34	0.79	0.42	631
	H1	4.41	42.1	24.0	19.2	3.71	36.9	21.0	16.3	535
	H5	4.69	44.6	25.9	18.5	26.5	238	138	101	557
	J4	0.48	4.16	2.37	1.98	14.7	151	85.8	63.0	478
	M7	1.50	14.3	7.73	7.87	8.15	82.5	44.6	38.5	520
	C3	3.81	35.7	20.0	16.6	2.87	29.2	16.3	13.3	261
	C17	6.41	65.5	36.7	29.1	9.89	99.9	55.9	45.4	602
	T3	2.45	25.7	14.9	11.4	4.12	46.7	27.1	20.3	373
	C03	1.85	19.3	10.8	9.03	9.04	92.0	51.5	39.5	355

	G6	4.78	48.8	26.4	23.6	10.9	113	61.1	51.3	249
Mean		***3.18 ± 1.90^{a}***	***31.4 ± 19.1^{a}***	***17.7 ± 10.7^{a}***	***14.3 ± 8.54^{a}***	***9.00 ± 7.53^{a}***	***96.2 ± 71.9^{a}***	***50.2 ± 39.6^{a}***	***38.9 ± 29.2^{a}***	***499 ± 133^{b}***
Peach	H10	5.28	53.7	27.4	27.5	15.0	147	74.8	72.2	190
	J1	3.37	32.5	15.9	16.0	21.2	219	107	113	251
	J7	3.39	35.7	17.1	19.0	26.7	260	125	139	240
	S2	12.1	127.2	61.1	68.9	9.03	92.3	44.3	44.1	324
	M5	1.97	20.7	9.8	12.3	6.97	70.2	33.0	38.3	246
	C16	3.25	34.3	17.2	18.0	3.21	32.6	16.3	17.5	276
	D8	2.32	22.5	10.6	12.0	13.8	142	66.9	74.6	563
	D9	1.67	16.8	7.89	9.08	14.0	140	63.0	77.8	500
	G2	5.30	54.2	23.9	31.1	10.0	112	49.4	63.8	436
Mean		***4.30 ± 3.19^{ab}***	***55.8 ± 48.7^{b}***	***21.2 ± 16.3^{a}***	***23.8 ± 18.4^{c}***	***13.5 ± 6.97^{a}***	***112 ± 58^{a}***	***64.4 ± 34.5^{a}***	***71.1 ± 37.3^{ab}***	***334 ± 133^{a}***
Pineapple										
	K3	0.73	8.21	4.11	4.00	0.88	8.98	4.49	4.69	222
	H2	3.30	33.7	17.5	15.2	3.70	36.4	18.9	18.5	481
	H14	5.47	53.9	27.5	26.7	44.2	442	226	219	427
	J8	2.52	26.6	13.3	14.3	12.0	123	61.7	64.0	173
	J10	15.0	147	75.2	73.5	10.9	187	95.8	93.5	406
	M6	0.90	8.86	4.34	5.04	8.22	83.5	40.9	43.0	390
	C9	1.93	19.2	9.40	10.4	7.06	71.0	34.8	34.6	182
	C10	1.70	16.0	8.33	8.73	3.89	38.1	19.8	19.2	292
	C15	0.60	5.57	3.06	2.32	4.42	43.4	23.9	18.5	361
	T6	1.30	13.8	6.61	7.71	17.1	172	82.8	90.6	248
	D10	2.51	24.4	12.7	12.4	13.5	133	69.4	65.0	260
	D11	2.98	28.5	13.7	12.4	17.5	180	86.4	96.5	340
	G5	4.34	44.3	21.7	24.0	10.0	100	49.0	49.9	423
Mean		***3.36 ± 3.78^{ab}***	***22.9 ± 14.4^{a}***	***16.7 ± 19.0^{a}***	***16.7 ± 19.0^{a}***	***12.7 ± 10.7^{a}***	***124 ± 112^{a}***	***62.6 ± 56.8^{a}***	***62.9 ± 56.3^{ab}***	***328 ± 100^{a}***
Tomato	A1	0.53	5.21	2.29	2.56	1.95	20.2	8.89	11.9	158
	S3	3.03	31.3	13.8	19.0	35.2	362	159	202	469
	G1	0.71	7.23	2.82	4.32	6.01	60.8	23.7	37.6	423
Mean		***1.43 ± 1.39^{c}***	***14.6 ± 14.5^{c}***	***6.29 ± 6.47^{c}***	***8.61 ± 9.00^{b}***	***14.4 ± 18.1^{a}***	***148 ± 186^{a}***	***64.00 ± 83.0^{a}***	***83.8 ± 103^{ab}***	***350 ± 167^{a}***
Freshly squeezed juices										
Apple		6.55 ± 1.26^{a}	67.6 ± 5.43^{a}	49.4 ± 3.95^{a}	19.4 ± 1.43^{a}	0.39 ± 0.09^{a}	3.24 ± 1.97^{a}	2.37 ± 1.28^{a}	0.76 ± 0.27^{a}	121 ± 34^{a}
Mango		3.95 ± 1.07^{b}	40.2 ± 4.35^{b}	20.1 ± 1.67^{b}	19.3 ± 1.39^{a}	8.40 ± 2.49^{b}	85.6 ± 7.35^{b}	42.8 ± 4.51^{b}	41.3 ± 4.33^{b}	126 ± 39^{a}
Pineapple		5.43 ± 1.13^{ab}	55.4 ± 4.88^{c}	25.5 ± 2.02^{b}	30.4 ± 2.25^{b}	6.06 ± 2.46^{b}	62.0 ± 25.5^{b}	28.5 ± 3.51^{c}	34.2 ± 3.78^{b}	275 ± 46^{b}

[1] Different letters within the column indicate statistically significant differences $p < 0.05$. Antioxidant activity (ABTS, GAR_{ABTS}, FRAP and GAR_{FRAP}) expressed as mmol trolox/L and polyphenols expressed as mg gallic acid/L. The number of decimal figures is based on the significant figures.

3.1.2. Assessment of antioxidant capacity with GAR method: the importance of insoluble antioxidants

Antioxidant species are generally found in foods both in free and bound forms (Açar, Gökmen, Pellegrini & Fogliano, 2009). These insoluble antioxidant compounds bound to the food matrix possibly play a role in human health (Chandrasekara & Shahidi, 2010) since their antioxidant effects are exerted for longer times than free ones (Cömert & Gökmen, 2017). Thus, the interaction between insoluble and soluble antioxidant compounds was proposed as the "regeneration concept" by Çelik, Gökmen and Skibsted (2015), in which the balance between soluble and insoluble antioxidant compounds in the gut provides a healthy antioxidant environment.

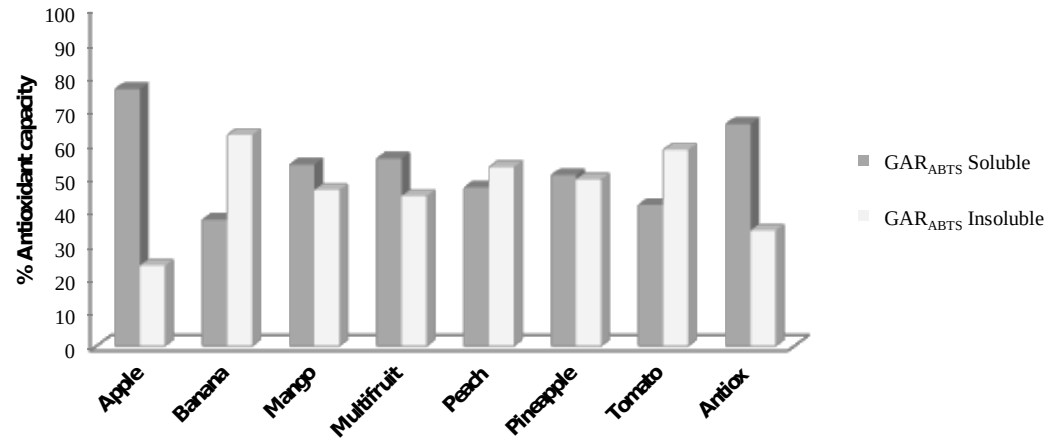
The use of chemical extracts for the analysis of the antioxidant activity is the common procedure applied in almost every paper for different kind of foods, including juices (Santhirasegaram et al., 2015; Włodarska et al., 2016; Bamidele & Fasogbon, 2017). In the present study, to take into account the activity of all fractions obtained after *in vitro* digestion and not only that present in a chemical extract, the antioxidant capacity of non-citric juices was analyzed with the GAR protocol (Pastoriza et al., 2011). The GAR method was developed to determine the underestimated insoluble antioxidant capacity of foods thanks to the QUENCHER approach discovered by Serpen, Capuano, Fogliano and Gökmen (2007). In the case of the ABTS method, the overall antioxidant capacity (GAR_{ABTS}) was around 10-times higher than that measured with the conventional method (**Table 29**), ranging 4.16 to 147 mmol trolox/L for samples J4 and J10, respectively. The analysis of juices by type of fruit revealed that peach and mango juices had higher antioxidant capacity ($p<0.05$) than antiox, apple, multifruit and pineapple juices, which in turn had higher antioxidant capacity than tomato ones (values ranging from 14.6 to 55.86 mmol trolox/L for tomato and peach

juices, respectively). The antioxidant capacity measured with the GAR method for commercial citrus juices (Álvarez et al., 2014) showed that such juices are, in general, less antioxidant than non-citric juices. In the case of freshly-squeezed juices, apple and pineapple juices but not mango showed higher antioxidant power than their commercial counterparts, and such antioxidant capacity significantly increased in the following order ($p<0.05$): mango < pineapple < apple. In a previous work, our research team observed that recently prepared citrus juices had up to 25 mmol trolox/L higher antioxidant capacity than non-citrus juices (Álvarez et al., 2014).

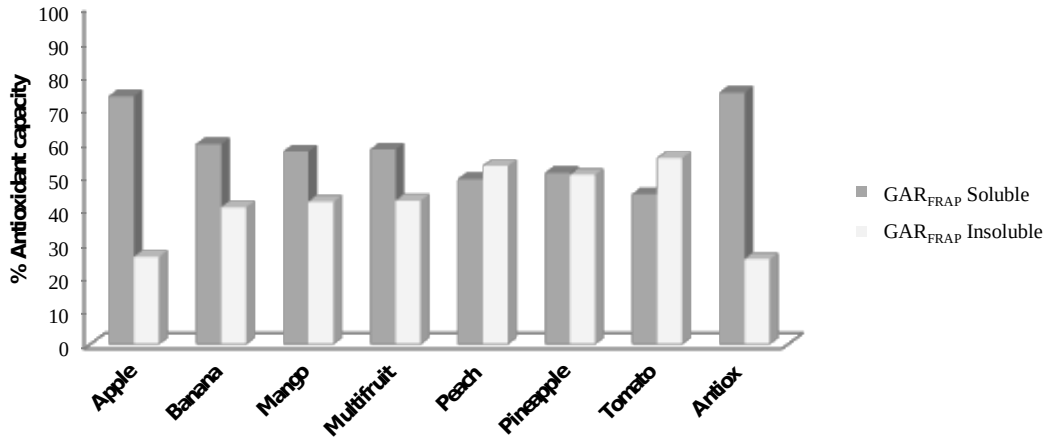
The GAR method allowed the calculation of the antioxidant capacity of both the bioaccesible (soluble) and non-bioaccesible (insoluble) fractions obtained from non-citric juices (**Figure 50A**). In general, the soluble fraction accounted for 53.5% (mean value among groups) in the overall antioxidant capacity, while the insoluble fraction reached a 46.5%. In particular, juices like banana and tomato had a higher antioxidant capacity in the insoluble fraction (62.6 and 58.5%, respectively) than in the soluble one, a portion usually discarded with the usual procedures. Thus, almost 50% of the antioxidant capacity escapes digestion, reaching the colon where a strong antiradical activity can be developed. The values obtained for the soluble fraction (53.5%) are lower than those obtained previously (Álvarez et al., 2014) for citric juices (68.9%), while the opposite was found for the insoluble fraction. However, the values obtained for the insoluble fraction of non-citric juices are in line with those reported by Pastoriza et al (2011) for kiwifruit. It is also noteworthy to mention that other authors (Cömert & Gökmen, 2017) have found that a 27-24% of phenolic compounds are bound to dietary fiber in apple and oranges, respectively. As depicted in **Figure 50A**, a 24% of the total antioxidant capacity (measured with the ABTS method) was located in the insoluble fraction after digestion, which is in line with the presence of polyphenols.

Figure 50. Contribution of soluble and insoluble fractions to the overall antioxidant capacity of commercial juices by the ABTS (50A) and FRAP methods (50B).

50A



50B



As stated above, the values obtained for the reducing capacity of commercial non-citric juices (GAR_{FRAP}) were much higher than for their antiradical capacity (GAR_{ABTS}) (**Table 29**), being the banana group the most antioxidant in terms of reducing capability ($p<0.05$). The values ranged from 1.34 to 952 mmol trolox/L (for samples K5 and H11, respectively) the last one probably reflecting the addition of ascorbic acid or other reducing compounds as preservatives. In general, the values

obtained for the GAR_{FRAP} method were higher than those previously obtained for citric juices (Álvarez et al., 2014). As depicted in **Figure 50B**, the mean antioxidant capacity of the soluble fraction of commercial juices was 58.2%, while the insoluble fraction account for a 41.8%. Thus, a higher portion of reducing capacity was present in the soluble fraction, which could reinforce once again the hypothesis on the enrichment with reducing chemical species. A similar increase was found for the GAR_{FRAP} method in citric juices (Álvarez et al., 2014), although the antioxidant capacity of the soluble fraction of these juices was more active (75%) than that of non-citric juices found in the present study (58.2%). Finally, as in the case of freshly squeezed juices, the antioxidant capacity of both soluble and insoluble fractions was lower (around a 40-50%) than that of commercial juices. Even more, the antioxidant capacity of the soluble fraction of home-made apple juice reached only a 3% of that found for commercial ones. All of these findings point to a very possible addition of vitamin C in commercial juices.

Least, but not last, the amount of total polyphenols in non-citric juices was also measured, since these chemical species are responsible in a large extension of the antioxidant activity of these foodstuffs. The polyphenols content ranged from 116 to 869 mg gallic acid/L (samples K4 and C6, respectively) in commercial juices (**Table 29**). Banana, mango and multifruit juices showed the highest amount of phenols ($p<0.05$). These values are in line for those stated by other authors for apple (Schempp, Christof, Mayr & Treutter, 2016; Wlodarska et al., 2016), mango (Naresh et al., 2015) and pineapple (Laorko, Tongchitpakdee & Youravong, 2013; Rodríguez et al., 2017) juices. The amount of polyphenols found in freshly squeezed juices was lower to that found for commercial apple, mango and pineapple. In this sense, as documented for mango juice, the degree of maturity of the fruit could play a role in the amount of

polyphenols (Gámez, Caballero-Montoya, Ledesma, Sáyago-Ayerdi, de Lourdes, von Wettberg & Montalvo-González, 2017).

The last trends in juice consumption in Spain (Mercasa, 2017) shows a 26% increase on the consumption of commercial cooled juices, which are perceived by consumers as a healthier option. Thus, we also assessed if the storage under refrigeration plays a role on the antioxidant characteristics of commercial multifruit juices (**Table 30**). There was no statistically significant differences ($p>0.05$) on the antiradical capacity of cooled and not cooled multifruit juices (ABTS and GAR_{ABTS} methods), but cooled juices were less reducing ($p<0.05$) than not cooled multifruit juices, for both the FRAP and GAR_{FRAP} methods: 3.57 *Vs.* 11.3 and 81.6 *Vs.* 152 mmol trolox/L in cooled *Vs.* not cooled juices for FRAP and GAR_{FRAP} methods, respectively. As stated above and described also for citric juices (Álvarez et al., 2014) this could be related to the addition of ascorbic acid as preservative in not cooled multifruit juices, as well as the formation of antioxidant compounds coming from the Maillard reaction, due to thermal treatment and/or storage (see discussion below in physicochemical properties). No differences on polyphenols were found.

Table 30. Statistical analysis of the different commercial multifruit juices grouped by storage conditions[1].

Analytical assay	Cooled	Not cooled
ABTS	3.56 ± 1.00[a]	3.02 ± 2.24[a]
GAR_{ABTS}	34.5 ± 8.3[a]	30.1 ± 28.8[a]
FRAP	3.57 ± 0.64[a]	11.3 ± 7.99[b]
GAR_{FRAP}	81.6 ± 135[a]	152 ± 60.8[b]
Polyphenols	390 ± 137[a]	406 ± 108[b]
HMF	1.42 ± 1.07[a]	4.24 ± 2.09[b]
Furfural	0.92 ± 0.44[a]	1.11 ± 0.60[a]
*L**	43.6 ± 2.3[a]	35.4 ± 2.2[b]
*a**	1.2 ± 3.4[a]	-1.1 ± 2.0[b]
*b**	13.7 ± 1.6[a]	25.2 ± 6.4[b]
Fluorescence	20 ± 12[a]	12 ± 5[a]

[1] Different letters between cooled-not cooled indicate statistically significant differences $p < 0.05$. Antioxidant capacity (ABTS, GAR_{ABTS}, FRAP and GAR_{FRAP}) expressed as mmol trolox/L, polyphenols expressed as mg gallic acid/L, HMF and furfural expressed as mg/L, L*, a* and b* adimensional units and fluorescence expressed as absorbance units $x10^3$. The number of decimal figures is based on the significant figures.

Finally, we analyzed the contribution of peach and pineapple juice consumption to the overall antioxidant capacity and polyphenols intake in the Spanish diet. In Spain, the mean antioxidant capacity intake range from 3549 to 6014 μmol Trolox equivalents/ day for the ABTS and FRAP methods, respectively (Saura-Calixto & Goñi, 2006). The consumption of peach and pineapple commercial juices in Spain in 2016 was 2.2 and 1.1 mL/inhabitant/day, respectively (MERCASA, 2017). Such consumption means a contribution of a 0.1-0.3% of the daily antioxidant activity intake for the ABTS method (**Table 31**) and a 0.4-0.9% for the FRAP method. However, when the GAR_{ABTS} method is used to calculate the antioxidant capacity, then the contribution increase till 0.8-3.6% for pineapple and peach commercial juices, and 4-7% for GAR_{FRAP}. Although such contribution is calculated taking into account the mean intake in Spain, a realistic approach could be the calculation based in the intake of antioxidant capacity per serving

(200 mL). Then, for the standard ABTS method the contribution increased up to 25% of the daily intake and a 78% for the FRAP method (**Table 31**). When the same calculation is applied to the GAR method, the contribution exceeds the 100% daily intake (133 and 325 for pineapple and peach for GAR_{ABTS} and 724 and 649 for GAR_{FRAP}). Thus, one serving provides a high amount of antioxidant capacity as far as 4500-25000 μmol trolox. These discrepancies are caused by the underestimation of the antioxidant capacity when chemical extraction procedures are used.

In the case of polyphenols, the daily intake of polyphenols was 1171 mg gallic acid/day (Saura-Calixto & Goñi, 2006). Thus, taking into account the mean daily intake of peach and pineapple juices (2.2 and 1.1 mL/day, respectively) they contribute only to a 0.04-0.06% of the daily polyphenols intake. However, if a serving (200 mL) is used for calculation, then contribution of juices to the daily intake increases up to 5.7%, since each serving provides around 67 mg of polyphenols.

Table 31. Contribution of juice consumption to the daily antioxidant activity (AOX) and polyphenols intake in the Spanish diet.

Type of juice	**Analytical assay**	**AOX/daily intake**[1] (μmol trolox/day)	**Contribution to daily antioxidant capacity intake** (%)	**AOX/serving intake**[2] (μmol trolox/serving)	**Contribution to daily antioxidant capacity intake** (%)
Peach	*ABTS*	9.41	0.27	859	25
	GAR_{ABTS}	122.26	3.56	11157	325
	FRAP	29.51	0.86	2693	78
	GAR_{FRAP}	244.29	7.11	22291	649
Pineapple	*ABTS*	3.68	0.11	672	20
	GAR_{ABTS}	25.12	0.73	4584	133
	FRAP	13.87	0.40	2531	74
	GAR_{FRAP}	136.27	3.97	24870	724
Type of juice	**Analytical assay**	**Polyphenols/daily intake**[1] (mg/day)	**Contribution to daily polyphenols intake** (%)	**Polyphenols/serving intake**[2] (mg/serving)	**Contribution to daily polyphenols intake** (%)
Peach	*Folin-Ciocalteu*	0.74	0.06	66.8	5.7
Pineapple	*Folin-Ciocalteu*	0.36	0.04	65.6	5.6

[1]Considering juice consumption for a whole year.

[2]Consideing the complete serving ingested a particular day.

3.2. Physicochemical characteristics of juices

The food industry used different processing methods to preserve the sensory and nutritional quality of juices (Quitao-Texeira, Odriozola-Serrano, Soliva-Fortuny, Mota-Ramos & Martín-Belloso, 2009) while using minimal chemical preservatives. (Naresh et al., 2015). Among these methods thermal treatment extends the self-life of juices, although it leads to the formation of HMF, which correlates with the alteration of fresh odor by formation of detrimental off-flavours (Rattanathanalerk et al., 2005). Therefore, the use of HMF and furfural as indicators to control inappropriate heat processing and storage is quite extended (Kadakal, Sebahattin & Poyrazoglu, 2002; Tulek & Yilmaz, 2006).

The mean HMF values in commercial juices (**Table 32**) ranged from 1.06 to 9.55 mg/L for tomato and mango juices, respectively. Mango and antiox juices had a statistically significant ($p<0.05$) higher concentration of HMF while peach and tomato juices had the lowest ones ($p<0.05$). In the case of individual samples the HMF levels were comprised between not detected to 24.6 mg/L for sample H12. These values are in line with those reported by other authors for apple (Kadakal et al., 2002; Tulek & Yilmaz, 2006), peach (Calin et al., Ionel, 2011) and pineapple (Rattanathanalerk et al., 2005). Furfural levels were also evaluated and ranged from 0.21 to 4.67 mg/L for tomato and pineapple juices, respectively. As in the case of HMF, we found a statistically significant ($p<0.05$) difference in furfural levels, which increased in the following order: tomato = banana = peach = antiox = multifruit = apple < mango < pineapple. Regarding individual samples, furfural concentration was comprised between not detected to 21.0 for sample J10. For both thermal treatment indicators, some outliers were found (samples H12 and D14 for HMF and samples J10 and H2 for furfural), which could be related with an intense sterilization process or inadequate storage that

are reported to increase the concentration of these by-products (Kadakal et al., 2002; Rattanathanalerk et al., 2005; Tulek & Yilmaz, 2006). As expected, neither HMF nor furfural were found in freshly squeezed juices. Finally, it was also studied the effect of refrigeration over HMF and furfural content in multifruit juices (**Table 30**). Thus, not cooled juices had an HMF value statistically higher ($p<0.05$) than refrigerated ones while furfural values were stable. The different thermal treatment performed during juice production could be responsible for these HMF levels, since more drastic conditions are applied during the UHT sterilization of not cooled multifriut juices whereas cooled juices undergo pasteurization, a softer procedure. Consistently, HMF and furfural were undetectable in freshly squeezed juices.

Thus, it can be calculated the amount of HMF provided by the intake of peach and pineapple juices, since their daily intakes and HMF content are known: in peach juices 0.8 and 9.9 L/inhabitant/year 2016 for adult population and children, respectively; in pineapple juices 0.4 and 9.9 L/inhabitant/year 2016 for adult population and children, respectively; HMF content of 0.55 and 4.67 mg/L for peach and pineapple juices. In this sense, peach juice give rise to an overall HMF intake of 1.2 μg/day for adult population and 14.9 μg/day in the case of children. The intake increases up to 5.1 μg/day for adults and 126 μg/day for children in the case of pineapple. Such intakes, taken together, mean a 0.07% of the total intake of HMF for the Spanish adult population (Rufián-Henares & de la Cueva, 2008) and 1.01% for children (Pastoriza et al., 2017). However, the HMF intake could increase up to and 23.1 and 567 μg HMF for those adults and children, respectively, drinking the pineapple juice identified as J10. When the usual serving (200 mL) is used to perform the calculation in a more realistic way, an intake of 110 and 934 μg HMF for peach and pineapple juices is found, representing a 1.14-9.65% of the daily adult intake (9.68 mg/day) and 0.79-6.71% of daily children intake (13.92 mg/day).

Even more, a glass of pineapple juice J10 means an amount of 4200 μg HMF, which means a 43.4 and 30.2% of the daily intake of adults and children, respectively. Therefore, the consumption of certain commercial juices could be an objective for diminishing the overall HMF intake at the population/individual level if the sanitary authorities declare dietary HMF in the future as a possible carcinogenic substance like acrylamide or furan.

The results obtained for color measurement are reported in **Table 32**. Lightness (L*) of individual juices were comprised between 19.3 and 50.3, but L* values of different juice groups ranged from 22.4 to 47.5 for antiox and banana juices, respectively. Banana juices were darker ($p<0.05$) that the rest of the juices, while antiox and apple juices were the most light ($p<0.05$). These values are in line with those reported by other authors for apple (Włodarska et al., 2016), banana (López-Nicolás, Pérez-López, Carbonell-Barrachina & García-Carmona, 2007a), mango (Naresh et al., 2015), peach (López-Nicolás, Pérez-López, Carbonell-Barrachina & García-Carmona, 2007b; Rao, Guo, Pang, Tan, Liao & Wu, 2014) and pineapple (Laorko et al., 2013) juices. Regarding redness (a*), there was a large variation in individual values (ranging from -4.42 to 15.5) even within each group of juices (**Table 32**). The group values statistically increased redness ($p<0.05$) as follows: pineapple = mango < apple = multifruit = banana = peach < antiox < tomato. It is logical that tomato and antiox juices are the redder juices due to tomato color and the use of red berries on their composition. The last color parameter assessed was greenness (b*), which showed also a large dispersion among samples (from 0.58 to 32.4) even within the same juice group. Such dispersion has been previously reported by other authors (Laorko et al., 2013; Rao et al., 2014; Naresh et al., 2015; Włodarska et al., 2016). Antiox and apple juices were those with lower values ($p<0.05$). Finally, it was found a statistically significant decrease on

a* and b* values of not cooled multifruit juices (**Table 30**) and a not significant ($p = 0.054$) but tending to significance decrease of L*. Taking all these results together, the color of not cooled juices is darker than that of refrigerated ones, which in turn reinforces the idea that UHT sterilization favors the development of the Maillard reaction, giving rise to a darkening and production of compounds like HMF or furfural.

Fluorescent compounds are related with sensorial and antioxidant properties of heat-processed juices (Włodarska et al., 2016), being most of them generated along the Maillard reaction (Rufián-Henares, Guerra-Hernández & García-Villanova, 2002; Rufián-Henares, García-Villanova & Guerra-Hernández, 2004). Individual samples had a fluorescence intensity ranging from 2594 to 39557 units (**Table 32**). In the case of group juices, multifruit juices had the highest fluorescent intensity of all the assessed fruit groups ($p<0.05$). There was no statistical differences in fluorescence intensity of cooled *Vs.* not cooled multifruit juices (**Table 30**), which could be explained taking into account the large SD found in such group (the largest of all the studied juice types, **Table 32**). These results are in line with those reported previously by Álvarez et al. (2014) for orange juices.

Table 32. Physicochemical characteristics of commercial juices[1].

Juice	Code	HMF	Furfural	Color			Fluorescence
				L*	a*	b*	
Commercial juices							
Antiox	J6	1.15	0.12	19.3	0.97	0.59	5213
	M1	2.10	1.64	21.1	3.17	1.41	8498
	M2	6.77	0.78	21.0	1.80	0.91	4680
	M3	0.36	0.09	31.4	4.11	11.8	14898
	H9	9.31	0.13	24.7	8.10	3.02	4771
	K4	7.96	1.22	21.4	3.15	0.98	3700
	D13	7.02	0.98	22.3	4.72	1.68	6437
	D14	14.3	2.65	20.6	1.88	1.02	4894
	G7	0.49	0.00	19.4	1.71	0.92	3238
Mean		***5.50 ± 4.80^{a}***	***0.85 ± 0.89^{ab}***	***22.4 ± 3.75^{a}***	***3.29 ± 2.18^{a}***	***2.47 ± 3.55^{a}***	***6259 ± 3588^{a}***
Apple	J3	5.82	1.28	29.9	-4.42	4.00	5721
	C4	0.43	0.14	25.9	-0.92	1.08	2594
	C21	3.76	0.43	22.0	0.05	3.58	3471
	D12	1.37	0.51	22.1	0.23	4.35	3452
	G10	7.49	3.14	21.6	0.45	3.53	4252
Mean		***3.77 ± 2.95^{ab}***	***1.10 ± 1.21^{a}***	***24.3 ± 3.58^{a}***	***-0.93 ± 2.02^{b}***	***3.31 ± 1.29^{a}***	***3898 ± 1176^{b}***
Banana	K1	5.33	0.47	47.4	-2.66	4.88	5848
	H11	3.98	0.21	49.1	-1.76	4.69	4559
	C8	0.47	0.73	44.2	6.77	13.5	7785
	G9	0.04	0.06	49.4	-3.37	3.43	7818
Mean		***2.46 ± 2.60^{b}***	***0.37 ± 0.29^{b}***	***47.5 ± 2.4^{b}***	***-0.25 ± 4.72^{b}***	***6.61 ± 4.60^{b}***	***6502 ± 1589^{b}***
Mango	H12	24.6	1.51	36.0	-2.78	18.5	3734
	C6	2.73	0.73	50.3	-0.15	32.4	15216
	CO1	1.36	5.02	38.1	-0.99	22.6	4609
Mean		***9.55 ± 13.0^{a}***	***2.42 ± 2.28^{c}***	***41.5 ± 7.73^{c}***	***-1.31 ± 1.34^{c}***	***24.5 ± 7.13^{c}***	***7853 ± 6391^{ab}***
Multifruit	K5	5.83	1.71	35.1	-2.10	7.07	6047
	H1	0.84	1.10	43.9	1.82	27.0	9202
	H5	3.87	0.72	39.0	2.83	23.9	10131
	J4	5.81	2.28	34.1	-1.73	6.08	9653
	M7	6.77	0.49	33.4	-0.53	12.5	21978

	C3	0.75	0.42	45.7	-2.47	24.0	39557
	C17	3.14	0.77	35.4	2.31	17.5	11751
	T3	2.66	1.25	41.2	0.53	24.6	10072
	C03	1.02	0.93	36.5	4.12	20.7	9995
	G6	6.32	4.03	33.4	-1.63	16.7	16986
Mean		***3.47 ± 2.24***^{a}	***1.06 ± 0.54***^{a}	***37.7 ± 4.33***^{c}	***-0.46 ± 2.46***^{b}	***16.8 ± 7.60***^{c}	***13613 ± 9247***^{c}
Peach	H10	0.00	0.00	37.3	-0.96	17.0	4552
	J1	2.28	0.50	32.4	-0.77	11.7	7473
	J7	0.48	0.06	19.4	0.92	0.58	6156
	S2	0.54	0.33	37.2	1.19	19.1	6369
	M5	0.58	0.39	35.8	0.03	15.7	5054
	C16	0.39	0.17	32.8	-0.96	14.4	8451
	D8	1.40	0.69	35.8	-1.13	15.1	5549
	D9	4.23	2.42	36.3	0.54	17.7	7626
	G2	0.90	0.36	40.6	-0.33	22.6	5398
Mean		***1.20 ± 1.32***^{c}	***0.55 ± 0.73***^{ab}	***34.2 ± 6.04***^{c}	***-0.16 ± 0.87***^{b}	***13.7 ± 7.13***^{c}	***6292 ± 1312***^{a}
Pineapple	K3	0.07	0.00	29.5	-1.83	3.58	3165
	H2	0.31	15.2	35.8	-4.08	10.4	4663
	H14	0.53	0.00	25.2	-0.98	1.24	3330
	J8	0.77	4.04	29.2	-1.90	2.77	5083
	J10	2.90	21.0	34.0	-2.53	12.1	10439
	M6	2.17	3.54	26.3	-0.89	3.42	6803
	C9	1.83	6.46	31.6	-2.78	7.03	6863
	C10	6.43	1.92	26.3	-1.30	2.81	6186
	C15	10.3	2.97	27.9	-1.46	4.17	10171
	T6	0.09	0.77	32.8	-2.42	7.40	6623
	D10	3.88	1.86	23.7	-0.60	2.41	6056
	D11	0.69	7.75	29.7	-1.95	4.91	7269
	G5	0.49	1.72	29.0	-1.73	2.35	4408
Mean		***2.44 ± 3.05***^{ac}	***4.67 ± 6.31***^{d}	***28.6 ± 3.06***^{c}	***-1.66 ± 0.87***^{c}	***5.15 ± 3.24***^{ab}	***6449 ± 2382***^{a}
Tomato	A1	0.00	0.00	37.3	10.6	21.0	4174
	S3	0.00	0.00	29.0	12.1	11.3	10894
	G1	3.19	0.62	30.5	15.5	13.6	13374
Mean		***1.06 ± 1.84***^{c}	***0.21 ± 0.36***^{b}	***32.3 ± 4.44***^{c}	***12.7 ± 2.54***^{d}	***15.3 ± 5.08***^{c}	***9480 ± 4760***^{bc}

[1] Different letters within the column indicate statistically significant differences $p < 0.05$. HMF and furfural expressed as mg/L, L*, a* and b* adimensional units and fluorescence expressed as absorbance units x10^3. ND = Not detected. The number of decimal figures is based on the significant figures.

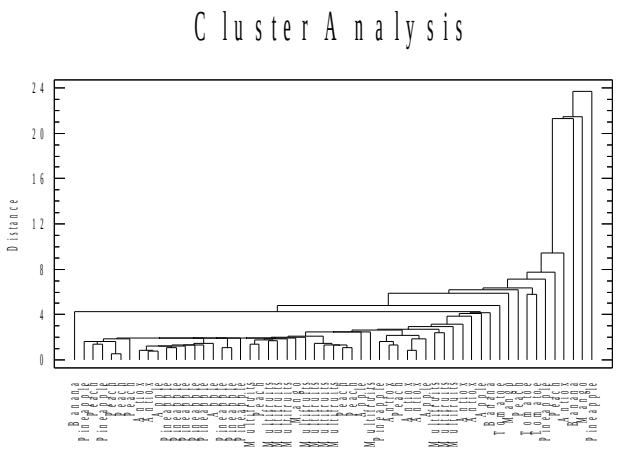
3.3. Statistical relationship among antioxidant and physicochemical properties

There is a link between the sensory quality of juices and some physicochemical parameters related with thermal treatment like HMF or furfural (Roig, Bello, Rivera & Kennedy, 1999), that is the reason why we studied the linear relationship among all the assessed variables. When all the juices were compared at the same time, the regression coefficient was quite low and statistically signification ($p<0.05$) was reached only between HMF-Furfural ($r^2 = 0.4491$) and L*-b* ($r^2 = 0.6361$). We next performed the correlation study taking into account the type of juice, excluding antiox and multifruit juices due to their variability in composition, which makes difficult to obtain any relationship among them. For apple and peach juices, statistically significant linear correlations ($p<0.05$) between ABTS-b* ($r^2 = 0.6627$) and FRAP-a* were found, indicating that the antioxidant capacity could be quickly estimated from color measurement. In those juices sugar-rich (banana, mango and pineapple juices) it was obtained a significant ($p<0.05$) linear correlation between ABTS-HMF (r^2 from 0.7880 to 0.9923) and ABTS-Furfural ($r^2 = 0.5339$), which could indicate that part of the antioxidant capacity of these juices is linked to Maillard reaction products generated during the thermal processing. Such relationships were also found previously in citric juices (Álvarez et al., 2014), which reinforce a direct relationship between thermal processing and antioxidant capacity.

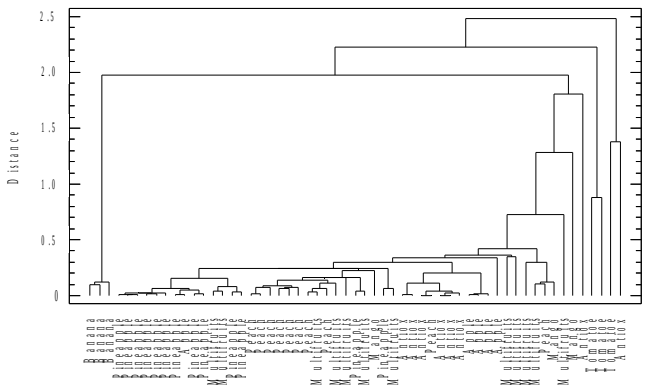
We subsequently analyzed the relationship among groups of juices by multivariate analysis. The first step consisted on grouping the samples depending on their physicochemical (HMF, furfural, L*. a*, b*) and antioxidant (ABTS, FRAP, polyphenols) characteristics. When all the parameters were taken together, it was not obtained a clear grouping of samples, being some types of juices like antiox or banana abnormally distributed along the samples (**Figure 51A**), indicating some problems with

Figure 51. Cluster analysis of commercial juices characterized by their antioxidant-physicochemical characteristics (A) or color parameters (B) and principal component analysis (PCA) of commercial juices without mixed juices (antiox and multifruit juices) (C).

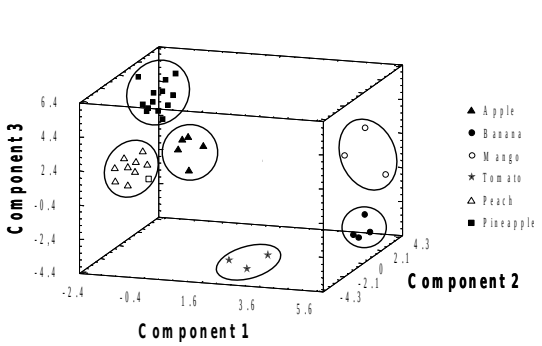
51A



51B



51C



the data used for cluster analysis. However, when only the color parameters L*, a* and b* were used for classification, almost a complete grouping was obtained (**Figure 51B**). Two types of incorrect classification were found: individual samples were not grouped with those of their groups since they were outliers, i.e. mango (C6) and peach (J7) for L*, apple (J3) and mango (H12) for a* and peach (J7) for b*. In the case of the antiox and multifruits groups, some of their fruits were broadly dispersed since they are composed by a mixture of different juices, so that it is quite difficult to describe such groups as a mathematical combination of different parameters. Then, we performed a Principal Component Analysis (PCA) in order to obtain a little number of linear combinations of the 8 parameters that explain as much as possible the data variability. When all the samples were taken together we found that the combination of 3 components explains a 61.8% of the original data variability, obtaining a clear separation of banana, mango and tomato juices (data not shown). As stated above, both antiox and multifruits juices are a variable combination of different juices, so that they are incorrectly classified: multifruits juices belonging to the apple, peach and pineapple groups (the main ingredients of multifruit juices) and antiox juices including also tomato as ingredient. Thus, we removed the antiox and multifruits groups and obtained a clear distinction of 100% of apple, banana, mango, tomato, peach and pineapple juices (**Figure 51C**). Consequently, it is possible to distinguish these types of juices according to their antioxidant and physicochemical profiles, however is not possible to distinguish antiox and multifruits juices.

4. Conclusions

As we did before in the case of citrus juices, the information compiled in this paper reinforces that non-citrus juices are a great source of antioxidant compounds with the novelty of establishing the suitability of the GAR method, including an *in vitro* gastrointestinal digestion, to consider the global antioxidant potential trapped in this food matrix. Our findings suggest the importance of considering the non-soluble fraction in the protocols for measuring the antioxidant activity since in some cases it could contain more antioxidant species than that found in the soluble one. Besides HMF and furfural were detected in many of the samples analyzed, stored non-citrus juices do not seem to have an important contribution to the daily HMF and furfural intake in the Spanish diet. Finally, it is stated that mathematical models based on the consideration of the antioxidant and physicochemical parameters of stored single no-citrus juices can be a suitable tool to distinguish them according with their nature or their storage conditions. However, they are not useful in the case of antioxidant and multifruit juices.

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Relationship between composition and bioactivity of persimmon and kiwifruit

Las frutas tropicales cada vez están más presentes en la dieta española. Por ello, se decidió llevar a cabo una revisión bibliográfica de dos de sus mayores representantes en España, el caqui y el kiwi. En este trabajo se detallan las principales propiedades sobre la salud de ambas frutas, la mayoría de ellas relacionadas con la capacidad antioxidante. Además, se describe la composición en compuestos bioactivos de cada una de ellas, destacando los compuestos fenólicos, íntimamente ligados con la capacidad antioxidante de estas frutas. Dada la elevada presencia de ambas frutas en la dieta española, y que la mayoría de sus propiedades saludables se deben a sus compuestos antioxidantes, este paper proporciona una información muy importante en la presente tesis doctoral.

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Relationship between composition and bioactivity of persimmon and kiwifruit

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ABSTRACT

Fruits are foods plenty of vitamins, minerals and some bioactive phytochemicals like polyphenols. Thus, fruits may exert many different functional properties on human health, some of them directly related with their antioxidant capacity like cancer or atherosclerosis. Thanks to globalization, consumers have a wide repertory of fruits along the year. Among them, tropical and subtropical fruits are steadily expanding, as well as the studies about them. In this sense, this is a timely review focusing on the nutritional value and chemical composition of persimmon and kiwifruit, two tropical fruits with a protective role on different chronic diseases. Thus, this review focuses in presence of bioactive compounds such as polyphenols, tannins, carotenoids, vitamin C, etc. and different functional properties (i.e. antioxidant capacity, antithrombotic activity, decrease of plasmatic lipids, etc.) arising from the presence of such biologically active molecules. Finally, the effect of genotype and ripening stage on antioxidant capacity and the content of bioactive compounds is also discussed.

KEYWORDS: Persimmon, kiwifruit, antioxidant capacity, polyphenols, functional properties.

1. Introduction

Antioxidant capacity is becoming more and more object of interest as possible treatment or as a mean to prevent many diseases. Oxidation is directly related to the damage caused to biological molecules such as DNA, proteins, lipids, etc., all of them essential parts of cells. Hydroxyl radicals are for example one of the most common and harmful free radicals. These free radicals can be generated as a result of unhealthy habits such smoking but also regular respiration produces them and are free to attack and damage cell components (Lee et al., 2004). In this sense, oxidative stress is known to be one of the causes of several chronic diseases such as cancer, liver disease, inflammation, diabetes, Alzheimer´s disease, Parkinson´s disease, atherosclerosis and aging (Moon & Shibamoto, 2009). Therefore, antioxidants consumption is thought to be an important mean to delay such diseases and some kind of foods, especially vegetables and fruits (Roginsky & Lissi, 2005). This healthy property depends on food composition and specifically on their content in bioactive compounds such as phenolics, vitamins, carotenoids, etc. (Patil, Jayaprakasha, Murthy, & Vikram, 2009).

Several studies have shown that fruit consumption could have a beneficial effect on health and a protective role on some chronic diseases such as atherosclerosis and cancer (Kim, Lee, Lee, & Lee, 2002). These properties have been attributed to bioactive compounds with antioxidant capacity which can avoid or make slower the oxidative damage (Shi, Noguchi & Niki, 2001). Thus, the nutritional composition and functional properties of fruits depend on several factors: species and variety, crop conditions, ripening, treatment with phytosanitary products, conditions and storage time, etc. Moreover, ripening can occur either in the tree or not, but in both cases it involves complex processes that transforms their components (Abellan, García-Villanova & Ruiz, 2010).

Nowadays, thanks to globalization, exotic tropical and subtropical fruits that some years ago were not available outside their climate zones can be found in almost any market around the world. There are many of these kinds of fruits but this review will focus on kiwifruit (*Actinidia spp*.) and persimmon (*Diospyros kaki*) and their functional properties, especially the antioxidant capacity. Both of them are important in markets all around the world, especially kiwifruit, which in fact has lost some of that exoticism it had when it first arrived at some markets (Illescas, Bacho & Ferrer, 2007). On the contrary, persimmon consumption and cultivation is not so spread than that of kiwifruit, but is a subtropical fruit with many bioactive compounds. Since these fruits are a well-known source of antioxidants, having a potential protective role on oxidative stress related diseases, their nutritional composition and effect on human health will be discussed further along this review.

2. Persimmon

2.1 Persimmon production

In the world, persimmon production in 2014 was of 5190624 tonnes and the area harvested was 1025989 ha. Persimmon production in the world is 0.75% of total fruit production (Food and Agricultural Organization of the United Nations 2016). Most persimmon production is in Asia, 91% of world production followed by Europe with a 5% of the global production. Moreover, most of the production comes from China with a 73%. On the other hand, Spain contributes to world persimmon production with a 4.7% (Food and Agricultural Organization of the United Nations 2016).

Persimmon production has grown greatly in the las 15-20 years. If we just take into account world production, it has been multiplied by 5 in that period of time. On the other hand, focusing on Spain, in the earlies 90s production was basically absent but has

grown to 250k tonnes a year which gives an idea of how important this crop has become. The same way as production, production value has also grown greatly in the last years, not only considering the whole world but in Spain as consequence of such increase in production (Food and Agricultural Organization of the United Nations 2016). This growth in production as well as in value has make this fruit of great interest in the last years and consequently lot of research has focused on it.

During 2015, in Spain 4424.4 millions of kilograms were consumed which meant an expenditure of 5973.2 million of euros. Per capita consumption was 99.2 kg and the expenditure was 133.9 € (MERCASA, 2016).

Table 33. Generic composition of persimmon and kiwfruit (MAPAMA, 2016; Barea-Alvarez et al., 2016; United States Department of Agriculture, Agricultural Research Service, Food Composition Database, 2017).

	Kiwifruit		**Persimmon**	
	100 g of edible portion	**By unit (100g)**	**100 g of edible portion**	**By unit (100g)**
Energy (Kcal)	59.00	49.00	71.50	114.40
Proteins (g)	1.06	0.87	0.64	1.02
Total fats (g)	0.39	0.32	0.25	0.39
Carbohydrates (g)	13.20	10.95	17.30	27.67
Fiber (g)	1.65	1.35	2.60	4.16
Water content (g)	84.17	70.34	80.86	129.38
Calcium (mg)	21.00	17.75	8.00	12.80
Iron (mg)	0.31	0.24	0.20	0.31
Magnesium (mg)	13.50	11.45	9.25	14.80
Zinc (mg)	0.09	0.08	0.11	0.17
Sodium (mg)	3.50	2.70	2.50	4.00
Potassium (mg)	302.50	252.00	175.50	280.80
Phosphorus (mg)	30.00	25.05	19.50	31.20
Selenium (µg)	0.60	0.50	0.60	0.96
Thiamine (mg)	0.01	0.01	0.03	0.04
Riboflavin (mg)	0.05	0.05	0.03	0.05
Niacin equivalents (mg)	0.42	0.34	0.20	0.32
Vitamin B_6 (mg)	0.11	0.10	0.10	0.16
Vitamin B_{12} (µg)	0.04	0.03	0.00	0.00
Vitamin C (mg)	110.15	90.70	11.75	18.80
Vitamin A: Retinol Eq. (µg)	2.00	1.80	119.50	191.20
Vitamin D (µg)	0.00	0.00	0.00	0.00
Folate (mg)	31.00	25.00	7.50	12.00

2.2 Composition and nutritional value of persimmon

The composition of persimmon can change depending on the variety. **Table 33** shows the generic composition of persimmon and kiwifruit. Regarding macronutrients, persimmon has a low protein and fat content and around a 16% of carbohydrates, mainly sugars. These sugars are mostly fructose, glucose and sucrose, which can be found in higher quantities than in other commonly consumed fruits. Moreover, persimmon has pectin and mucilagues as part of the soluble fiber and a large amount of insoluble fiber (Spanish Ministry of Agricultur, Fisheries, Nutrition and Environment (MAPAMA) 2016). Regarding micronutrients, persimmon is an exceptional source of provitamin A as β-carotene (with 160 μg/100 g of fresh weight) and also a good source of vitamin C (16 mg/100g of fresh weight). Persimmon has important amounts of potassium but rather low quantities of other minerals such as magnesium and phosphorus (MAPAMA, 2016). Persimmon has also an important content in tannins which gives them astringency. In this sense, persimmon cultivars can be divided in astringent and non-astringent. However, astringency decreases in both cultivars during ripening due to their transformation in their insoluble forms (Pei, Zhang, Guo, & Luo, 2013). The differences between such cultivars rely on the large amount of tannins still present in the astringent cultivars even in mature state (Yaqub et al., 2016). During ripening there is also an increase in sugars, glucose and fructose due to the activity of the invertase enzyme, which hydrolyzes sucrose (Del Bubba et al., 2009). On the other hand, there is also a decrease of vitamin C content during fruit growing and ripening due to its use in the Kreb´s cycle (Antoniolli, de Camargo, Kluge & Filho, 2002).

Phytochemicals are an important fraction of persimmon fruit comprising proanthocyanidins, flavonoid oligomers, tannins, phenolic acids and carotenoids. In fact, persimmon has 160-250 mg of polyphenols/100 g of fresh weight and 2 mg of

carotenoids/100 g of fresh weight, which is a high amount compared with the protein content (640-1300 mg of proteins/100 g of fresh weight) (Butt et al., 2015).

Carotenoids. Carotenoids are responsible of the color of the fruit and also responsible for some of their antioxidant capacity. Their content increases as the fruit matures except for lutein and lycopene. Their content is very variable depending on the cultivar, but usually the most abundant carotenoid is β-cryptoxanthin (Yaqub et al., 2016).

Tannins. Tannins are an important fraction of persimmon. These compounds are responsible of astringency. As stated above, persimmon cultivars can be divided in two groups regarding astringency: astringent and non-astringent. The first group has a higher content in soluble tannins than the second. However, during ripening, astringency decreases as soluble forms of tannins are transformed into their insoluble ones. According to some authors (Gu et al., 2008; Matsui, 2015), persimmon tannins are composed of epicatechin gallate, epigallocatechin gallate, epigallocatechin and an unknown monomer. These compounds are commonly called catechins and comprise a group of bioactive compounds with strong antioxidant capacity involving many others chronic diseases based on oxidative stress. These catechins are higher in astringent persimmons than in non-astringent ones.

Phenolic compounds. According to some authors, total polyphenols are around 1.45 mg/100 g of fresh weight (Butt et al., 2015). However, phenolic content is very variable among cultivars due to the different climate conditions, crop characteristics, harvest time, processing, nutrients available, etc. In persimmon phenolics can be divided into low molecular and high molecular groups. In the first one phenolic acids, catechins and hydrolyzed tannins are included. In the high molecular weight group tannins and proanthocyanidins can be found (Yaqub et al., 2016).

Proanthocyanidins. These compounds increase during the early stages of growing, giving the fruit resistance against different types of aggressions. They are composed of condensed flavan-3-ols. However, their content decreased during maturation, which results in a decreased antioxidant capacity (Yaqub et al., 2016).

2.3 Effect of genotype on antioxidant capacity and bioactive compounds of persimmon

The presence of antioxidant compounds such as carotenoids, ascorbic acid, tannins, catechins and other phenolic molecules makes persimmon a great source of antioxidants. This means that persimmon consumption could help preventing or reducing diseases related to oxidative stress (Butt et al., 2015). In this sense, **Table 34** shows the data available in bibliography about antioxidant phytochemicals (total polyphenols, total carotenoids, α- and β-carotene, zeaxanthin and β-cryptoxanthin) for a total of 21 genotypes of *Dyospiros kaki* (Pu, Ren, & Zhang, 2013; Veberic, Jurhar, Mikulic-Petkovsek, Stampar, & Schmitzer, 2010).

The total content of polyphenols was analyzed by means of the Folin-Ciocalteu method in different cultivars (Veberic et al., 2010; Pu et al., 2013). Phenolic content is very variable depending on the genotype. Results extracted from bibliography show that the genotype with higher phenolic content is *D. kaki. var. silvestris M*, with 1520 mg GAE/100 g of dry weight (**Table 34**). However, the genotype with the lowest amount of total phenols is *D. kaki cv. Mopan*, with 32 mg GAE/100 g of dry weight. Such huge difference (50 times higher) is just a sample of how phenolic content could vary. Not surprisingly, the wild genotype *D. kaki. var. silvestris M* was also the one with statistically significant higher values of total flavonoids and flavanols compared with commercial varieties (Veberic et al., 2010). In the case of individual polyphenols, different types such as p-coumaric acid, catechin, epicatechin, epigallocatechin,

condensed proanthocyanidins, quercetin or kaempferol can be found in persimmon (**Table 35)**. All these compounds could play a protective role against oxidative stress and, in extension, with diseases related to such condition.

Other family of important bioactive compounds in persimmon is carotenoids, which are responsible of their pigmentation. These molecules have shown antioxidant capacity and therefore are able to decrease oxidative stress (Yaqub et al., 2016). Carotenoids are able to protect biological membranes from oxidation and therefore they are potentially able to slow down cellular aging protecting against degenerative deseases (Suzuki, Someya, Hu, & Tanokura, 2005). Total carotenoid content did not vary that much compared to polyphenols (**Table 34**). Results for 11 genotypes showed a range between 490-936 mg/kg of fresh weight. Since the amount of carotenoids is up to 25-times lower than that of polyphenols, it could be hypothesized that the antioxidant properties of persimmon could rely more on the phenolic content. When individual carotenes were measured, β-carotene was the predominant species, with a 4-5 higher content than α-carotene. In this sense, β-carotene content ranged from 259 to 459 mg/kg of fresh weight while α-carotene was in the range of 73-160 mg/Kg of fresh weight. The content on two other carotenoids, such as zeaxanthin and β-cryptoxanthin, are in the same range (**Table 34**).

Regarding vitamin C, one fruit can fulfill around 46% of vitamin C requirements (Rao & Rao, 2007) since the vitamin C content ranges from 0.25-2.14 mg/ g of fresh weight (**Table 34**). In general, vitamin C levels in non-astringent cultivars are significantly higher (10-times) than those of the astringent cultivars (Giordani, Doumett, Nin, & Del Bubba, 2011). Vitamin C exists in persimmon under two forms: L-ascorbic acid and its oxidized product, L-dehydroascorbic acid. Although both chemical species are important compounds thanks to their antioxidant and anti-radical activities, they do

not exert the same activity since L-ascorbic acid is more active than the oxidized form (Gregory, 2007). Around 2/3 of total vitamin C in persimmon is available as L-ascorbic acid (Giordani et al., 2011).

Information about the relationship between cultivar and antioxidant capacity is available for three antioxidant methods: ABTS, FRAP and DPPH. Regarding the ABTS method, *D. kaki cv. Jiro, Zenjimaru, Xingyangshuishi, Zhouqumomoshi, Xiuningbianshi* and the wild genotype *D. kaki var. silvestris M* were compared (Pu et al., 2013). For this method results ranged from 0.5 to 37 μmol Trolox/g of fresh weight for *Jiro* and wild genotype, respectively. In the case of the FRAP method, the most antioxidant genotype with significant difference was again the wild one whereas the lowest reducing capacity was obtained by *Zenjimaru* cultivar (9.6 and 0.9 μmol Trolox/g of fresh weight, respectively). Finally, regarding DPPH method, the results showed the same relation. Thus, the antioxidant capacity of such cultivars decreased in the following order: *silvestris* >> *Zhouqumomoshi* > *Xiuningbianshi* > *Xingyangshuishi* > *Zenjimaru* > *Jiro*. Other study found that irrespective the antioxidant method used, the antioxidant capacity of persimmon genotypes decreases in the following order: *Seochonjosaeng* >> *Bongok* > *Dogeunjosaeng* > *Cheongdobansi* (Jang, Oh, Ahn, Lee, & Lee, 2011). Finally, (Park et al., 2015) found that the antioxidant capacity of persimmon is usually higher in astringent varieties than in non-astringent ones (Yaqub et al., 2016). In addition, whatever the variety assessed, the antioxidant capacity of persimmon is higher than that found in banana, durian, grape, grapefruit and lemon, but lower than strawberry, apple pulp, kiwifruit and mangosteen.

Table 34. Bioactive compounds found in persimmon and kiwifruit.

Astringent (A)/Not astringent (NA)	**Persimmon Genotypes/ Kiwifruit Cultivar**	**Total polyphenols** mg GAE g^{-1} DW[a]	**Total chlorophylls** mg g^{-1} DW	**Anthocyanins** mg CGE kg^{-1} DW	**Vitamin C** mg g^{-1} FW[b]	**Zeaxanthin** mg Kg^{-1}FW	**β-Cryptoxanthin** mg Kg^{-1} FW	**α-Carotene** mg Kg^{-1} FW	**β-Carotene** mg Kg^{-1} FW	**Total carotenoids** mg Kg^{-1} FW	**Ref.**
	Persimmon										
NA	*D. kaki* cv. Amankaki	1.40	NF	NF	NF	52	287	73	391	921	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
NA	*D. kaki* cv. Cal Fuyu	2.00	NF	NF	2.13	35	47	76	303	490	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007; Giordani et al. 2011)
A	*D. kaki* cv. Fuji	3.00	NF	NF	0.45	35	80	78	314	507	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007; Giordani et al. 2011)
NA	*D. kaki* cv. Hana Fuyu	1.90	NF	NF	2.14	87	257	83	403	727	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007; Giordani et al. 2011)
NA	*D. kaki* cv. O'Gosho	1.75	NF	NF	0.25	82	148	83	459	763	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007; Giordani et al. 2011)
NA	*D. kaki* cv. Tenjin O'Gosho	2.25	NF	NF	NF	58	154	160	360	758	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
NA	*D. kaki* cv. Thiene	2.15	NF	NF	NF	98	206	92	384	795	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
NA	*D. kaki* cv. Tipo	1.60	NF	NF	104	53	210	86	468	936	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007; Giordani et

											al. 2011)
A	*D. kaki* cv. Tone Wase	1.55	NF	NF	NF	37	236	80	305	814	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
NA	*D. kaki*. cv. Zenjimaru	3.48	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki*. cv. Xingyangshuishi	5.38	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki*. cv. Zhouqumomoshi	9.05	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki*. cv. Xiuningbianshi	10.55	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
Wild	*D. kaki. var. silvestris* M	15.20	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki* cv. Triumph	1.45	NF	NF	NF	53	262	113	448	920	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki cv.* Bongok	8.39	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki cv.* Cheongdobansi	7.75	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
A	*D. kaki cv.* Dogeunjosaeng	7.36	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
NA	*D. kaki* cv. Seochonjosaeng	4.23	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et

											al., 2007; Veberic et al., 2007)
A	*D. kaki* cv. Mopan	0.32	NF	NF	NF	NF	NF	NF	NF	NF	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007)
NA	*D. kaki*. cv. Jiro	1.68	NF	NF	2.12	49	178	121	259	576	(Pu et al., 2013; Jang et al., 2011; Chen et al., 2007; Veberic et al., 2007; Giordani et al. 2011)
	Kiwifruit										
	Bingo	15.0	121	60.1	0.15	0.26	NF	NF	0.96	5.21	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	M1	19.3	117.8	NF	0.19	0.21	NF	NF	0.59	6.23	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	Anna	14.2	142	22	0.11	0.24	NF	NF	1.1	8.35	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	Weiki	10.2	202.4	7.2	0.07	0.57	NF	NF	2.47	14.65	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	Jumbo	11.1	190.5	70.2	0.08	1.03	NF	NF	1.51	11.11	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	Geneva	14.3	139	20.1	0.15	0.58	NF	NF	1.18	10.06	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	Hayward	6.0	84.5	NF	0.18	NF	NF	NF	NF	6.73	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
	Bidan	27.2	328.8	NF	0.97	NF	NF	NF	1.11	25.26	(Leontowicz et al., 2016; Park et al., 2011; Park et al.,

										2013)
Daheung	3.8	NF	NF	0.14	NF	NF	NF	NF	NF	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
Haenam	4.2	NF	NF	0.07	NF	NF	NF	NF	NF	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
Hort16A	6.4	NF	NF	NF	NF	NF	NF	NF	NF	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
SKK12	9.8	NF	NF	NF	NF	NF	NF	NF	NF	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)
Hwamei	8.8	NF	NF	NF	NF	NF	NF	NF	NF	(Leontowicz et al., 2016; Park et al., 2011; Park et al., 2013)

[a]DW: dry weight
[b]FW: fresh weight
NF: Not found

2.4. Functional properties of persimmon

Persimmon functional properties are related to its content in bioactive compounds, which exerts a protective role on hypercholesterolemia, diabetes, cancer, hypertension and some dermic disorders (Park et al., 2015). Such bioactivity is related with their chemical composition on specific nutrients, described in **Table 36**. In a wide sense, the functional properties of persimmon have been related with its antioxidant capacity. For example, a randomized controlled trial studied the plasmatic antioxidant capacity and urinary excretion of 8-isoprostane in individuals with a high intake of persimmon vinegar (Mure, Takeshita, Morioka & Arita, 2007). A significant increase in plasma antioxidant activity and reduced excretion of 8-isoprostane (an urinary biomarker of oxidative stress) were found.

Diabetes mellitus. Due to the large amount of antioxidants found in persimmon, it is able to prevent at some extent diabetes or decrease the oxidative damage caused by the free radicals released due to this condition (Yaqub et al., 2016). Additionally, pancreatic β-cells are more sensible to oxidative stress due to the lower amounts of antioxidants compare to other tissues (Prasath, Sundaram, & Subramanian, 2013). Moreover, its fiber content could help reducing appetite and blood sugar by trapping glucose in the intestine. A study to elucidate the role of proanthocyanidins found in persimmon peel on diabetes was performed in streptozotocin-induced diabetic rats (Lee, Kim, Cho, & Yokozawa, 2007). A protective modulation of hyperglycemia was obtained thanks to the modulation of glucose and protein glycation. In addition, lipid peroxidation in kidney and serum was decreased in comparison with the control group. Furthermore, reactive oxygen species blockage was higher in the proanthocyanidins group as well as the ratio reduced-gluthatione/oxidized-gluthatione. Finally, proanthocyanidins had a protective role in inflammation due to their activity regulating

the expression of some proinflammatory factors such as iNOS, COX-2, NF-κB p65, and IκB (Lee et al., 2007). According to some authors, following a supplemented diet in persimmon peel could reduce blood sugar, cholesterol and triglycerides in diabetics (Gorinstein et al., 2001).

Table 35. Individual phenolics found in persimmon and kiwifruit.

Phenolic compounds	**Persimmon**	**Kiwifruit**	**Ref.**
(+)-Catechin	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Anisic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Bergapten		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Caffeic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Caffeic acid-*O*-hexoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Caffeoylhexoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Caffeoyl-quinic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Chlorogenic acid	x	x	(Leotowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013; Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Cinnamoyl glucose		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Cirsimaritin		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
cis-*p*-Coumaroyl quinic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Cryptochlorogenic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Cyanidin-3-*O*-sambubioside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Di-O-caffeoylquinic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Epigallocatechin gallate		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Epigalocatechin	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Ferulic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Feruloylquinic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Gallic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Genistin		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Kaempferol-3-*O*-(acetyl-rhamnoside)-(1 → 6) -galactoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Kaempferol-3-*O*-(acetyl-rhamnoside)-(1 → 6)		x	(Leontowicz et al., 2016; Wojdyło et al.,

-glucoside			2017; Park et al., 2011; Park et al., 2013)
Keampferol-3-*O*-(acetyl)-glucoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Keampferol-3-*O*-galactoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Keampferol-3-*O*-glucoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Keampferol-3-*O*-rutinoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Methyl (epi)afzelechin-3-O-gallate		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Neochlorogenic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
n-Triacontanol		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
o-Phthalic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
p-Coumaric acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Phenylnaringenin		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Phloridzin	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
p-Hydroxybenzoic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Protocatechuic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Quercetin-3-*O*-(acetyl)-galactoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-(acetyl)-rutinoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-(acetyl-rhamnoside)-(1 → 6)-galactoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-(acetyl-rhamnoside)-(1 → 6)-glucoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-galactoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-glucoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-hexoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-rutinoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-rutinoside-7-*O*-glucoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quercetin-3-*O*-rutinoside-7-*O*-rhamnoside		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Quinic acid		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Rutin	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Sinenstin		x	(Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Syringic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)
Tangeretin		x	(Leontowicz et al., 2016; Wojdyło et al.,

trans-p-Coumaroyl quinic acid		x	2017; Park et al., 2011; Park et al., 2013) (Leontowicz et al., 2016; Wojdyło et al., 2017; Park et al., 2011; Park et al., 2013)
Vanillic acid	x		(Pu et al., 2013; Chen et al., 2007; Veberic et al., 2010)

On the other hand, persimmon fruit polyphenols (after extraction) were tested on rats and humans in order to observe the effect on postpandrial glucose levels (Kometani & Takemori, 2016). In this sense, persimmon polyphenols extract showed significantly lower blood sugar levels in the 10 subjects administered than in the placebo group. It has been suggested that inhibition of alpha amylase as well as reduction of absorption are behind this fact (Kometani & Takemori, 2016). Accordingly, it could be suggested that persimmon could help controlling postpandrial blood sugar levels and be part of the primary prevention in diabetes (Kometani & Takemori, 2016).

Prasath, Sundaram, & Subramanian, (2013) carried out an experiment in Male Albino Wistar rats to observe the effect of Fisetin, a flavonoid present in persimmon but also in strawberries, over diabetes. In this sense, rats were streptozotocin-diabetic induced and were administered with 10 mg of Fisetin/kg of body weight for 30 days. Rats treated with Fisetin showed a significant reduce in blood sugar levels as well as in glycosylated hemoglobin. NF-κB p65 unit in pancreas and IL-8β in plasma as well as nitric oxide (NO) also showed significantly reduced levels whereas insulin levels were increased. On the other hand, antioxidant status also improved in pancreas and plasma of diabetic rats. Fisetin showed radical scavenging capacity which could be of help in radical mediated pathological processes. Improved insulin levels could be due to pancreas protection exerted thanks to fisetin antioxidant capacity and therefore protection against oxidative stress derived from hyperglycemia. A general improvement in antioxidant status was observed in treated rats, enzymes levels (SOD, CAT, GPx, and GST) and lipid peroxides and hydroperoxydes. Additionally, the observed reduced

levels of NF-kB p65 unit along with IL-8b and NO shows a potentially anti-inflammatory action reducing pancreatic damage (Prasath, Sundaram, & Subramanian, 2013).

Table 36. Biological properties of bioactive compounds found in persimmon and kiwifruit.

Bioactive compound	Biological activity	Reference
Vitamin C	Antioxidant against free radicals and maintaining glutathione reduced	(Gregory, 2007)
Phenolic acids	Antioxidants and exhibit potential antifungal, antibacterial, anti-inflammatory, and anti-cancer activity	(Pu et al., 2013)
Flavonoids	Antioxidant capacity against free radicals and reduce risk of cancer. Examples found in bibliography: -**Rutin:** Flavonol that could protect against spatial memory impairment accompanying hippocampal pyramidal neuron loss. -**Quercetin**: Seems to participate in vitamin E recovery after acting as antioxidant. Moreover, quercetin could protect against inflammation, tumorogenesis and other damages that can occur to cell structures due to oxidation. -**Catechins**: Antioxidant, antiangiogenic, chemopreventive and anti cancer, induction/inhibition of some enzymes, antiinflammatory, obesity, diabetes, antimicrobial, antiviric, anticariogenic, anti-osteoporosis, anti-allergenic, photoprotective, antiestrogenic, neuroprotective.	(Yaqub et al., 2016; Park et al., 2015; Abuajah et al., 2015; Pu et al., 2013)
Tannins	Improve urinary tract health; Reduce risk of cardiovascular disease, antidiabetic, anti obesity	(Yaqub et al., 2016; Pu et al., 2013; Lee et al., 2007; Mure et al., 2007; Abuajah et al., 2015)
Phloridzin	Dihydrochalcone with potentially antidiabetic activity thanks to its ability to reduce glucose absorption in the small intestine through a competitive mechanism via sodium D-glucose cotransporter 1.	(Pu et al., 2013)
Carotenoids	Neutralization of free radicals (antioxidant), reduce risk of muscular degeneration, reduce risk of some cancer, cellular aging protection	(Yaqub et al., 2016; Rao and Rao, 2007; Abuajah et al., 2015)

Atherosclerosis and lipid metabolism is another condition closely related to oxidative stress. The oxidation of LDL initiates the atheromatous plaque, being the oxidative species in the vessels the main cause of this problem. Due to its high content in antioxidants, persimmon could be of help reducing or preventing LDL oxidation and thus the developing of atherosclerosis (Yaqub et al., 2016). In addition, tannins have shown ability to trap bile acids (Gato, Kadowaki, Hashimoto, Yokoyama, & Matsumoto, 2013) which could lead to lower cholesterol levels in plasma resulting in reduced cardiovascular disease risk. According to some authors, Wistar rats fed with an hypercholesterolemic diet enriched in persimmon (7%) had lower values of plasmatic lipids (cholesterol, triglycerides, LDL) after a 4-weeks period compared to control rats (Dembitsky et al., 2011). Persimmon is known to have a high amount of tannins, especially astringent cultivars. As said above, tannins have showed capacity to behave as fiber and trap bile acids which would be convenient in hypercholesterolemia treatment (Gato, Kadowaki, Hashimoto, Yokoyama, & Matsumoto, 2013). Accordingly, Gato, Kadowaki, Hashimoto, Yokoyama, & Matsumoto, (2013) performed a randomized control trial double-blinded (40 subjects) to shed some light into the hypocholesterolemic effect of persimmon tannins. The study lasted for 12 weeks and it was comprised of a placebo group, low-dose group (3 g three times per day) and high-dose group (5 g three times per day). These authors found that in both low- and high-dose groups total plasmatic cholesterol was significantly lower than in the placebo groups while low density lipoproteins levels were only lower in the high-dosed group. Accordingly, persimmon could be of used in hypercholesterolemic patients.

Antiatherogenic and antioxidant capacity has also been assessed in persimmon wine. The process of vinification is known to improve polyphenol preservation and make them more bioavailable (Suh et al., 2011). Accordingly, Suh et al. (2011)

compared persimmon wine against grape wine (Merlot) in order to expose their effects on hypercholesterolemic hamsters. In this sense, they observed as both wines improved total cholesterol, low density lipoprotein, triglycerides and glucose levels but with no significant difference among both types of wines. They also prove to protect endothelial function from damage related to such dyslipidemic status (Suh et al., 2011).

In addition, a reduced oxidative stress (lower oxidized LDL and lipid peroxides) was observed. These properties were attributed to the persimmon content in soluble fiber and polyphenols. In another study, rats fed with a hypercholesterolemic diet were separated into two groups: one supplemented with whole dry persimmon and the other with polyphenol free dry persimmon (Gorinstein et al., 2011). Both groups showed reduced levels of plasmatic lipids but only the whole persimmon group showed an improvement in the antioxidant status, proving that antioxidant properties are mostly due to the polyphenol content. In a different study, rats were fed with either a standard cholesterol-rich diet or the same diet supplemented with two persimmon cultivars for 47 days (Gorinstein et al., 2011). The results showed that that diet supplemented with persimmon (whatever the cultivar used) gave to lower lesions in aorta compared to the control group.

Obesity is a pathology characterized not only with fat accumulation (adipocyte hypertrophy) but also related with adipocyte hyperplasia, which in turn reveals preadipocytes differentiation. In this sense, it was stated that persimmon tannins have the potential to be an antiadipogenic bioactive compounds (Zou, Ge, Zhu, Xu, & Li, 2015). Persimmon tannins can inhibit *in vitro* 3T3-L1 preadipocyte differentiation and reduce the expression of adipogenic transcription factors such as PPAR-γ and C/EBP-α in the early stages of adipogenesis.

Inflammatory bowel disease. Direito et al. (2017) tested the anti-inflammatory effects of persimmon phenolic extracts over colitis by using mice with TNBS-induced colitis. The experiment lasted 4 days. Persimmon administration achieved several improvements in TNBS-induced colitis mice; visible injuries less spread and visible (ulcers), diarrhea less severe, mortality rate reduced, mucosal hemorrhage reduced and general inflammation status improvement in the colon. As possible mechanisims behind this finding, these authors found mainly two: cyclooxygenase 2 (COX-2) and nitric oxide synthesa (iNO) expression reduction. COX-2 overexpression is known to be realted with inflammatory bowel disease and also with colorectal cancer progression. Accordingly, colitis-induced mice showed increased levels of COX-2 whereas the administration of the persimmon phenolics led to a significant decrease. On the other hand, NO is now known to act have a role in inflammation and, accordingly, its production was found to be higher in colitis-induced mice. Persimmon administration also reduced the levels (Direito et al., 2017).

Cancer. Persimmon has been found to have protective effect against some types of cancer such as prostate, breast, oral carcinoma or lymphoid leukemia due to its high carotenoids content, which are known to regulate cell growth and differentiation (Yaqub et al., 2016). Direito et al. (2017) also carried out an *in vitro* experiment in HT-29 colon carcinoma cells to observe the possible antiproliferative effect of persimmon phenolics. They impaired cell proliferation and invasion suggesting a possible therapy via. Moreover, 28-oxoallobetulin , a compound isolated from persimmon calyx has proven to have cytotoxic effects on HT-29 colon cancer cells (Lee, Koo, & Park, 2014).

Moreover, aside from the scavenging activity mentioned above, fisetin has shown to have anti-cancer activity both *in vitro* and *in vivo* through interfering in signaling pathways related to cell survival, growth and proliferation (Syed, Adhami,

Khan, & Mukhtar, 2016). Fisetin has shown to decrease growth and proliferation of prostate cancer cells. It has also showed capacity to inhibit PI3K/Akt and mTOR pathways in non-small cells lung cancer which is important since both of them are some of the major signaling pathways implicated (Syed, Adhami, Khan, & Mukhtar, 2016). Fisetin was also negatively correlated with melanoma cells growth not only *in vitro* but also *in vivo.* It was observed fewer lung metastases in athymic mice treated with fisetin along with sorafenib than in mice treated only with sorafenib showing therefore anti-invasive effects (Pal et al., 2015).

The effects of persimmon have been also studied over human lymphoid leukemia Molt 4B cells. In this sense, a persimmon extract and some polyphenols were investigated (catechin, epicatechin, epigallocatechin and epicatechin gallate) (Achiwa, Hibasami, Katsuzaki, Imai, & komiya, 1997). They found that persimmon extract as well as epigallocatechin and epicatechin gallate inhibited the growth of these cells in a dose dependant manner. After 3 days of treatment they observe severe damage on the cells as DNA fragmentation. These findings could indicate a possible use for therapeutic purposes though as always this needs to be scale up to be able to extrapolate conclusions.

Additionally, as part as a case-control study about thyroid cancer in Korean women, it was found an inverse correlation among persimmon consumption and malignant as well as bening thyroid cancer risk (Jung, Kim, Tae, Kong, & Kim, 2013). This study included 111 cases of malignant cancer and 115 of bening cases.

3. Kiwifruit

3.1 Kiwifruit production

Kiwifruit world production is around 3447604 tons which suppose around 0.5% of total fruit production around the world. The main producer of kiwifruit is China with 1840000 tons and a 53.4% of global kiwifruit production. New Zealand is another important producer with 410746 tons and 12% of kiwifruit production. However Spain production is only 20881 tons, a 0.61% of world production. Asia is the main producer followed by Europe, Oceania, America and finally Africa with an almost inexistent production (Food and Agricultural Organization of the United Nations 2017 http://www.fao.org/faostat/en/#data/QC). In New Zealand, kiwifruit production is a really important crop since it supposes a 29% of fruit production being therefore an important factor for their economy. For other countries, kiwifruit is not one of the main crops with percentages below one.

In the last three decades, kiwifruit production has grown greatly. World production has become around 3.5 times greater. This tendency shows how kiwifruit is becoming more and more popular, probably being introduced as an exotic fruit but also due to their health benefits. Accordingly, world production value has also grown becoming more than twice the value in 1990.

In Spain, in 2015 kiwifruit production was around 21000 tons. In Spain, kiwifruit production is mostly focused on the north of Spain, Asturias and Galicia though Basque Country, Navarra, Catalonia and Comunidad Valenciana also contribute with kiwifruit production. In 2015, the surface dedicated to this crop was around 1200 ha and exportations were close to 20000 tons. It is mostly commercialized between October and March, though there are years where commercialization starts in September

and ends in April (MAPAMA, Spanish Ministry of Agriculture, Fisheries, Nutrition and Environment).

In Spain, kiwifruit production has evolved irregularly in the las three decades with increases in production but also decreases. However, overall, kiwifruit production has grown becoming around 4 times greater in the last three decades. As it happened with production, the value has also grown greatly and has passed from less than 5 to around 25 million dollars in the last three decades (MAPAMA, Spanish Ministry of Agriculture, Fisheries, Nutrition and Environment).

3.2 Composition and nutritional value of kiwifruit

Kiwifruit (*Actinidia sp.)* is an important source of vitamin C, also with high levels of fiber, potassium, vitamin E and folic acid **(Table 33)**. However, the nutritional composition of kiwifruit, especially regarding vitamin C, can vary depending on the cultivar. Usually, “SunGold” and “Sweet Green” cultivars have the highest contents in vitamin C (Sivakumaran, Huffman, Sivakumaran, & Drummond, 2018). Thus, one kiwifruit can fulfill 85% or vitamin C requirements. During kiwifruit ripeness the acid content decreases and aromatic compounds appear (Perera, 1998). Moreover, sugars such as glucose, fructose and sucrose increase, improving the taste of this fruit (Park et al., 2006). Glucose and fructose are the main sugars in kiwifruit, but some cultivars like “hardy kiwifruit” are richer in sucrose (Latocha, Łata, & Stasiak, 2015). Among the organic acids, citric and quinic acids are the most abundant (Latocha et al., 2015).

There are several species of kiwifruit, being the most popular *A. deliciosa* (Hayward), *A. chinensis* and *A. eriantha* (*Bidan*). However, there are also other species that are grown in colder climates such as *A. arguta* (Hardy kiwifruit), *A. kolomikta* and *A. purpurea.*(Leontowicz et al., 2016). *A. arguta* includes several different cultivars

such as *Bingo* (hybrid between *A. arguta* and *A. purpurea)*, *M1* (select arguta), *Anna*, *Weiki*, *Jumbo* and *Geneva* (Leontowicz et al., 2016).

Kiwifruit is considered an important source of antioxidants species (**Table 36**) (Wojdyło, Nowicka, Oszmiański, & Golis, 2017) due to its content in bioactive compounds like polyphenols (**Table 35**) and vitamin C (**Table 34**), existing a positive correlation among both chemical species (Leontowicz et al., 2016). Vitamin C is an important compound in kiwifruit since its contribution to antioxidant capacity is equal to that of polyphenols (Park et al., 2011). In addition, chlorophyls are also important antioxidant compounds, which play a role on the characteristic green color of kiwifruit. Chlorophyls are also important as regulators of inflammatory processes, microbial infections, ageing and atherosclerosis (Leontowicz et al., 2016). Moreover, other pigments like lutein, zeaxantine and β-carotene are present in kiwifruit. These compounds are going to contribute also to their functional properties, especially to those related with antioxidant capacity such as neutralization of free radicals, reduction of muscular degeneration, cancer risk or cellular aging (Abuajah, Ogbonna, & Osuji, 2015).

Another important contribution of kiwifruit to diet is related with its fiber content (**Table 37**) (Wojdyło et al., 2017). Total dietary fiber ranges from 9 to 27% of dry weight, being the highest fraction insoluble fiber (65-90% to total fiber). Insoluble fiber can trap glucose, cholesterol, bile acids and dietetic carcinogens, reducing their absorption and improving conditions such as diabetes, dyslipidemia or hypercholesterolemia (Abellan et al., 2010). On the other hand, soluble fiber increases satiety, slow down gastric emptying and stimulates intestinal motility, being used as treatment for constipation. In addition, soluble fiber can be fermented by the gut microbiota, producing butyric acid that lowers colorectal cancer (Wojdyło et al., 2017).

The amount of all these bioactive compounds is very variable among species and cultivars. Not only they vary among species but also, according to several authors, these quantities can depend on fertilization conditions, weather, etc. (Park et al., 2013; Park et al., 2014). For example, a comparison among organic and conventionally grown cultivars showed that kiwifruit grown under organic conditions had a higher antioxidant capacity, although such it was not statistically significant in all the cultivars (Park et al., 2013).

Table 37. Fiber content of different kiwifruit cultivars (Leontowicz et al., 2016; Park et al., 2011; United States Department of Agriculture, Agricultural Research Service, Food Composition Database, 2017).

	TDF[a], % DW[b]	**IDF[c], %DW**	**SDF[d], % DW**	**IDF/SDF**
Bingo	25.86	20.43	5.43	3.76
M1	27.11	21.40	5.71	3.75
Anna	24.75	18.26	6.50	2.81
Weiki	24.58	17.61	6.97	2.53
Jumbo	19.78	14.02	5.76	2.43
Geneva	19.86	14.09	5.77	2.44
Hayward	9.39	6.08	3.31	2.01
Bidan	9.95	7.01	2.93	2.38
Daheung	8.05	5.64	2.41	2.34
Haenam	8.04	5.63	2.41	2.34
***Actinidia deliciosa* (mean)**	11.11	8.33	2.78	2.99
***Actinidia chinensis* (mean)**	7.40	5.55	1.85	3

[a]TDF: total dietary fiber
[a]DW: dry weight
[c]IDF: insoluble dietary fiber
[a]*SDF: soluble dietary fiber*

3.3 Effect of genotype on antioxidant capacity and bioactive compounds of kiwifruit

The antioxidant capacity and bioactive compounds content of several cultivars of kiwifruit have been described elsewere: *Bidan*, *M1*, *Bingo*, *Geneva*, *Anna*, *Jumbo*, *Weiki*, *SKK12*, *Hwamei*, *Hort16A*, *Hayward*, *Haenam* and *Daheung* (Park et al., 2011; Drzewiecki et al., 2016; Leontowicz et al., 2016). These papers studied the antioxidant capacity of kiwifruit with several antioxidant methdos such as FRAP, ABTS, DPPH and CUPRAC. In the FRAP method, *Bidan* cultivar gave the highest antioxidant value, which was almost three times more antioxidant than the second (*Haenam*). In general, the reducing capacity measured by the FRAP method ranged from 7 to 63 μmol Trolox/ g of fresh weight; the lowest activity corresponded to *Hort16A* and *Hayward* cultivars. The ABTS method showed that the *M1* cultivar was the most antioxidant cultivar, with 112 μmol Trolox/g of fresh weight. On the other hand, the lowest value corresponded to the *Hayward* cultivar, with 17 μmol Trolox/g of fresh weight. In the case of DPPH method, *Bidan* again was the most antioxidant cultivar with 84 μmol Trolox/g of fresh weight, being the lowest value that of the *Hayward* cultivar, with 7 μmol Trolox/g of fresh weight. Finally, the CUPRAC assay showed that the *M1* and *Bidan* cultivars were the most antioxidant, with 105 μmol Trolox/g of fresh weight. Again, the *Hayward* cultivar showed the lowest value with 15 μmol Trolox/g of fresh weight. In conclusion, the antioxidant capacity of kiwifruit decreases in the following order: *Bidan* >> *Bingo* > *Anna* > *Geneva* > *Jumbo* > *Weiki* > *Haenam* > *SKK12* > *Hwamei* > *Daheung* > *Hort16A* > *Hayward*.

As stated above, ascorbic acid and polyphenols are the most important chemical species contributing to the antioxidant capacity of kiwifruit (Park et al., 2011). Thus, the knowledge about the content of such bioactive compounds is essential in order to give clear dietetic recommendations. According to the scientific literature (**Table 34**), the

concentration of vitamin C is kiwifruit ranges from 0.07 to 0.97 mg/ g of fresh weight. The highest amounts of vitamin C are present in the *Bidan* cultivar, followed by the *Bingo*, *M1*, *Geneva* and *Hayward* genotypes. However, *Anna*, *Weiki*, *Jumbo*, *Daheung* and *Haenam* varieties had the lowest values.

Accordingly to the findings about antioxidant capacity and vitamin C, the kiwifruit cultivar with the highest content of total phenolics is the *Bidan* genotype with 27 mg gallic acid/g of dry weight. On the contrary, the *Daheung* cultivar showed the lowest content, with 4 mg gallic acid/g of dry weight. In general, polyphenols content decreases on kiwifruit cultivars in the following order: *Bidan* > *M1* > *Bingo* > *Geneva* > *Anna* > *Jumbo* > *Weiki* > *SKK12* > *Hwamei* > *Hort16A* > *Hayward* > *Haenam* > *Daheung*. Finally, statistically significant correlations were found among the four antioxidant methods and total phenolics, reinforcing the idea that these chemical species are probably the most important compounds for the antioxidant capacity of any kiwifruit cultivar (Park et al., 2011; Park et al., 2013; Leontowicz et al., 2016).

Flavonoids, flavanols and tannins have been also reported (**Table 35**), since they are an important fraction of total polyphenols. In the case of flavonoids, the highest content was reported for the *Daheung* cultivar, with 5 mg catechin equivalents/g of dry weight. On the contrary, the lowest value was found in the *Bidan* genotype, with 0.3 mg catechin equivalents/g of dry weight. In the case of flavanols, the content ranged from 17 to 0.6 mg catechin equivalents/g of dry weight for the *Hort16A* and *Bingo* cultivars. The values of tannins were distributed again between *Bingo* and *Hort16A* cultivars, with values ranging from 9 to 1 mg catechin equivalents/g of dry weight, respectively. From these data it could be deduced that flavonoids, flavanols and tannins are not the main contributors to antioxidant capacity in some kiwifruit cultivars such as *Bidan* or *M1*. These genotypes showed a high antioxidant capacity but the amount of different

polyphenols classes were quite low. Thus, in such cultivars the antioxidant capacity could be related more specifically with other chemical species such as vitamin C, carotenoids, chlorophyll, xanthophylls, anthocyanins, etc, (Rao & Rao, 2007).

3.4 Functional properties of kiwifruit

Due to the large amount of bioactive compounds found in kiwifruit, some functional properties derived from its intake could be expected. Among them, effects over platelet aggregation, plasma antioxidant capacity, plasmatic lipids, hypertension, inflammation or insulin resistance have been demonstrated among others.

Platelet aggregation. A randomized cross-over study with healthy volunteers was carried out in order to unravel whether kiwifruit consumption could have any effect on platelet aggregation (Duttaroy & Jørgensen, 2004). Kiwifruit was able to inhibit ADP-induced aggregation in a dose dependent manner and reducing collagen-induced aggregation. In addition, kiwifruit also inhibited arachidonic-induced aggregation. Such platelet inhibition could be related with a decrease on TXB2 synthesys (Dizdarevic et al., 2014). In this sense, a margarine supplemented with kiwifruit extracts resulted in decreased platelet aggregation just two hours after consumption (Dizdarevic et al., 2014).

Plasma antioxidant capacity/ROS protection. Kiwifruit consumption also resulted in an increased antioxidant capacity of plasma, measured with the FRAP method of volunteers consuming 2-3 kiwifruits per day (Duttaroy & Jørgensen, 2004). Moreover, plasmatic levels of vitamin C and E also increased significantly after 4 and 8 weeks of kiwifruit consumption while decreasing LDL oxidation (Chang & Liu, 2009). Kiwifruit has also proven its ability to reduce DNA damage due to oxidation. In this sense, kiwifruit juice administrated to individuals demonstrated to reduce significantly

DNA damage in lymphocytes (Collins, Horská, Hotten, Riddoch, & Collins, 2001). Since kiwifruit juice was more active than vitamin C supplementation, it was suggested that part of the induced antioxidant protection of kiwifruit should come not only from ascorbic acid but also from other compounds such as polyphenols.

Apart from vitamin C and polyphenols, the antioxidant capacity of kiwifruit is also related with the presence of an antioxidant peptide called kissper, a 39 residue peptide with pore-forming ability (Ciardiello et al., 2008). The antioxidant potential of kissper peptide on intestinal mucosa inflammation was studied by using Caco-2 cells and colonic mucosa from healthy and Chron´s disease individuals (Ciacci et al., 2014). Kissper peptide was able to reduce or modulate the damage induced by ROS derived from LPS challenge in Caco-2 cells as well as in CD mucosa. Moreover, kissper was also able to increase cell viability. In addition, the kissper peptide has showed ability to reduce TG2 levels in Caco-2 cells and colonic mucosa. This is an important result since TG2 is a protein closely involved in mitochondrial functionality, and its expression is considered a link between oxidative stress and inflammation (Ciacci et al., 2014). Finally, some authors found statistical association between the antioxidant capacity of kiwifruit and its ability to reduce proliferation in two cell lines of human carcinoma (lung and stomach) (Park et al., 2012).

Plasmatic lipids. Kiwifruit also play a role on the levels of plasmatic lipids. A human intervention during 28 days (daily intake of 2 kiwifruits) showed no effect on cholesterol concentration (LDL, HDL or total cholesterol) but a decrease on plasmatic triglycerides (Duttaroy & Jørgensen, 2004). When the study was repeated with hyperlipidemic volunteers, there was not any difference in total cholesterol, triglycerides, or LDL cholesterol (Chang & Liu, 2009). However, HDL-cholesterol increased in a significant manner as well as the ratio HDL-C/LDL-C and HDL-C/TC. A

different cross-sectional study called EVIDENT investigated in healthy individuals whether consuming one or more kiwifruit units per week had any effect on plasmatic lipids, fibrinogen and insulin sensitivity (Recio-Rodriguez et al., 2015). Individuals who consumed at least one kiwifruit per week had significantly higher values of HDL-c and lower triglycerides, fibrinogen values insulin resistance was found. No differences were detected in total cholesterol and LDL-c. Accordingly to all these findings, kiwifruit might have a beneficial impact in inflammatory processes, atherogeneis and thrombogenesis, exerting also hypolipidemic activity and therefore being beneficial in diabetes development. Interestingly, these findings were only found for kiwifruit and not for other fruits according to the EVIDENT study (Recio-Rodriguez et al., 2015).

Antihypertensive activity. According to some authors, kiwifruit extracts showed inhibitory activity of human and rabbit serum angiotensin-converting enzyme (ACE) in a dose-dependent manner, whereas orange extracts did not showed any activity (Dizdarevic et al., 2014).

Inflammation. The kissper peptide was also able to control the release and expression of proinflammatory cytokines TNF-α, ICAM-1 and COX-2 in a mixed *in vivo-in vitro* study (Ciacci et al., 2014).

Insulin resistance. Many metabolic effects of insulin are mediated by signaling pathways involving the phosphorylation of insulin receptor substrate proteins, and the activation of PI3K, Akt and protein kinase B. The activated Akt can enter the cytoplasm leading to phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3). Glycogen synthase (GS) is the substrate of GSK-3 which catalyzes glycogen synthesis. Phosphorylation of GS by GSK-3 inhibits glycogen synthesis; therefore the inactivation of GSK-3 by Akt promotes glucose storage as glycogen. Defects at any one of these factors may contribute to insulin resistance states (Kim, Kim, Kim, & Lee, 2013). In

this sense, Kiwifruit extracts were able to increase the phosphorylation of GSK-3/Atk, which in turn reduces the phosphorylation of GS. Moreover, transportation of GS to the membrane was also increased. All together indicates that kiwifruit improves insulin resistance through this pathway.

Large intestine health. Kiwifruit has an important content of fiber, which is helpful to maintain large intestine health as stated above (Wojdyło et al., 2017). A study to unravel the effect of kiwifruit in large intestine health and functionality was carried out in Sprague-Dawley rats with a diet enriched in kiwifruit (10%) (Paturi, Butts, Bentley-Hewitt, & Ansell, 2014). After a 6-weeks treatment, the production of short chain fatty acids increased, colonic barrier function improved and the microbial populations of *Lachnospiraceae* increased. All this suggests that kiwifruit could be beneficial for gut microbiota and in extension for large intestine health (Paturi et al., 2014). Kiwifruit pectin (MonoK) was also tested in order to unravel their potential healthy properties for the gut (Parkar et al., 2010). This pectin was compared with citrus pectin, inulin and guar gum. Kiwifruit pectin was the most efficient fiber in increasing the adhesion of *Lactobacillus rhamnosus* and decreasing the adhesion of *Salmonella typhimorium* to colonic cells (Parkar et al., 2010).

Effects on bone health. Kiwifruit effects on bone resorption were tested in ovariectomized mice (Katsumata et al., 2015). Kiwifruit could have a protective effect against bone resorption through reducing the expression of the receptor activator of NF-kβ ligand (RANKL) mRNA in ovariectomized mice. A different study was conducted in order to study the effect of combining daidzein and kiwifruit on bone density and equol production in ovariectomized rats (Tousen et al., 2014). According to this study, kiwifruit could have a small effect in reducing bone loss due to oestrogen deficiency, although equol production is not affected.

Insomnia. Kiwifruit was tested in a randomized control trial focused on whether this fruit has any effect in reducing insomnia (Nødtvedt, Hansen, Bjorvatn, & Pallesen, 2017). A slight association between kiwifruit consumption before going to sleep (1 hour before) and a relative improvement in two out of 12 variables studied was found.

4. Conclusions

This review includes the composition and nutritional value of persimmon and kiwifruit in general, although it describes deeper the presence of bioactive compounds such as polyphenols, tannins, carotenoids, vitamin C, etc. In addition, the functional properties of these fruits (i.e. inhibition of platelet aggregation, ROS protection, decrease of plasmatic lipids and cholesterol, antihypertensive activity, increase on insulin sensitivity, etc.) and effects on different pathologies is described. Among such health benefits, both persimmon and kiwifruit possess high antioxidant potential due to the high amounts of bioactive compounds. In this sense, the effect of genotype and ripening stage play an important role on antioxidant capacity and the content of bioactive compounds. Taking into account such nutritional profile and large content of bioactive compounds, it can be concluded that the supplementation of diets with the reviewed fruits positively affects human health.

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Effect of home cooking on the antioxidant capacity of vegetables: relationship with Maillard reaction indicators

En este trabajo se estudió el efecto de diferentes tratamiento culinarios sobre la capacidad antioxidante de los vegetales mas frecuentemente consumidos en España, tras ser digeridos y fermentados *in vitro.* Así se determinó el efecto del tratamiento térmico sobre dicha capacidad antioxidante. El contenido de este paper hace que esté estrechamente relacionado con la presente tesis doctoral.

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Effect of home cooking on the antioxidant capacity of vegetables: relationship with Maillard reaction indicators

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ABSTRACT

Vegetables are health-promoting foods due to their content on a wide range of phytochemicals, being involved in antioxidant protection. However, such bioactivity can be modified during cooking and also along the digestion-fermentation process. Thus, the aim of the paper is to establish a relation among the type of processing (raw, boiled, steamed, grilled, roasted, and fried), time of processing (raw, usual time and well-done), antioxidant capacity and the development of the Maillard reaction (measured though the analysis of furosine and HMF) of 23 widely consumed vegetables. Antioxidant capacity was measured with three methods ($TEAC_{ABTS}$, $TEAC_{FRAP}$, $TEAC_{OH}$) after submitting vegetables to an *in vitro* digestion followed by and *in vitro* fermentation process. Furosine and HMF were useful indicators to control both cooking time and heat intensity of common vegetables, being correlated with antioxidant capacity. Those samples cooked with aggressive techniques (frying, grilling or breading) showed the higher antioxidant values.

KEYWORDS: vegetables, cooking, Maillard reaction, furosine, HMF, antioxidant capacity.

1. Introduction

Vegetables and fruit consumption is strongly linked to a lower risk of several chronic diseases and according to dietary guidelines (Liu, 2013). Vegetables provide a wide range of phytochemicals involved in antioxidant protection (Liu, 2013; Abuajah, Ogbonna & Osuji, 2015). All these compounds have been positively related to chronic diseases such as cancer, diabetes type II, atherosclerosis, etc. (Abuajah et al., 2015).

Most vegetables are consumed after being cooked under different conditions, involving water or oil mediums and a wide range of temperatures. Thus, the heat treatment will become very important since it will affect directly the final composition of vegetables. Several cooking methods are widely used in vegetables depending on the temperature (Bello, 1999); boiling and steaming are less aggressive, compared to frying, roasting or grilling. On the other hand, the cooking medium (water or oil) is also important since the use of boiling water could decrease the concentration of certain hydrosoluble compounds, whereas the use of oil can increase their antioxidant capacity due to an improvement of the phytochemicals content (Ramírez-Anaya, Samaniego-Sánchez, Castañeda-Saucedo, Villalón-Mir, & de la Serrana, 2015). However, thermal treatment can also improve bioactive compounds availability by braking down cell structures (Miglio, Chiavaro, Visconti, Fogliano & Pellegrini, 2008). Accordingly, the phytochemical composition of vegetables changes during culinary processing (Podsędek, 2007; Bunea et al., 2008; Soares, Carrascosa & Raposo, 2017).

The Maillard reaction is also a source of compositional changes in vegetables during cooking, since the carbonyl group of a reducing sugar or other molecule with carbonyl groups (vitamin C, oxidized lipids, etc.) reacts with the free amino group of a protein, amino acid or peptide (de la Cueva, Seiquer, Mesías, Rufián-Henares, & Delgado-Andrade, 2017). Both processing time and temperature are important variables

to take into account for the development of the Maillard reaction. This reaction is favored at temperatures above 50ºC and long cooking times (Rufián-Henares & de la Cueva, 2008). Thus, aside from the cooking technique used, it is important to establish the effect of processing time. Therefore, an aggressive long cooking procedure could result in a loss of bioactive compounds (Rufián-Henares, Guerra-Hernández, & García-Villanova, 2013). In order to control the development of the Maillard reaction, different chemical indicators have been used during thermal processing in food manufacturing companies (Rada-Mendoza, García-Baños, Villamiel, & Olano, 2004). In this sense, furosine is a product of the acidic hydrolysis of Amadori compounds, and has been used as an indicator of the initial stages of Maillard reaction, where organoleptic changes are still not present. On the other hand, 5-hydroxymethylfurfural (HMF) is produced during intermediate stages of Maillard reaction and therefore used as an indicator of middle advanced Maillard reaction (Rufián-Henares et al. (2008). Accordingly, furosine and HMF have been used for evaluating the intensity of heating in several vegetable products such as onion, garlic, potato, carrots, among others (Rufián-Henares et al. 2008; Rufián-Henares et al., 2013). There are also several papers which study the antioxidant properties of Maillard reaction products from several sources (Dittrich et al., 2009; Carvalho, Correia, Lopes & Guido, 2014; Pastoriza & Rufián-Henares, 2014; de la Cueva et al., 2017). In this sense, vegetable processing could increase antioxidant capacity even after losing some phytochemicals due to either loss in boiling water or to heat during processing.

Therefore, the main objective of this paper is to establish a relation among the type of processing (raw, boiled, steamed, grilled, roasted, and fried), time of processing (raw, usual time and well-done), antioxidant capacity and the development of the Maillard reaction (measured through the analysis of furosine and HMF) of 23 widely

consumed vegetables. Antioxidant capacity will be measured after submitting vegetables to an *in vitro* digestion followed by and *in vitro* fermentation process, in order to mimic as much as possible physiological conditions. The main novelty of this work is not the relation among antioxidant capacity and the cooking technique, but the effect that thermal damage (monitored through furosine and HMF determination) has over antioxidant capacity. Another novelty is the use of an *in vitro* digestion-fermentation process to better simulate the physiological extraction and transformation of bioactive compounds. This would provide valuable information about how culinary treatments and their degree of intensity can affect antioxidants available at the small and large intestine.

2. Materials and methods

2.1. Chemicals

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), potassium persulphate, sodium hydroxide, hydrochloric acid, iron (III) chloride hexahydrate, sodium acetate, potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide, resazurin, salivary alpha-amylase, pepsin from porcine, bile acids (porcine bile extract), ethanol, hydrochloric acid, acetonitrile, and HMF standard were from Sigma-Aldrich (Darmstadt, Germany). Pancreatin from porcine pancreas was purchased from Alpha Aesar (United Kingdom). Furosine standard from Neosystem Laboratories (Strasbourg, France).

2.2. Vegetable samples and cooking conditions

Fresh chard (*Beta vulgaris* subsp. *Vulgaris)*, garlic *(Allium sativum)*, artichoke (*Cynara cardunculus* var. *scolymus*), eggplant (*Solanum melongena*), zucchini (*Cucurbita pepo*), pumpkin (*Curcubita moschata*), onion (*Allium cepa* L.), mushroom (*Agaricus bisporus*), cabbage (*Brassica olaracea* var. *gemmifera*), cauliflower (*Brassica olaracea* var. *botrytis*), asparagus (*Asparagus oficinalis*), spinach (*Spinacia oleracea*), peas (*Pisum sativum*), broad beans (*Vicia faba*), beans (*Phaseolus vulgaris*), lettuce (*Lactuca sativa*), potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*), parsley (*Petroselinum crispum*), red pepper (*Capsicum annuum*), leek (*Allium ampeloprasum* var. *porrum*), tomato (*Solanum lycopersicum*) and carrot (*Daucus* carota) were purchased in local markets. Vegetables were washed and, peeled when applicable. Each one of them was submitted to the usual culinary processes common for each vegetable (**Table 38**) following traditional recipes. Vegetables had to be cut in different sizes to achieve the same texture in the same cooking time. Carrots, zucchini, pumpkin, onion, red pepper, asparagus, and leek were cut in fine dices (brunoise cutting). Green leaf vegetables were shredded (chiffonade cutting). Eggplant was cut in sticks (julienne cutting). Potatos, tomato, cucumber, were cut to obtain broad an thin slices (parallel cutting). Garlic was crushed, and parsley was minced. Additionally, for each culinary process, two processing times were applied; the usual one (normal; N) and a longer processing time consisting on a 50% more of exposure (well done; WD). The culinary treatments chosen were steaming, water boiling, grilling, roasting, frying and breading. Extra virgin olive oil (EVOO) was used as medium for grilling and frying. Steaming and boiling were carried out at 100ºC for 20 minutes (N) and 30 minutes (WD) with a proportion water:vegetable in the case of boiling of 5:1. Grilling was carried out at 220-250ºC for 3 minutes on each side (N) and 4.5 minutes each side (WD)

with a proportion oil:vegetable of 0.5:1. Roasting was carried out at 180ºC for 10 minutes (N) and 15 minutes (WD). Frying and breading were carried out at 180ºC for 8 minutes (N) and 12 minutes (WD) with a proportion oil:vegetable of 5:1. Breaded vegetables were covered with flour prior to be fried. The same amount of flour was used for each vegetable. The utensils used for sample preparation were the following: stainless steel spoons, forks, and knives, frying pan, saucepan, household size steam cooking machine and a portable oven (1500W). All these utensils were purchased from Centro Hogar Sanchez (Granada, Spain). Cooking times and medium proportions were acquired from Ramírez-Anaya et al. (2015) and adapted to our own equipment and laboratory conditions. Samples were homogenized and stored under nitrogen atmosphere at -80ºC in order to avoid oxidations. All analyses were carried out in triplicate.

Table 38. Culinary treatment and degree of intensity for each vegetable studied.

Vegetable	Culinary treatment	Degree of intensity
Chard	Boiled	N
Chard	Boiled	WD
Chard	Steamed	N
Chard	Steamed	WD
Garlic	Roasted	N
Garlic	Roasted	WD
Garlic	Raw	R
Garlic	Fried	N
Garlic	Fried	WD
Garlic	Grilled	N

Garlic	Grilled	WD
Artichoke	Boiled	N
Artichoke	Boiled	WD
Artichoke	Fried	N
Artichoke	Fried	WD
Artichoke	Steamed	WD
Artichoke	Steamed	N
Eggplant	Roasted	N
Eggplant	Roasted	WD
Eggplant	Fried	N
Eggplant	Fried	WD
Eggplant	Grilled	N
Eggplant	Grilled	WD
Eggplant	Breaded	N
Eggplant	Breaded	WD
Zucchini	Roasted	N
Zucchini	Roasted	WD
Zucchini	Boiled	N
Zucchini	Boiled	WD
Zucchini	Grilled	N
Zucchini	Grilled	WD
Zucchini	Steamed	N
Zucchini	Steamed	WD

Pumpkin	Roasted	N
Pumpkin	Roasted	WD
Pumpkin	Boiled	N
Pumpkin	Boiled	WD
Pumpkin	Grilled	N
Pumpkin	Grilled	WD
Pumpkin	Steamed	N
Pumpkin	Steamed	WD
Onion	Roasted	N
Onion	Roasted	WD
Onion	Raw	R
Onion	Fried	N
Onion	Fried	WD
Onion	Grilled	N
Onion	Grilled	WD
Mushroom	Roasted	N
Mushroom	Roasted	WD
Mushroom	Grilled	N
Mushroom	Grilled	WD
Cabbage	Boiled	N
Cabbage	Boiled	WD
Cabbage	Raw	R
Cabbage	Grilled	N

Cabbage	Grilled	WD
Cabbage	Steamed	N
Cabbage	Steamed	WD
Cauliflower	Boiled	N
Cauliflower	Boiled	WD
Cauliflower	Breaded	N
Cauliflower	Breaded	WD
Cauliflower	Steamed	N
Cauliflower	Steamed	WD
Asparagus	Roasted	N
Asparagus	Roasted	WD
Asparagus	Boiled	N
Asparagus	Boiled	WD
Asparagus	Grilled	N
Asparagus	Grilled	WD
Asparagus	Steamed	N
Asparagus	Steamed	WD
Spinach	Boiled	N
Spinach	Boiled	WD
Spinach	Steamed	N
Spinach	Steamed	WD
Peas	Boiled	N
Peas	Boiled	WD

Peas	Raw	R
Peas	Grilled	N
Peas	Grilled	WD
Peas	Steamed	N
Peas	Steamed	WD
Broad beans	Raw	R
Broad beans	Grilled	N
Broad beans	Grilled	WD
Beans	Boiled	N
Beans	Boiled	WD
Beans	Steamed	N
Beans	Steamed	WD
Lettuce	Raw	R
Potato	Roasted	N
Potato	Roasted	WD
Potato	Boiled	N
Potato	Boiled	WD
Potato	Fried	N
Potato	Fried	WD
Potato	Steamed	N
Potato	Steamed	WD
Cucumber	Raw	R
Parsley	Raw	R

Red pepper	Roasted	N
Red pepper	Roasted	WD
Red pepper	Raw	R
Red pepper	Fried	N
Red pepper	Fried	WD
Red pepper	Grilled	N
Red pepper	Grilled	WD
Leek	Boiled	N
Leek	Boiled	WD
Leek	Steamed	N
Leek	Steamed	WD
Tomato	Roasted	N
Tomato	Roasted	WD
Tomato	Raw	R
Tomato	Grilled	N
Tomato	Grilled	WD
Carrot	Roasted	N
Carrot	Roasted	WD
Carrot	Boiled	N
Carrot	Boiled	WD
Carrot	Raw	R
Carrot	Grilled	N
Carrot	Grilled	WD

Carrot	Steamed	N
Carrot	Steamed	WD

2.3. In vitro digestion

All vegetables were subjected to an *in vitro* digestion process followed by an *in vitro* fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out according to the protocol described by Pérez-Burillo, Rufián-Henares, & Pastoriza (2018a). The gastrointestinal *in vitro* digestion was composed of an oral phase (5 minutes at 37ºC with alpha-amylase 75 U/mL, pH 7.0), a gastric phase (2 hours at 37ºC with pepsin 2000U/mL at pH 3.0) and an intestinal phase (2 hours at 37ºC with pancreatin 13.37 mg/mL at pH 7.0).

2.4. In vitro fermentation

The *in vitro* fermentation was carried out according to the protocol described by Pérez-Burillo et al. (2018a). *In vitro* fermentation was carried out using faecal samples from three healthy donors (not taking antibiotics, people with body mass index within the “normal weight range”, mean Body Mass Index = 21.3). The solid residue obtained after *in vitro* gastrointestinal digestion plus 10% of the digestion supernatant was fermented (500 mg).

After *in vitro* gastrointestinal digestion and *in vitro* fermentation three different fractions were obtained: digestion supernatant (fraction available for absorption at the small intestine), fermentation supernatant (fraction available for absorption at the large intestine) and fermentation solid residue (fraction not available for absorption and excreted with feces).

2.5. Antioxidant capacity

The antioxidant capacity of three fractions was assessed: the supernatant obtained after gastrointestinal digestion, the supernatant derived from fermentation and the solid residue remaining after fermentation. Three different methods were used to analyse the antioxidant capacity of foods. All antioxidant capacity values for all three methods were corrected taking into account their respective blanks (enzymes, chemicals and inoculum).

$TEAC_{OH}$ method: In this method, performed to unravel the scavenging activity against OH· radicals, carmin indigo was used as the redox indicator, following the method of Pérez-Burillo, Rufián-Henares & Pastoriza (2018b). It is carried out at physiological pH (7.24). The results obtained are expressed as mmol Trolox equivalents per kg of sample.

$TEAC_{ABTS}$ assay: This method measures the activity of the samples against ABTS· radicals. The ABTS assay was conducted as described by Re et al., (1999) with slight modifications. Results are expressed as mmol equivalents of Trolox per kg of sample.

$TEAC_{FRAP}$ assay: The ferric reducing ability of the extract of each sample was estimated following the procedure described by Benzie and Strain (1996) with minor modifications. Results are expressed as mmol equivalents of Trolox per kg of sample (Benzie & Strain, 1996).

2.6. Furosine assay

Furosine determination was performed following the method described by Rufián-Henares, Guerra-Hernández & García-Villanova, (2013). The analysis was performed in duplicate and the results are expressed as mg of furosine/kg of sample.

2.7. HMF assay

HMF determination was performed following the method described by Rufián-Henares et al. (2008). The analysis was performed in duplicate and the results are expressed as mg of HMF/kg of sample.

2.8. Sugars and protein content

Sugars and protein content of all 23 vegetables were taken from the food composition database of the United States Department of Agriculture (USDA), available at https://ndb.nal.usda.gov/ndb/search/list?home=true.

2.9 Statistical analysis

The statistical significance of the data was tested by one-way analysis of variance (ANOVA), followed by the Duncan test to compare the means that showed significant variation ($p < 0.05$). As factors for ANOVA we used type of cooking (frying, grilling, roasting, boiling, steaming, breadind, and raw) and intensity of the cooking (raw, normal time or extended time [WD]). Statistical analysis was performed using raw vegetables as the reference group. Pearson correlation coefficient was calculated to show the lineal relation between antioxidant capacity and furosine at a p value < 0.05. All the statistical analyses were performed using Statgraphics Plus software, version 5.1.

3. Results and discussion

3.1. Furosine and HMF content

Furosine and HMF content were analyzed in 127 vegetable samples submitted to different cooking techniques (breaded, fried, grilled, roasted, boiled, steamed and raw) for two different periods of time; one of them would be the time described in traditional recipes (normal, N) and the other one involving cooking for a 50% more time to obtain the commonly known as "well done" form (WD). Therefore, it is possible to distinguish among type of treatment and intensity of the treatment (**Table 38**). Furosine and HMF, two sensitive markers of Maillard reaction, were used to unravel heat damage during vegetables cooking (**Figure 52**). The vegetables that showed higher furosine content were red pepper and eggplant, followed by artichoke and cauliflower (**Table 39).** In the case of HMF, these vegetables were eggplant followed by red pepper, cauliflower and onion (**Table 39).** Regarding the type of treatment (**Figure 52A and 52C**), breaded and fried vegetables showed the highest furosine and HMF values (86.3 and 82.1 mg of furosine/Kg sample, respectively and 140.8 and 50.1 mg of HMF/Kg sample, respectively), which is a logical result since the high temperature used during frying produce greater thermal damage due to the Maillard reaction. All culinary techniques but steaming showed significantly higher furosine content than the raw form ($p < 0.05$). However, HMF was not detected in boiled, steamed and raw vegetables, probably because HMF is an indicator of intermediate Maillard reaction stages and therefore such cooking techniques did not provide enough energy to develop the reaction further. These results are in accordance with those of other authors (Delgado-Andrade, Seiquer, Haro, Castellano & Navarro, 2010). In the case of breaded vegetables, the combination of the high temperatures used for frying and the presence of flour (high starch content) resulted in the highest values of furosine and especially of HMF. Grilled vegetables and

roasted vegetables were both submitted to similar temperatures (around 200ºC) and still their furosine and HMF values are much lower than in fried vegetables. The reason behind could be that, in the case of roasting, heat is transmitted through the air not having direct contact with the vegetable (resulting in a lower damage). On the other hand, grilled vegetables are in direct contact with the source of heat, but as a consequence, they are exposed to the heat source for a shorter period of time in comparison to frying. In this case, the use of high temperature (180ºC) and a liquid medium (olive oil) for heat transfer give rise to a high thermal damage.

Regarding treatment intensity (**Figure 52B and 52D**), WD vegetables had around twice as much furosine as N vegetables (37.2 *Vs.* 16.9 mg of furosine/Kg sample, respectively). In the case of HMF, raw vegetables did not show any HMF content whereas WD vegetables content was 28.3 mg of HMF/Kg sample and N vegetables was 8.1 mg of HMF/Kg sample. N and vegetables showed significantly higher furosine values than raw ones ($p < 0.05$) whereas WD vegetables showed significantly higher values of furosine and HMF than N vegetables. These results make sense since the Maillard reaction is favored by time and temperature (Rufián-Henares et al., 2008), concluding that overcooking vegetables could result in a greater development of Maillard reaction. However, though Maillard reaction development is also related to food composition (reducing sugars and protein content), in our case furosine and HMF content can be explained mainly by the type of treatment and intensity. The content of sugars (2.62 ± 1.48 g/100g) and proteins (2.91 ± 4.63 g/100g) are not exactly the same in all samples, but these differences seem to be insufficient to play a predominant role in Maillard reaction advance compared to the cooking technique. This conclusion is supported by the absence of statistically significant correlations between furosine or HMF and sugar (or protein content) in every foodstuff and type of treatment.

Accordingly, since furosine and HMF can be used as an indicator of thermal damage (Delgado-Andrade et al., 2010), for the ulterior multivariable analysis the culinary techniques were classified in descendent order regarding their thermal damage: breaded > fried > grilled > roasted > boiled > steamed > raw.

Table 39. Antioxidant capacity of cooked vegetables

	ABTS	FRAP	TEACOH		
	Total antioxidant capacity	**Total antioxidant capacity**	**Total antioxidant capacity**	**Furosine**	**HMF**
	µmol Trolox/g	**µmol Trolox/g**	**µmol Trolox/g**	**(µg/g of vegetable)**	**(µg/g of vegetable)**
Boiled chard N	6.87 ± 0.77	1.71 ± 0.12	0.00 ± 0.00	11.16 ± 0.05	0.00 ± 0.00
Boiled chard WD	7.89 ± 0.11	1.74 ± 0.10	59.70 ± 2.64	9.94 ± 0.07	0.00 ± 0.00
Steamed chard N	12.88 ± 0.27	2.55 ± 0.05	20.39 ± 3.32	6.18 ± 0.02	0.00 ± 0.00
Steamed chard WD	11.98 ± 0.35	3.97 ± 0.57	37.82 ± 1.10	4.74 ± 0.01	0.00 ± 0.00
Roasted garlic N	13.56 ± 0.41	1.78 ± 0.13	2.39 ± 3.27	9.85 ± 0.1	7.60 ± 0.36
Roasted garlic WD	12.99 ± 2.19	2.32 ± 0.07	35.06 ± 4.37	12.19 ± 0.09	24.44 ± 0.00
Raw garlic	13.61 ± 1.53	3.73 ± 0.12	17.64 ± 1.02	6.62 ± 0.08	0.00 ± 0.00
Fried garlic N	13.71 ± 1.12	1.67 ± 0.11	210.48 ± 3.60	6.66 ± 0.07	3.41 ± 0.22
Fried garlic WD	11.10 ± 1.11	3.08 ± 0.58	61.49 ± 1.37	22.71 ± 0.2	6.94 ± 0.26
Grilled garlic N	11.75 ± 0.74	2.50 ± 0.67	109.50 ± 1.05	10.60 ± 0.11	2.92 ± 0.08
Grilled garlic WD	12.44 ± 0.24	2.18 ± 1.25	274.37 ± 0.70	0.00 ± 0	7.79 ± 0.00
Boiled artichoke N	12.63 ± 1.52	4.38 ± 0.32	320.62 ± 1.76	0.00 ± 0	0.00 ± 0.00
Boiled artichoke WD	9.76 ± 2.38	7.28 ± 0.11	229.69 ± 0.00	1.19 ± 0.01	0.00 ± 0.00

Fried artichoke N	22.15 ± 1.21	7.25 ± 0.17	201.92 ± 0.77	117.16 ± 1.56	46.62 ± 6.83
Fried artichoke WD	20.45 ± 0.18	4.06 ± 0.05	323.91 ± 4.17	130.45 ± 1.78	78.57 ± 1.26
Steamed artichoke N	12.05 ± 0.49	4.58 ± 0.12	254.38 ± 4.91	4.15 ± 0.02	0.00 ± 0.00
Steamend artichoke WD	11.00 ± 0.62	3.38 ± 0.19	176.92 ± 0.15	0.34 ± 0.01	0.00 ± 0.00
Roasted eggplant N	8.80 ± 0.69	3.27 ± 0.02	28.70 ± 0.62	7.25 ± 0.05	0.74 ± 0.27
Roasted eggplant WD	12.41 ± 0.24	1.93 ± 0.10	48.43 ± 0.55	6.12 ± 0.03	1.97 ± 0.05
Fried eggplant N	7.56 ± 1.47	3.79 ± 0.39	56.30 ± 4.01	112.29 ± 2.63	85.37 ± 2.52
Fried eggplant WD	13.94 ± 0.05	3.69 ± 0.34	172.59 ± 0.27	125.56 ± 5.41	182.15 ± 4.35
Grilled eggplant N	8.04 ± 0.30	1.80 ± 0.07	146.99 ± 3.65	11.45 ± 1.02	5.65 ± 1.75
Grilled eggplant WD	7.27 ± 0.65	1.66 ± 0.04	82.34 ± 0.43	30.07 ± 2.04	12.97 ± 3.53
Breaded eggplant N	7.21 ± 0.53	1.70 ± 0.03	77.64 ± 0.97	2.20 ± 0.01	103.54 ± 0.03
Breaded eggplant WD	7.22 ± 4.40	2.23 ± 0.11	287.56 ± 3.72	202.03 ± 17.58	211.04 ± 7.33
Roasted zucchini N	15.95 ± 3.17	1.51 ± 0.03	10.94 ± 0.00	13.77 ± 1.21	4.98 ± 0.67
Roasted zucchini WD	8.06 ± 0.62	2.63 ± 0.02	1.80 ± 1.31	17.76 ± 1.05	9.28 ± 0.80
Boiled zucchini N	9.01 ± 0.22	1.40 ± 0.11	0.96 ± 0.00	1.09 ± 0.02	0.00 ± 0.00
Boiled zucchini WD	7.69 ± 0.74	1.58 ± 0.08	38.49 ± 1.37	4.19 ± 0.03	0.00 ± 0.00
Grilled zucchini N	7.06 ± 0.16	1.90 ± 0.03	195.31 ± 1.13	19.44 ± 1.01	8.68 ± 1.73
Grilled zucchini WD	5.42 ± 1.31	4.18 ± 0.82	279.93 ± 0.86	68.97 ± 2.36	49.75 ± 5.48
Steamed zucchini N	9.91 ± 0.81	1.51 ± 0.02	3.21 ± 0.00	0.00 ± 0	0.00 ± 0.00
Steamed zucchini WD	8.47 ± 0.63	1.80 ± 0.50	12.68 ± 0.00	0.00 ± 0	0.00 ± 0.00

Roasted pumpkin N	10.19 ± 0.43	1.68 ± 0.09	12.50 ± 0.00	0.00 ± 0	0.00 ± 0.00
Roasted pumpkin WD	11.56 ± 0.64	2.32 ± 0.30	16.00 ± 0.97	22.43 ± 1.36	11.72 ± 4.01
Boiled pumpkin N	8.83 ± 0.74	1.64 ± 0.02	2.47 ± 0.67	0.00 ± 0	0.00 ± 0.00
Boiled pumpkin WD	11.46 ± 0.51	1.86 ± 0.01	16.70 ± 0.19	1.30 ± 0.01	0.00 ± 0.00
Grilled pumpkin N	7.23 ± 0.21	2.49 ± 0.04	155.85 ± 1.20	23.99 ± 2.87	10.71 ± 1.34
Grilled pumpkin WD	8.02 ± 1.27	3.76 ± 0.49	90.61 ± 1.42	41.65 ± 3.14	30.04 ± 3.07
Steamed pumpkin N	9.18 ± 1.20	1.88 ± 0.25	17.14 ± 0.00	0.00 ± 0	0.00 ± 0.00
Steamed pumpkin WD	8.91 ± 0.60	1.90 ± 0.09	26.93 ± 0.04	11.93 ± 1.03	0.00 ± 0.00
Roasted onion N	10.95 ± 0.65	2.22 ± 0.05	22.10 ± 2.35	0.98 ± 0.01	4.78 ± 0.00
Roasted onion WD	10.04 ± 1.28	1.53 ± 0.03	33.93 ± 6.85	49.14 ± 2.47	97.22 ± 12.98
Raw onion	6.58 ± 0.29	1.91 ± 0.07	51.65 ± 2.02	9.00 ± 1.65	0.00 ± 0.00
Fried onion N	8.34 ± 1.18	1.94 ± 0.13	137.20 ± 11.15	15.09 ± 2.14	3.45 ± 1.58
Fried onion WD	7.97 ± 0.97	2.35 ± 0.03	372.12 ± 2.62	38.82 ± 2.01	54.65 ± 2.64
Grilled onion N	10.99 ± 0.64	1.83 ± 0.02	165.18 ± 1.12	10.27 ± 1.02	8.16 ± 5.69
Grilled onion WD	9.40 ± 0.78	2.33 ± 0.11	498.17 ± 13.57	41.90 ± 3.47	133.87 ± 7.22
Roasted mushroom N	15.92 ± 1.06	2.29 ± 0.28	89.33 ± 0.77	0.00 ± 0	0.00 ± 0.00
Roasted mushroom WD	11.53 ± 1.12	2.38 ± 0.05	81.54 ± 0.45	1.12 ± 0.01	0.59 ± 0.00
Grilled mushroom N	11.05 ± 0.27	2.39 ± 0.06	142.86 ± 0.78	1.24 ± 0.02	0.55 ± 0.05
Grilled mushroom WD	11.61 ± 2.06	1.88 ± 0.21	435.31 ± 1.27	4.44 ± 0.9	3.20 ± 0.38
Boiled cabbage N	8.30 ± 0.66	1.32 ± 1.15	5.53 ± 0.18	0.00 ± 0	0.00 ± 0.00

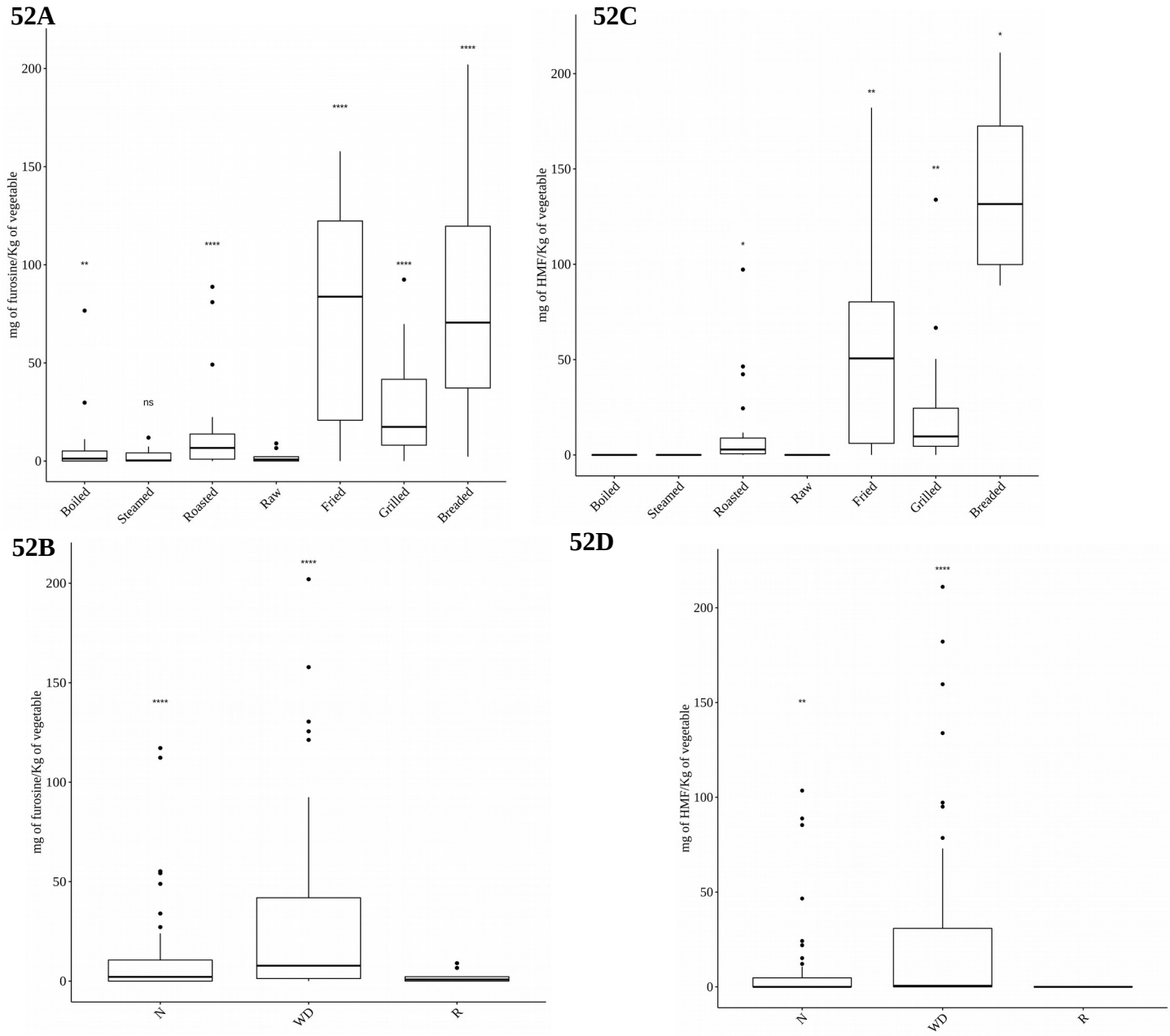
Boiled cabbage WD	5.71 ± 0.30	2.27 ± 0.12	15.75 ± 0.08	0.00 ± 0	0.00 ± 0.00
Raw cabbage	5.22 ± 0.58	2.86 ± 0.20	25.48 ± 0.29	0.00 ± 0	0.00 ± 0.00
Grilled cabbage N	6.26 ± 0.19	2.14 ± 0.28	44.64 ± 4.66	54.23 ± 3.78	24.22 ± 20.14
Grilled cabbage WD	6.05 ± 0.51	3.56 ± 0.17	46.64 ± 0.00	92.44 ± 9.03	66.68 ± 0.38
Steamed cabbage N	7.10 ± 0.45	1.91 ± 0.08	33.38 ± 0.28	0.12 ± 0.01	0.00 ± 0.00
Steamed cabbage WD	7.13 ± 1.09	2.40 ± 0.40	17.27 ± 0.12	0.35 ± 0.02	0.00 ± 0.00
Boiled cauliflower N	12.78 ± 0.31	2.03 ± 0.35	15.28 ± 0.00	0.00 ± 0	0.00 ± 0.00
Boiled cauliflower WD	15.87 ± 0.60	1.37 ± 0.03	20.20 ± 0.00	76.63 ± 4.98	0.00 ± 0.00
Breaded cauliflower N	15.51 ± 0.31	3.95 ± 0.04	39.01 ± 0.59	48.89 ± 5.62	88.85 ± 10.21
Breaded cauliflower WD	17.13 ± 0.62	3.45 ± 0.17	56.34 ± 0.00	92.20 ± 6.37	159.65 ± 11.03
Steamed cauliflower N	7.20 ± 0.53	1.77 ± 0.06	13.93 ± 4.37	3.06 ± 0.3	0.00 ± 0.00
Steamed cauliflower WD	13.33 ± 0.87	2.07 ± 0.10	19.25 ± 0.75	7.40 ± 0.9	0.00 ± 0.00
Roasted asparagus N	7.99 ± 0.93	1.95 ± 0.10	23.94 ± 0.23	4.81 ± 0.09	1.74 ± 0.03
Roasted asparagus WD	18.73 ± 0.34	3.76 ± 0.09	162.62 ± 17.58	80.95 ± 7.15	42.31 ± 37.55
Boiled asparagus N	12.99 ± 0.30	1.86 ± 0.06	17.20 ± 0.42	0.00 ± 0	0.00 ± 0.00
Boiled asparagus WD	7.93 ± 3.26	2.64 ± 0.46	27.28 ± 4.40	2.40 ± 0.05	0.00 ± 0.00
Grilled asparagus N	25.96 ± 0.35	2.85 ± 0.60	98.00 ± 0.40	27.15 ± 2.69	12.12 ± 1.24
Grilled asparagus WD	12.09 ± 2.19	2.75 ± 0.04	92.06 ± 0.81	43.17 ± 4.87	31.14 ± 6.80
Steamed asparagus N	14.36 ± 3.56	2.46 ± 0.03	151.91 ± 2.59	0.00 ± 0	0.00 ± 0.00
Steamed asparagus WD	12.37 ± 0.17	2.48 ± 0.05	18.42 ± 7.51	1.10 ± 0.08	0.00 ± 0.00

Boiled spinach N	18.59 ± 1.03	1.60 ± 0.04	8.36 ± 0.06	5.14 ± 0.48	0.00 ± 0.00
Boiled spinach WD	13.69 ± 0.55	1.72 ± 0.03	7.54 ± 0.72	29.75 ± 2.05	0.00 ± 0.00
Steamed spinach N	15.14 ± 3.48	2.60 ± 0.13	96.97 ± 4.10	3.56 ± 0.65	0.00 ± 0.00
Steamed spinach WD	15.80 ± 0.64	3.66 ± 0.04	187.82 ± 12.36	5.63 ± 0.47	0.00 ± 0.00
Boiled peas N	7.59 ± 0.08	0.44 ± 0.53	10.08 ± 0.00	0.00 ± 0	0.00 ± 0.00
Boiled peas WD	14.96 ± 0.13	1.77 ± 0.00	53.30 ± 0.00	1.92 ± 0.11	0.00 ± 0.00
Raw peas	6.14 ± 0.26	2.47 ± 0.12	1.78 ± 6.03	0.00 ± 0	0.00 ± 0.00
Grilled peas N	10.66 ± 0.19	1.63 ± 0.41	160.19 ± 39.81	0.00 ± 0	0.00 ± 0.00
Grilled peas WD	13.71 ± 0.45	1.41 ± 0.08	0.00 ± 3.31	0.82 ± 0.03	0.14 ± 0.00
Steamed peas N	12.18 ± 0.95	2.22 ± 0.06	283.66 ± 15.16	0.00 ± 0	0.00 ± 0.00
Steamed peas WD	10.70 ± 0.69	1.60 ± 0.09	29.92 ± 0.00	0.00 ± 0	0.00 ± 0.00
Raw broad beans	13.41 ± 0.29	4.27 ± 0.07	50.95 ± 2.34	0.89 ± 0.04	0.00 ± 0.00
Grilled broad beans N	15.09 ± 0.33	4.24 ± 0.17	59.83 ± 1.61	10.73 ± 1.03	4.79 ± 5.50
Grilled broad beans WD	17.15 ± 1.18	5.75 ± 0.14	114.56 ± 0.62	34.13 ± 3.05	24.62 ± 0.28
Boiled beans N	5.68 ± 1.32	3.47 ± 0.08	5.82 ± 0.00	1.56 ± 0.08	0.00 ± 0.00
Boiled beans WD	9.49 ± 0.52	1.71 ± 0.09	14.96 ± 0.22	5.49 ± 0.65	0.00 ± 0.00
Steamed beans N	8.07 ± 0.49	1.58 ± 0.01	22.98 ± 1.19	2.04 ± 0.03	0.00 ± 0.00
Steamed beans WD	9.37 ± 0.35	1.77 ± 0.18	30.00 ± 1.55	4.29 ± 0.06	0.00 ± 0.00
Raw lettuce	11.70 ± 0.80	1.55 ± 0.07	35.53 ± 1.66	0.53 ± 0.02	0.00 ± 0.00
Roasted potato N	8.26 ± 0.28	1.76 ± 0.10	15.89 ± 9.87	3.50 ± 0.07	1.26 ± 0.13

Roasted potato WD	8.79 ± 1.38	1.38 ± 0.14	209.98 ± 0.49	11.22 ± 1.26	5.87 ± 2.48
Boiled potato N	8.50 ± 1.49	3.18 ± 0.10	0.34 ± 7.14	0.00 ± 0	0.00 ± 0.00
Boiled potato WD	11.26 ± 0.43	1.61 ± 0.87	8.54 ± 0.05	3.33 ± 0.06	0.00 ± 0.00
Fried potato N	18.82 ± 1.14	1.47 ± 0.14	285.46 ± 0.00	0.00 ± 0	0.00 ± 0.00
Fried potato WD	7.11 ± 0.67	1.88 ± 0.02	102.31 ± 28.89	121.27 ± 10.45	73.04 ± 4.99
Steamed potato N	8.67 ± 1.00	2.12 ± 0.23	85.05 ± 3.60	0.00 ± 0	0.00 ± 0.00
Steamed potato WD	11.55 ± 0.31	1.81 ± 0.10	4.34 ± 0.00	3.40 ± 0.56	0.00 ± 0.00
Raw cucumber	7.45 ± 0.52	1.69 ± 0.56	10.64 ± 1.05	1.91 ± 0.15	0.00 ± 0.00
Raw parsley	11.96 ± 0.28	2.02 ± 0.15	21.15 ± 2.21	0.81 ± 0.06	0.00 ± 0.00
Roasted red pepper N	12.51 ± 0.47	2.76 ± 0.20	4.80 ± 0.49	10.27 ± 1.09	3.71 ± 1.43
Roasted red pepper WD	10.72 ± 0.31	4.35 ± 0.08	151.50 ± 0.82	88.78 ± 5.62	46.40 ± 2.11
Raw red pepper	9.35 ± 0.57	4.93 ± 0.27	18.28 ± 0.98	2.22 ± 0.05	0.00 ± 0.00
Fried red pepper N	7.89 ± 0.71	3.14 ± 0.21	44.15 ± 0.37	55.21 ± 3.12	21.97 ± 1.05
Fried red pepper WD	3.08 ± 0.41	4.46 ± 0.12	285.66 ± 0.00	157.83 ± 12.46	95.06 ± 22.10
Grilled red pepper N	11.40 ± 0.43	3.53 ± 0.07	18.92 ± 18.90	33.97 ± 2.98	15.17 ± 9.64
Grilled red pepper WD	3.96 ± 0.33	2.54 ± 1.37	2.38 ± 0.35	69.80 ± 5.78	50.35 ± 6.26
Boiled leek N	11.21 ± 0.30	1.41 ± 0.02	131.76 ± 18.33	0.00 ± 0	0.00 ± 0.00
Boiled leek WD	10.42 ± 0.74	1.35 ± 0.01	33.66 ± 3.90	7.27 ± 1.02	0.00 ± 0.00
Steamed leek N	7.00 ± 0.58	1.46 ± 0.10	48.59 ± 1.27	0.00 ± 0	0.00 ± 0.00
Steamed leek WD	13.00 ± 1.43	1.34 ± 0.03	9.66 ± 0.72	0.00 ± 0	0.00 ± 0.00

Roasted tomato N	7.43 ± 0.41	1.50 ± 0.19	7.87 ± 1.94	0.53 ± 0.01	0.19 ± 0.02
Roasted tomato WD	11.58 ± 1.34	1.60 ± 0.07	57.24 ± 1.01	1.11 ± 0.03	0.58 ± 0.01
Raw tomato	9.19 ± 0.51	2.16 ± 0.03	19.92 ± 1.26	0.00 ± 0	0.00 ± 0.00
Grilled tomato N	11.62 ± 0.34	1.46 ± 0.07	212.12 ± 2.72	10.01 ± 1.45	4.47 ± 7.74
Grilled tomato WD	7.15 ± 0.43	2.35 ± 0.64	208.62 ± 1.37	15.36 ± 1.07	11.08 ± 0.10
Roasted carrot N	24.70 ± 0.39	1.61 ± 0.02	11.71 ± 4.31	0.00 ± 0	0.00 ± 0.00
Roasted carrot WD	10.63 ± 1.09	2.41 ± 0.03	68.05 ± 0.94	0.00 ± 0	0.00 ± 0.00
Boiled carrot N	9.45 ± 0.33	1.86 ± 0.21	23.46 ± 2.67	0.00 ± 0	0.00 ± 0.00
Boiled carrot WD	16.28 ± 2.00	1.93 ± 0.03	55.95 ± 1.73	0.00 ± 0	0.00 ± 0.00
Raw carrot	7.29 ± 0.24	1.49 ± 1.18	44.03 ± 1.09	0.00 ± 0	0.00 ± 0.00
Grilled carrot N	8.54 ± 2.37	4.01 ± 0.14	156.83 ± 0.48	6.55 ± 0.05	2.93 ± 0.11
Grilled carrot WD	10.52 ± 1.00	2.61 ± 0.14	170.04 ± 4.90	8.09 ± 0.07	5.84 ± 0.17
Steamed carrot N	6.77 ± 0.47	1.50 ± 0.24	17.19 ± 8.79	0.00 ± 0	0.00 ± 0.00
Steamed carrot WD	6.66 ± 1.08	2.03 ± 0.05	26.85 ± 5.51	0.00 ± 0	0.00 ± 0.00

Figure 52. Furosine content of processed vegetables (mg/Kg of vegetable) depending on the cooking technique (52A) or heat treatment intensity (52B). HMF content of processed vegetables (mg/Kg of vegetable) depending on the cooking technique (52C) or heat treatment intensity (52D). Statistical analysis was performed through ANOVA using raw vegetables as the reference group. Values are the mean value of all vegetables for each cooking technique or treatment intensity. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.



3.2. Antioxidant capacity

For each sample, the antioxidant capacity of three fractions was assessed: the supernatant obtained after gastrointestinal digestion (which would be the antioxidant capacity available for absorption in the small intestine), the supernatant derived from fermentation (which would be the antioxidant capacity available for absorption in the large intestine) and the solid residue remaining after fermentation (which is not absorbed, but could exert some antioxidant protection on the large intestine walls). The sum of the three terms is the total antioxidant capacity (Pérez-Burillo et al., 2018a). Three different methods were used to analyse the antioxidant capacity of foods. All antioxidant capacity values for all three methods were corrected taking into account their respective blanks (enzymes, chemicals and inoculum).

- **Gastrointestinal digestion supernatant**. Regarding $TEAC_{ABTS}$, antioxidant capacity released during in vitro digestion was significantly ($p < 0.05$) higher in all cooking techniques, in comparison to raw vegetables, but in boiling which was not significant (**Figure 53A**). Regarding the degree of intensity, N and WD vegetables showed significantly higher antioxidant capacity than raw vegetables ($p < 0.05$) (**Figure 53B**). On the other hand, $TEAC_{FRAP}$ assay showed also that antioxidant capacity of digestion fraction was significantly ($p < 0.05$) higher in fried, grilled and breaded vegetables in comparison with the raw ones. However, boiling, steaming and roasting did not increase antioxidant capacity significantly in comparison with the raw form (**Figure 54A**). Moreover, WD vegetables showed to be significantly more antioxidant than the raw form (**Figure 54B**). $TEAC_{OH}$, as in $TEAC_{FRAP}$, only showed significantly higher antioxidant values than raw vegetables when they were fried, grilled or breaded (**Figure 55A**). Moreover, WD vegetables exerted higher antioxidant capacity than raw ones (**Figure 55B**). The explanation behind these findings could be that thermal

treatments (cooking) could break down cell structures making easier their digestion and therefore releasing more bioactive compounds available for absorption in the small intestine (Miglio, Chiavaro, Visconti, Fogliano & Pellegrini, 2008). In the case of $TEAC_{ABTS}$, boiling did not increase antioxidant capacity in comparison with the raw vegetables, which could be due to a solubilization of hydrosoluble compounds in the boiling water (Ramírez-Anaya et al., 2015). However, $TEAC_{FRAP}$ and $TEAC_{OH}$, only showed higher antioxidant capacity when vegetables were fried, grilled or breaded. Accordingly, even though cooking could help releasing antioxidant compounds during digestion, olive oil could play a more important role (Ramírez-Anaya et al. 2015). Additionally, this could mean that reducing compounds and compounds active against OH· radicals would only increase during digestion by adding olive oil. Nevertheless, thermal treatment could also help releasing bioactive compounds as WD vegetables were significantly more antioxidant than raw ones. N cooked vegetables were also more antioxidant but not significantly.

- **Fermentation supernatant**. Regarding $TEAC_{ABTS}$, only roasting increased antioxidant capacity in a significant manner with respect to the raw form ($p < 0.05$) during fermentation (**Figure 53A**). N cooked vegetables showed also significantly ($p < 0.05$) higher antioxidant capacity than raw ones (**Figure 53B**). In the case of $TEAC_{FRAP}$, fermentation supernatant antioxidant capacity of the raw form was significantly higher than all cooking techniques except for frying and breading which were not significant (**Figure 54A**). Moreover, N and WD vegetables showed significantly higher antioxidant capacity than raw ones (**Figure 54B**). Regarding $TEAC_{OH}$, each type of culinary technique produced higher antioxidant capacity than raw vegetables during fermentation, especially fried, grilled and breaded ones (**Figure 55A**). N and WD vegetables also gave significantly higher antioxidant capacity than raw ones (**Figure**

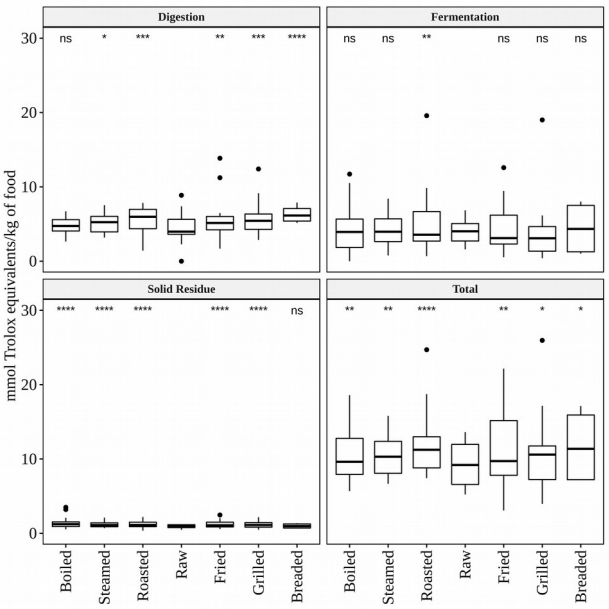
55B). In this step, gut microbial activity could play an important role (Pérez-Burillo et al., 2018b). As results show, according to $TEAC_{ABTS}$, there is no differences between most cooking techniques, which could indicate that the antioxidants active against ABTS· radicals released by gut microbial activity are not affected by the type of cooking. However, in the case of $TEAC_{FRAP}$, we see how reduction capacity released after gut microbial activity could be improve by adding olive oil. Otherwise, raw vegetables produce more bioactive compounds with reduction power than cooked ones. Finally, the activity against OH· released after microbial degradation of vegetables, greatly improves when they are cooked. This could be due to the metabolization of bioactive compounds from the vegetables that reach the colon in larger amounts thanks to an easier digestion but also to the degradation of melanoidins or other compounds that appear during the Maillard reaction. (Delgado-Andrade & Morales, 2005; Tagliazucchi et al., 2008; Dittrich et al., 2009; de la Cueva et al., 2017). The metaboilization of such compounds by gut microbes could produce a large amount of compounds able to block OH· radicals. On the other hand, frying, grilling and breading achieved the higher values, which could also indicate that olive oil greatly improves the ability to fight against OH· radicals.

- **Fermentation solid residue**. Regarding $TEAC_{ABTS}$, antioxidant capacity of the solid residue was significantly higher in all cooking techniques but in breading in comparison with the raw form ($p < 0.05$) (**Figure 53A**). N and WD were also significantly more antioxidant than raw vegetables (**Figure 53B**). $TEAC_{FRAP}$ on the other hand, did not show significant differences regarding cooking techniques but in the case of boiling and grilling which were significantly lower than the raw form (**Figure 54A**). N and WD were also significantly less antioxidant than the raw ones (**Figure 54B**). In the case of $TEAC_{OH}$, no significant differences were found neither in relation to

cooking techniques nor degree of cooking. The differences found here between $TEAC_{ABTS}$ and $TEAC_{FRAP}$ could be due to the different nature of the methods and the residue left after fermentation could be more active against ABTS· radicals.

Figure 53. Antioxidant capacity measured by the $TEAC_{ABTS}$ assay of each fraction depending of each cooking technique (53A) or heat intensity (53B). Statistical analysis was performed through ANOVA using raw vegetables as the reference group. Values are the mean value of all vegetables for each cooking technique or treatment intensity. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

53A



53B

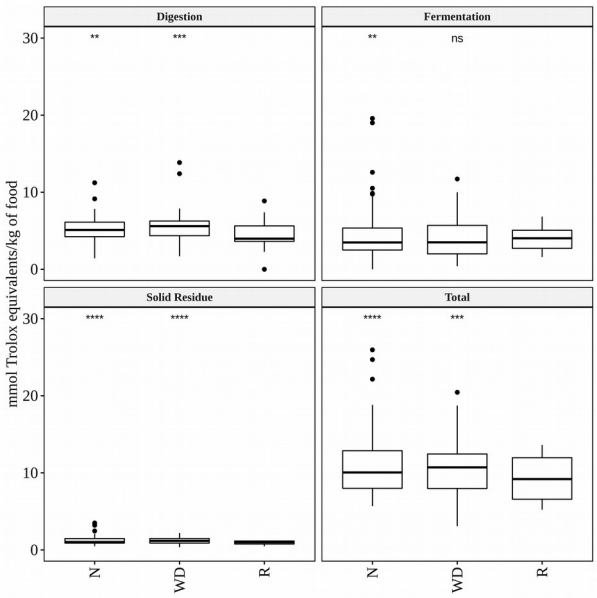
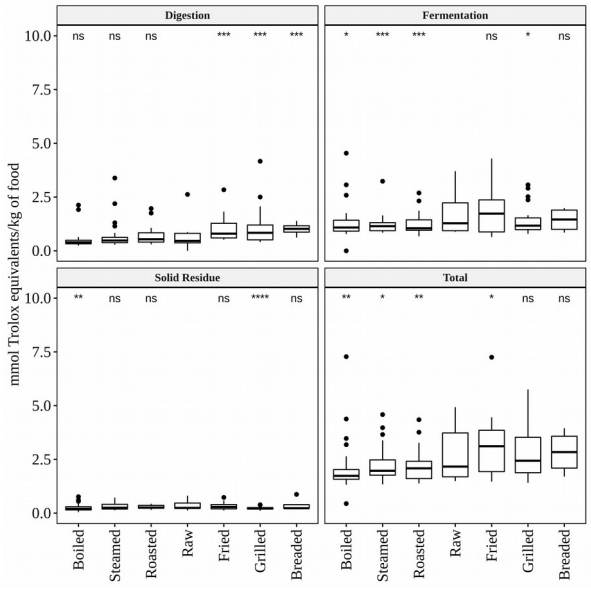


Figure 54. Antioxidant capacity measured by the $TEAC_{FRAP}$ assay of each fraction depending of each cooking technique (54A) or heat intensity (54B). Statistical analysis was performed through ANOVA using raw vegetables as the reference group. Values are the mean value of all vegetables for each cooking technique or treatment intensity. Statistic labels: *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001, ns: not significant.

54A



54B

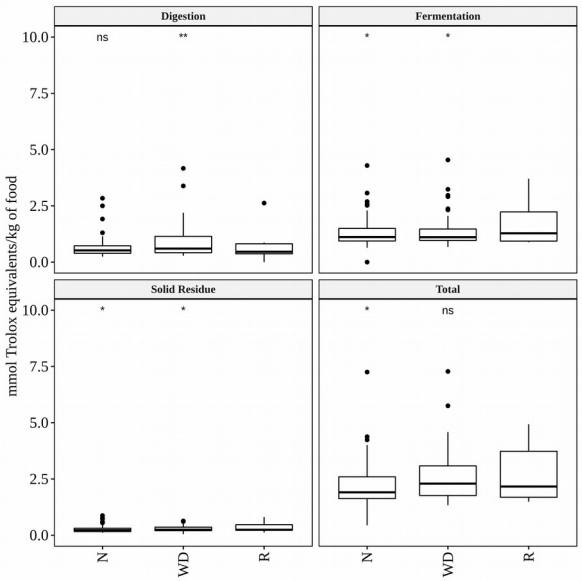
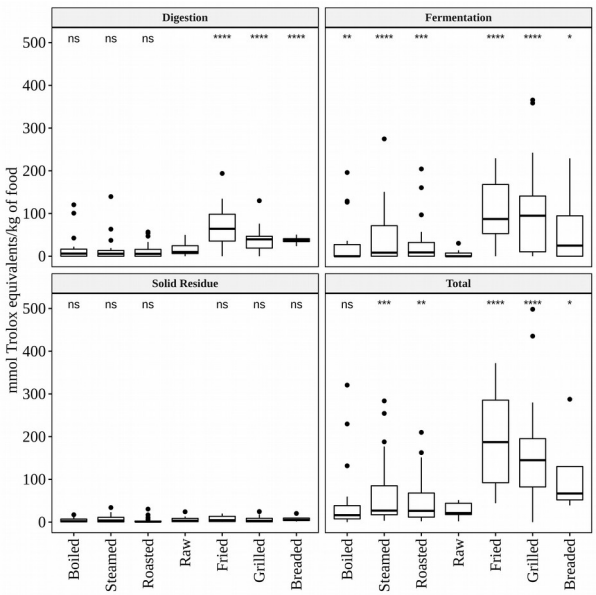
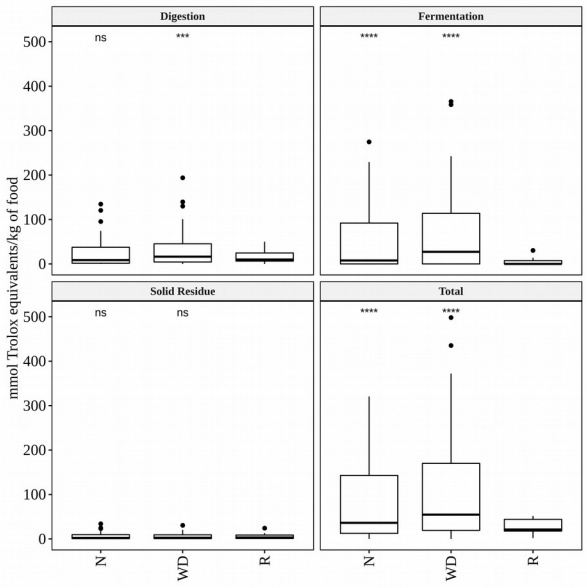


Figure 55. Antioxidant capacity measured by the $TEAC_{OH}$ assay of each fraction depending of each cooking technique (55A) or heat intensity (55B). Statistical analysis was performed through ANOVA using raw vegetables as the reference group. Values are the mean value of all vegetables for each cooking technique or treatment intensity. Statistic labels: *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001, ns: not significant.

55A

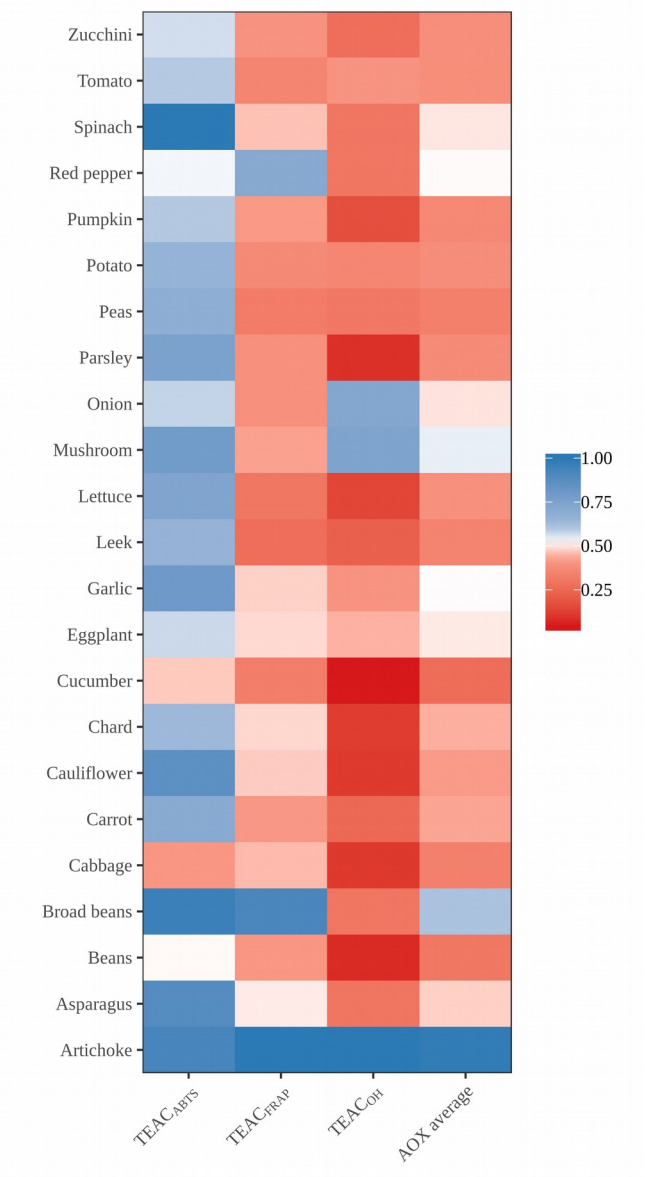


55B



-Total antioxidant capacity and contribution of each fraction to total antioxidant capacity. **Table 39** in supplemental information shows the antioxidant capacity values for each foodstuff per type of treatment and intensity. For $TEAC_{ABTS}$, he most antioxidant vegetable (mean of the different culinary treatments and degree of intensity) in this antioxidant assay (**Figure 56, Table 39**) was spinach whereas the lowest one was cabbage. For $TEAC_{FRAP}$, the most antioxidant vegetable was artichoke, followed by beans and red pepper (**Figure 56, Table 39**) and the less antioxidant leek. For $TEAC_{OH}$, the most antioxidant vegetal was artichoke and the less antioxidant was cucumber (**Figure 56, Table 39**).

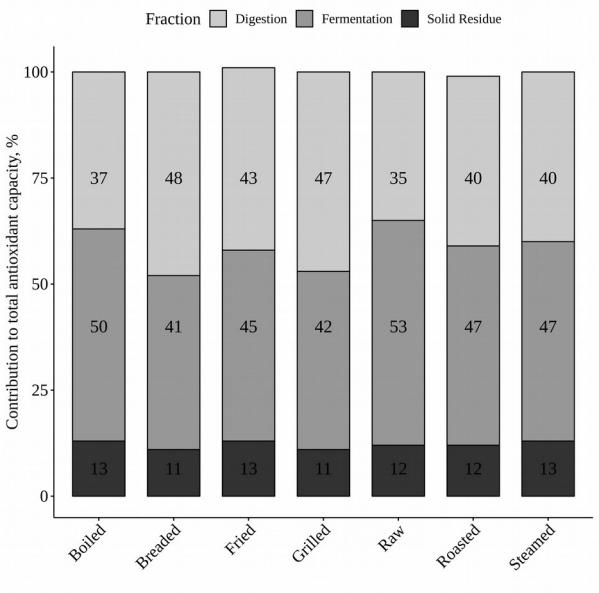
Figure 56. Heatmap of total antioxidant capacity obtained with each method for each vegetable. Vales have been scaled in a 0-1 scale for each method.



Tables 40 and **41** show the contribution of each fraction in each sample to total antioxidant capacity. Overall, digestion contribution to total antioxidant capacity was lower in boiled, steamed, roasted and raw vegetables whereas fermentation supernatant contribution was higher in such techniques. The solid residue contribution was also higher in boiled, steamed, raw and fried vegetables (**Figure 57A** and **57B**). Accordingly, taking the above information into account about the antioxidant capacity of cooked vegetables, it could be concluded that during digestion, antioxidant compounds are more easily released from those vegetables that have suffered greater thermal damage, whereas fermentation contributes with higher antioxidant capacity when vegetables are boiled, steamed, roasted or raw (less thermally damaged). Therefore, the cooking technique along with the degree of intensity could modify the substrates that reach the colon, which would influence differentially to gut microbiota. Additionally, for all methods tested those vegetables prepared with EVOO had the higher antioxidant capacity. Moreover, in most cases, cooking the vegetable results in an increase of the antioxidant activity, not only when using EVOO. In this sense, our results are in accordance with that found by other authors (Miglio et al., 2008; Bellail, Shaltout, Youssef & Gamal, 2012; Ramírez-Anaya et al., 2015). However, it could be also interesting for future research to study the effect that olive oil has on its own on antioxidant capacity after gastrointestinal digestion and microbial fermentation of vegetables. Accordingly, it would be interesting further research to elucidate the prebiotic effect of each type of cooking. Another field for future research could be the study of the specific compounds generated during gastrointestinal digestion, and microbial fermentation (including the ones found in feces) to better explain the differences found between antioxidant capacity methods.

Figure 57. Contribution to the total antioxidant capacity of the fractions obtained after digestion depending of each cooking technique (57A) or heat intensity (57B).

57A



57B

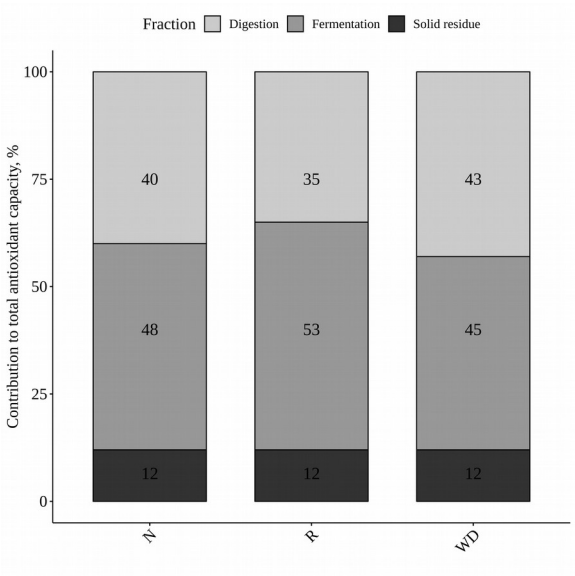


Table 40. Contribution percentage of each vegetable fraction to the global antioxidant capacity

	ABTS			FRAP			TEACOH		
	Digestion	Fermentation supernatant	Fermentation solid residue	Digestion	Fermentation supernatant	Fermentation solid residue	Digestion	Fermentation supernatant	Fermentation solid residue
		%			%			%	
Boiled artichoke N	43.80	41.18	15.02	43.77	38.77	17.45	37.56	61.11	1.34
Boiled artichoke WD	34.13	58.12	7.75	29.22	62.39	8.39	43.85	55.04	1.11
Boiled asparagus N	48.90	44.09	7.01	23.93	65.40	10.67	0.00	0.00	100.00
Boiled asparagus WD	75.13	16.39	8.49	15.47	66.28	18.24	66.22	0.00	33.78
Boiled beans N	49.89	27.61	22.50	6.98	88.38	4.64	0.00	0.00	100.00
Boiled beans WD	51.46	40.27	8.27	28.81	56.75	14.43	64.44	28.77	6.79
Boiled cabbage N	48.98	39.48	11.54	18.49	69.42	12.09	100.00	0.00	0.00
Boiled cabbage WD	46.40	31.03	22.57	20.87	64.78	14.35	98.93	0.00	1.07
Boiled carrot N	35.32	52.31	12.36	21.13	70.88	7.99	95.28	0.00	4.72
Boiled carrot WD	19.19	71.98	8.83	16.88	73.71	9.40	35.00	52.21	12.80
Boiled cauliflower N	33.28	41.68	25.04	19.64	53.23	27.12	31.55	0.00	68.45

Boiled cauliflower WD	28.19	62.99	8.82	25.76	56.81	17.44	30.00	0.00	70.00
Boiled chard N	68.77	14.92	16.32	37.71	55.75	6.54	0.00	0.00	0.00
Boiled chard WD	64.59	23.42	11.98	33.59	63.64	2.77	27.84	51.52	20.64
Boiled leek N	49.86	36.12	14.02	24.79	63.19	12.02	1.99	97.99	0.02
Boiled leek WD	53.27	37.89	8.84	26.87	64.78	8.35	18.75	80.98	0.27
Boiled peas N	88.46	0.00	11.54	72.84	0.00	27.16	70.17	0.00	29.83
Boiled peas WD	39.03	51.74	9.23	18.51	69.75	11.74	79.71	19.27	1.02
Boiled potato N	47.36	40.38	12.26	9.52	81.19	9.29	100.00	0.00	0.00
Boiled potato WD	50.54	34.86	14.60	19.81	64.92	15.27	81.17	0.00	18.83
Boiled pumpkin N	54.30	39.64	6.05	21.45	66.38	12.18	0.00	0.00	100.00
Boiled pumpkin WD	41.50	40.29	18.21	22.91	66.20	10.89	0.00	40.89	59.11
Boiled spinach N	24.58	56.60	18.82	37.62	51.68	10.70	73.35	0.00	26.65
Boiled spinach WD	42.37	46.41	11.22	31.90	49.33	18.77	90.38	0.00	9.62
Boiled zucchini N	49.94	42.94	7.12	22.70	67.17	10.13	0.00	0.00	100.00
Boiled zucchini WD	60.73	21.91	17.35	24.98	65.91	9.11	0.00	93.79	6.21
Breaded cauliflower N	43.92	47.29	8.80	27.62	50.28	22.10	96.44	0.00	3.56
Breaded cauliflower WD	46.04	46.82	7.14	40.40	54.01	5.60	89.56	0.00	10.44

Breaded eggplant N	76.01	14.15	9.84	36.33	49.86	13.82	30.42	64.08	5.50
Breaded eggplant WD	71.46	18.67	9.87	42.84	47.15	10.01	13.23	79.70	7.06
Fried artichoke N	50.68	42.68	6.64	39.16	59.20	1.65	66.66	27.25	6.08
Fried artichoke WD	67.74	26.80	5.46	44.92	49.48	5.61	30.83	65.14	4.03
Fried eggplant N	47.39	40.54	12.07	27.75	68.30	3.95	0.00	100.00	0.00
Fried eggplant WD	31.26	57.47	11.27	49.20	39.39	11.42	0.00	97.69	2.31
Fried garlic N	41.23	40.78	17.98	38.87	48.31	12.82	45.31	53.19	1.49
Fried garlic WD	58.35	28.35	13.30	25.42	66.54	8.04	75.52	0.00	24.48
Fried onion N	60.31	31.99	7.70	29.58	32.89	37.53	54.17	45.38	0.44
Fried onion WD	47.77	26.64	25.60	34.71	40.11	25.18	52.08	43.75	4.17
Fried potato N	27.96	66.90	5.14	37.04	53.39	9.57	18.01	80.31	1.68
Fried potato WD	82.51	7.49	10.00	32.20	47.94	19.86	52.88	45.54	1.58
Fried red pepper N	56.57	30.13	13.31	16.52	73.15	10.34	6.03	92.68	1.29
Fried red pepper WD	55.21	26.12	18.67	24.71	66.54	8.74	34.26	58.72	7.03
Grilled asparagus N	23.57	73.22	3.21	35.22	57.62	7.16	39.77	50.65	9.57
Grilled asparagus WD	52.50	38.66	8.85	47.89	46.89	5.22	49.20	50.80	0.00

Grilled broad beans N	60.57	33.43	6.00	59.02	35.73	5.24	80.15	0.00	19.85
Grilled broad beans WD	72.33	19.30	8.37	72.43	23.19	4.38	36.47	60.86	2.67
Grilled cabbage N	70.45	13.69	15.86	30.66	51.57	17.77	93.75	0.00	6.25
Grilled cabbage WD	66.34	12.60	21.07	57.98	34.63	7.39	91.93	0.00	8.07
Grilled carrot N	47.60	40.29	12.11	20.25	76.51	3.25	25.61	58.62	15.77
Grilled carrot WD	37.32	56.46	6.23	48.48	42.36	9.16	24.95	66.99	8.06
Grilled eggplant N	68.94	16.82	14.24	25.50	60.58	13.92	5.25	89.68	5.07
Grilled eggplant WD	44.54	45.38	10.09	25.07	59.38	15.55	35.85	59.10	5.05
Grilled garlic N	59.29	30.59	10.12	31.96	54.38	13.65	34.29	64.93	0.77
Grilled garlic WD	53.20	32.93	13.87	42.61	46.64	10.75	19.10	80.50	0.40
Grilled mushroom N	63.65	25.77	10.58	25.11	64.16	10.73	18.86	68.45	12.69
Grilled mushroom WD	57.79	25.91	16.30	41.21	45.43	13.37	17.48	82.37	0.15
Grilled onion N	57.30	28.78	13.92	33.32	54.06	12.62	33.80	65.35	0.85
Grilled onion WD	62.34	25.07	12.59	49.10	42.12	8.79	26.08	73.39	0.53
Grilled peas N	56.82	26.37	16.81	30.21	58.86	10.93	11.91	87.95	0.15
Grilled peas WD	45.68	44.84	9.47	33.56	55.99	10.45	0.00	0.00	0.00

Grilled pumpkin N	68.70	21.53	9.77	35.07	53.85	11.09	29.96	64.33	5.72
Grilled pumpkin WD	63.58	9.30	27.12	31.90	62.95	5.15	84.11	11.46	4.43
Grilled red pepper N	41.65	53.16	5.20	24.47	71.49	4.03	98.37	0.00	1.63
Grilled red pepper WD	72.25	10.17	17.58	54.70	36.64	8.65	0.00	0.00	100.00
Grilled tomato N	44.63	40.06	15.30	27.94	64.39	7.67	6.97	92.71	0.32
Grilled tomato WD	74.57	11.17	14.26	21.65	70.39	7.96	5.50	94.27	0.23
Grilled zucchini N	60.69	32.45	6.86	26.16	53.42	20.42	18.58	68.90	12.52
Grilled zucchini WD	71.44	8.40	20.16	22.41	69.66	7.93	11.05	86.59	2.36
Raw broad beans	66.14	30.38	3.48	61.42	26.83	11.75	98.03	0.00	1.97
Raw cabbage	43.48	35.74	20.77	16.13	76.13	7.74	5.04	0.00	94.96
Raw carrot	49.70	39.72	10.58	24.99	60.02	14.98	30.08	68.82	1.10
Raw cucumber	51.06	36.50	12.44	16.85	75.87	7.28	59.32	10.25	30.44
Raw garlic	54.28	37.22	8.50	16.93	61.33	21.74	53.87	0.00	46.13
Raw lettuce	34.00	58.47	7.53	23.93	60.38	15.69	100.00	0.00	0.00
Raw onion	59.15	24.22	16.63	28.98	46.44	24.58	47.69	27.00	25.32
Raw parsley	42.58	49.02	8.40	40.35	48.33	11.32	74.03	0.00	25.97

Raw peas	0.00	81.18	18.82	0.00	90.20	9.80	0.00	0.00	100.00
Raw red pepper	44.58	43.04	12.37	17.61	75.13	7.26	44.82	7.53	47.65
Raw tomato	61.28	32.37	6.35	17.73	69.97	12.30	49.56	37.46	12.99
Roasted asparagus N	82.00	8.63	9.37	37.18	54.99	7.83	29.34	0.00	70.66
Roasted asparagus WD	41.36	46.91	11.73	52.25	41.64	6.11	0.83	98.63	0.54
Roasted carrot N	17.21	79.26	3.53	30.67	53.34	15.99	96.25	0.00	3.75
Roasted carrot WD	46.10	45.65	8.25	28.36	59.74	11.90	16.09	83.91	0.00
Roasted eggplant N	38.16	56.13	5.71	10.71	82.35	6.94	0.00	98.77	1.23
Roasted eggplant WD	34.35	53.79	11.86	24.04	54.55	21.41	29.67	56.17	14.16
Roasted garlic N	55.19	37.66	7.14	26.98	58.76	14.26	0.00	0.00	100.00
Roasted garlic WD	57.32	39.94	2.74	40.93	43.58	15.50	0.00	99.03	0.97
Roasted mushroom N	49.23	45.14	5.64	36.67	52.04	11.29	63.60	35.08	1.33
Roasted mushroom WD	60.51	23.47	16.02	25.03	61.00	13.97	57.51	39.48	3.01
Roasted onion N	54.67	27.23	18.11	22.74	58.79	18.47	100.00	0.00	0.00
Roasted onion WD	60.10	28.13	11.77	37.33	48.32	14.35	98.43	0.00	1.57
Roasted potato N	47.15	41.93	10.92	25.70	56.58	17.72	61.69	0.00	38.31

Roasted potato WD	66.10	22.81	11.10	21.54	48.81	29.65	2.06	97.29	0.65
Roasted pumpkin N	66.52	24.54	8.94	33.86	53.22	12.92	0.00	93.64	6.36
Roasted pumpkin WD	63.56	23.65	12.78	46.11	43.97	9.91	100.00	0.00	0.00
Roasted red pepper N	11.38	78.04	10.58	33.93	50.80	15.27	54.04	0.00	45.96
Roasted red pepper WD	55.73	30.29	13.98	40.35	53.41	6.24	35.62	63.94	0.44
Roasted tomato N	72.54	11.68	15.78	25.89	64.00	10.11	14.01	76.60	9.39
Roasted tomato WD	51.59	31.61	16.80	25.39	60.73	13.88	1.04	45.62	53.34
Roasted zucchini N	27.43	61.77	10.80	20.09	62.72	17.19	0.00	0.00	100.00
Roasted zucchini WD	57.60	29.11	13.28	12.37	71.02	16.61	0.00	0.00	100.00
Steamed artichoke N	26.60	63.70	9.70	73.84	23.16	3.00	54.86	44.49	0.65
Steamed asparagus N	48.82	40.46	10.71	22.99	55.46	21.55	1.03	98.97	0.00
Steamed asparagus WD	57.95	34.88	7.16	33.71	46.59	19.70	69.41	0.00	30.59
Steamed beans N	47.91	32.71	19.38	33.83	55.73	10.44	58.64	0.00	41.36
Steamed beans WD	48.63	43.24	8.12	29.00	51.10	19.90	62.74	0.00	37.26
Steamed cabbage N	48.98	38.54	12.48	21.06	67.23	11.71	35.90	62.76	1.34
Steamed cabbage WD	44.69	44.83	10.48	31.05	42.62	26.33	0.00	0.00	100.00

Steamed carrot N	60.22	25.85	13.93	24.82	60.32	14.85	38.86	0.00	61.14
Steamed carrot WD	54.10	29.17	16.73	30.91	57.70	11.38	71.82	26.72	1.46
Steamed cauliflower N	54.02	29.97	16.01	18.88	64.46	16.66	23.72	6.18	70.10
Steamed cauliflower WD	42.35	47.29	10.35	14.20	79.32	6.49	23.37	0.00	76.63
Steamed chard N	45.75	37.87	16.37	22.29	64.47	13.24	46.38	46.44	7.18
Steamed chard WD	42.63	47.57	9.80	10.96	81.42	7.62	0.00	91.81	8.19
Steamed leek N	78.72	11.06	10.21	22.83	58.78	18.39	30.00	0.00	70.00
Steamed leek WD	46.18	45.53	8.29	26.61	63.27	10.12	52.49	0.00	47.51
Steamed peas N	53.36	38.23	8.41	20.84	52.36	26.80	2.43	96.80	0.77
Steamed peas WD	64.51	19.07	16.42	31.01	59.20	9.79	36.05	62.90	1.04
Steamed potato N	50.92	38.66	10.42	18.85	70.58	10.57	0.00	98.65	1.35
Steamed potato WD	52.22	33.59	14.20	20.92	50.37	28.72	0.00	0.00	100.00
Steamed pumpkin N	58.79	31.56	9.65	27.89	59.79	12.32	0.00	27.45	72.55
Steamed pumpkin WD	69.81	17.63	12.55	15.06	65.39	19.56	0.00	40.92	59.08
Steamed spinach N	38.47	55.55	5.98	44.23	47.59	8.18	2.37	73.67	23.96
Steamed spinach WD	47.71	44.35	7.94	60.00	35.83	4.17	19.80	80.20	0.00

Steamed zucchini N	39.79	42.17	18.05	25.79	61.68	12.53	84.48	0.00	15.52
Steamed zucchini WD	52.31	39.44	8.26	23.89	53.39	22.72	0.00	100.00	0.00
Steamend artichoke WD	38.48	48.71	12.81	38.55	40.23	21.22	35.81	63.22	0.97
Overall mean	51.56	36.54	11.90	30.27	56.92	12.81	37.66	38.37	22.39

Table 41. Mean contribution percentage of each vegetable fraction to the global antioxidant capacity

		Overall mean	Boiled	Breaded	Fried	Grilled	Raw	Roasted	Steamed	N	R	WD
ABTS	**Digestion, %**	51.56	48.08	59.36	52.25	57.61	46.02	50.72	50.54	50.98	46.02	53.19
	Fermentation supernatant, %	36.54	39.01	31.73	35.49	29.86	42.53	38.52	37.76	37.43	42.53	34.51
	Fermentation solid residue, %	11.90	12.91	8.91	12.26	12.53	11.44	10.77	11.71	11.59	11.44	12.31
	Total, %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
FRAP	**Digestion, %**	30.27	26.01	36.80	33.34	36.69	24.08	29.91	28.62	28.72	24.08	33.00
	Fermentation supernatant, %	56.92	61.41	50.32	53.77	53.57	62.78	56.11	56.46	58.10	62.78	54.64
	Fermentation solid residue, %	12.81	12.58	12.88	12.89	9.74	13.13	13.98	14.92	13.18	13.13	12.37
	Total, %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
$TEAC_{OH}$	**Digestion, %**	37.66	44.08	57.41	36.31	34.58	51.13	34.55	28.85	36.25	51.13	36.52
	Fermentation supernatant, %	38.37	22.37	35.95	59.14	53.00	13.73	40.37	39.28	38.13	13.73	43.28
	Fermentation solid residue, %	22.39	29.70	6.64	4.55	8.58	35.14	25.08	31.87	23.89	35.14	18.47
	Total, %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

-Correlations between antioxidant capacity, culinary treatment and thermal indicators (**Table 42**). The type of culinary treatment (breaded, fried, grilled, roasted, boiled, steamed and raw) correlated positively and significantly ($p < 0.05$) with the antioxidant values from TEA_{ABTS} and $TEAC_{FRAP}$ from digestion, total $TEAC_{FRAP}$, and digestion-fermentation $TEAC_{OH}$ (r from 0.19 to 0.43). Treatment intensity (normal, well done and raw) correlated with digestion $TEAC_{FRAP}$ and $TEAC_{OH}$ (r from 0.17 to 0.21). On the other hand, furosine correlated positively ($p < 0.05$) with $TEAC_{FRAP}$ and $TEAC_{OH}$ from digestion, fermentation and total antioxidant capacity (r from 0.26 to 0.38). Finally, HMF correlated also with digestion and fermentation $TEAC_{FRAP}$ and $TEAC_{OH}$ (r from 0.28 to 0.36). These correlations could support that an aggressive treatment (such as frying of grilling) could improve the availability of some molecules due to cell break down. Another possible explanation could be the development of Maillard compounds, which in turn contribute to the antioxidant capacity of other different types of foods (Delgado-Andrade & Morales, 2005; Tagliazucchi et al., 2008; Dittrich et al., 2009; Martín et al., 2009; Carvalho et al., 2014; de la Cueva et al., 2017).

Table 42. Lineal correlations between antioxidant capacity, digestion-fermentation fractions and cooking intensity. D: digestion; LF: fermentation supernatant; SF: solid residue from fermentation; Total: total antioxidant capacity.

	Type of Culinary treatment		**Intensity of the treatment**		**Furosine**		**HMF**	
		R value		**R value**		**R value**		**R value**
TEACABTS-D	*	0.24	-		-		-	
TEACABTS-LF	-		-		-		-	
TEACABTS-SF	-		-		-		-	
TEACABTS-Total	-		-		-		-	
TEACFRAP-D	*	0.24	*	0.17	*	0.37	*	0.30
TEACFRAP-LF	-		-		*	0.26	-	
TEACFRAP-SF	-		-		-		-	
TEACFRAP-Total	*	0.19	-		*	0.38	*	0.28
TEACOH-D	*	0.40	-	0.21	*	0.31	*	0.30
TEACOH-LF	*	0.38	*		*	0.28	*	0.33
TEACOH-SF	-		-		-		-	
TEACOH-Total	*	0.43	*	0.22	*	0.33	*	0.36
Furosine	*	0.57	*	0.28			*	0.82
HMF	*	0.64	*	0.35	*	0.82		

-Dietary antioxidants per serving and daily antioxidant intake

Every food consumed has an impact on the overall antioxidant capacity, with the corresponding effect on human health. The antioxidant capacity of foods does not come only from one source but from the synergistic effect of a great number of different molecules such as vitamins, phenolic compounds, Maillard reaction products, molecules generated during digestion or fermentation, etc. (Pastoriza, Delgado-Andrade, Haro & Rufián-Henares, 2011). Accordingly, the antioxidant capacity coming from the daily diet (including all solid and liquid foods) is called dietary antioxidant capacity (Saura-Calixto, Pérez-Jiménez, & Goñi, 2009). That would refer to the amount of antioxidants, expressed as antioxidant units, that undergoes digestion and fermentation and are susceptible to serve human beings as radicals scavengers. Accordingly, two different calculations could be assessed: first, the daily consumption of a given food (MAPAMA, 2018) along with the antioxidant capacity of such foodstuff allows to calculate the contribution of such item to the daily antioxidant intake; secondly, the usual serving size of each foodstuff (García-Arias & García-Fernández, 2003) along with their antioxidant capacity per gram, allows to calculate the antioxidant capacity per serving size, and the contribution of such serving size to daily antioxidant capacity.

Table 43 shows the contribution to daily antioxidant intake and antioxidant capacity per serving size depending only on the vegetable type (including those vegetables for which consumption data in Spain are available). The contribution to daily antioxidant intake and antioxidant capacity per serving size of each vegetable per culinary technique and degree of intensity is in **Table 44**. In order to perform the calculations, the mean antioxidant capacity intake in Spain was obtained from Saura-Calixto & Goñi (2006): 3549 and 6014 μmol Trolox equivalents/day for the ABTS and

FRAP methods, respectively. Regarding the ABTS method, the vegetables with higher contribution to the daily antioxidant intake were potato (18.3%) followed by tomato (10.4%) and onion (5.4%). Although these vegetables were not the most antioxidant ones (garlic, cauliflower or asparagus were more antioxidant) they are the main contributors to daily antioxidant intake due to their high consumption. However, when focusing on the antioxidant capacity per serving size, garlic, mushroom, asparagus and cauliflower showed the higher percentages of contribution (from 70.6 to 79.2%) due to their higher antioxidant capacity per gram. In the case of the FRAP method, the contribution to daily antioxidant intake was also higher in potato, tomato and onion due to their high consumption. However, the larger serving size of red pepper make this food as the most important contributor to the daily intake of reducing capacity.

Table 43. Contribution of vegetables consumption to the daily antioxidant activity (AOX) intake in the Spanish diet.

Vegetable	**Daily consumption** g/day/person	**AOX assay**	**Content** μmol Trolox/ g	**AOX daily intake[1]** μmol Trolox/day	**Contribution to daily intake** %	**AOX serving intake[2]** μmol Trolox/serving	**Contribution to daily intake** %
Green leaf vegetables	3.84	$TEAC_{ABTS}$	9.90	38.0	1.1	1981	55.8
Garlic	2.74		12.7	34.9	1.0	2547	71.8
Eggplant	4.66		9.06	42.2	1.2	1811	51.0
Zucchini	10.7		8.95	95.6	2.7	1790	50.4
Onion	20.8		9.18	192	5.4	1836	51.7
Mushroom	3.84		12.5	48.1	1.4	2506	70.6
Cabbage	4.93		6.40	32.2	0.9	1308	36.8
Cauliflower	4.93		13.4	67.3	1.9	2728	76.9
Asparagus	1.92		14.5	27.0	0.8	2811	79.2
Beans	6.85		8.15	55.9	1.6	1631	46.0
Lettuce	12.3		11.7	144	4.1	2340	65.9
Potato	62.7		10.4	650	18.3	2074	58.4
Cucumber	6.30		7.45	46.9	1.3	1490	42.0
Red pepper	14.0		8.42	118	3.3	1683	47.4
Tomato	39.2		9.39	368	10.4	1879	52.9
Carrot	9.9		11.2	111	3.1	2241	63.1
Green leaf vegetables	3.84	$TEAC_{FRAP}$	2.49	9.57	0.2	499	8.3
Garlic	2.74		2.46	6.75	0.1	493	8.2

Eggplant	4.66	2.51	11.7	0.2	502	8.3
Zucchini	10.7	2.06	22.1	0.4	413	6.9
Onion	20.8	2.02	42.0	0.7	403	6.7
Mushroom	3.84	2.23	8.56	0.1	447	7.4
Cabbage	4.93	2.35	11.6	0.2	471	7.8
Cauliflower	4.93	2.44	12.0	0.2	488	8.1
Asparagus	1.92	2.59	4.98	0.1	519	8.6
Beans	6.85	2.13	14.6	0.2	426	7.1
Lettuce	12.3	1.55	19.1	0.3	309	5.1
Potato	62.7	1.90	119	2.0	381	6.3
Cucumber	5.63	1.69	10.6	0.2	338	5.6
Red pepper	12.2	3.67	51.3	0.9	734	12.2
Tomato	6.04	1.82	71.1	1.2	363	6.0
Carrot	7.22	2.16	21.3	0.4	432	7.2

[1]Considering consumption for a whole year.
[2]Consideing the complete serving ingested a particular day.

Table 44. Contribution of vegetable consumption to the daily antioxidant capacity intake

	Culinary treatment	Intensity	AOX assay	Daily AOX intake, µmol Trolox/day	Content, µmol Trolox/ g	Daily intake, µmol Trolox/day	Contribution to daily intake, %	Content per serving intake, µmol Trolox/serving	Contribution to daily intake, %
Green leaf vegetables mean	Boiled	N	*ABTS*	3549	12.73	48.814	1.3754	2545.28	71.72
(Chard, Spinach)	Boiled	WD	*ABTS*	3549	10.79	41.372	1.1657	2157.25	60.78
	Steamed	N	*ABTS*	3549	14.01	53.730	1.5140	2801.66	78.94
	Steamed	WD	*ABTS*	3549	13.89	53.271	1.5010	2777.71	78.27
Garlic	Roasted	N	*ABTS*	3549	13.56	37.144	1.0466	2711.52	76.40
Garlic	Roasted	WD	*ABTS*	3549	12.99	35.581	1.0026	2597.43	73.19
Garlic	Raw	R	*ABTS*	3549	13.61	37.290	1.0507	2722.15	76.70
Garlic	Fried	N	*ABTS*	3549	13.71	37.553	1.0581	2741.35	77.24
Garlic	Fried	WD	*ABTS*	3549	11.10	30.407	0.8568	2219.73	62.55
Garlic	Grilled	N	*ABTS*	3549	11.75	32.200	0.9073	2350.58	66.23
Garlic	Grilled	WD	*ABTS*	3549	12.44	34.095	0.9607	2488.90	70.13
Eggplant	Roasted	N	*ABTS*	3549	8.80	40.988	1.1549	1760.07	49.59
Eggplant	Roasted	WD	*ABTS*	3549	12.41	57.802	1.6287	2482.09	69.94

Eggplant	Fried	N	*ABTS*	3549	7.56	35.216	0.9923	1512.20	42.61
Eggplant	Fried	WD	*ABTS*	3549	13.94	64.946	1.8300	2788.86	78.58
Eggplant	Grilled	N	*ABTS*	3549	8.04	37.426	1.0545	1607.12	45.28
Eggplant	Grilled	WD	*ABTS*	3549	7.27	33.865	0.9542	1454.19	40.97
Eggplant	Breaded	N	*ABTS*	3549	7.21	33.580	0.9462	1441.95	40.63
Eggplant	Breaded	WD	*ABTS*	3549	7.22	33.609	0.9470	1443.20	40.67
Zucchini	Roasted	N	*ABTS*	3549	15.95	170.434	4.8023	3190.17	89.89
Zucchini	Roasted	WD	*ABTS*	3549	8.06	86.158	2.4277	1612.69	45.44
Zucchini	Boiled	N	*ABTS*	3549	9.01	96.288	2.7131	1802.31	50.78
Zucchini	Boiled	WD	*ABTS*	3549	7.69	82.220	2.3167	1538.99	43.36
Zucchini	Grilled	N	*ABTS*	3549	7.06	75.461	2.1263	1412.48	39.80
Zucchini	Grilled	WD	*ABTS*	3549	5.42	57.962	1.6332	1084.94	30.57
Zucchini	Steamed	N	*ABTS*	3549	9.91	105.910	2.9842	1982.42	55.86
Zucchini	Steamed	WD	*ABTS*	3549	8.47	90.481	2.5495	1693.62	47.72
Onion	Roasted	N	*ABTS*	3549	10.95	227.906	6.4217	2189.10	61.68
Onion	Roasted	WD	*ABTS*	3549	10.04	209.133	5.8927	2008.78	56.60

Onion	Raw	R	*ABTS*	3549	6.58	136.988	3.8599	1315.81	37.08
Onion	Fried	N	*ABTS*	3549	8.34	173.552	4.8902	1667.01	46.97
Onion	Fried	WD	*ABTS*	3549	7.97	166.020	4.6779	1594.67	44.93
Onion	Grilled	N	*ABTS*	3549	10.99	228.775	6.4462	2197.45	61.92
Onion	Grilled	WD	*ABTS*	3549	9.40	195.804	5.5172	1880.75	52.99
Mushroom	Roasted	N	*ABTS*	3549	15.92	61.078	1.7210	3184.81	89.74
Mushroom	Roasted	WD	*ABTS*	3549	11.53	44.231	1.2463	2306.33	64.99
Mushroom	Grilled	N	*ABTS*	3549	11.05	42.378	1.1941	2209.71	62.26
Mushroom	Grilled	WD	*ABTS*	3549	11.61	44.546	1.2552	2322.74	65.45
Cabbage	Boiled	N	*ABTS*	3549	8.30	40.914	1.1528	1659.27	46.75
Cabbage	Boiled	WD	*ABTS*	3549	5.71	28.141	0.7929	1141.29	32.16
Cabbage	Raw	R	*ABTS*	3549	5.22	25.765	0.7260	1044.91	29.44
Cabbage	Grilled	N	*ABTS*	3549	6.26	30.868	0.8698	1251.85	35.27
Cabbage	Grilled	WD	*ABTS*	3549	6.05	29.848	0.8410	1210.51	34.11
Cabbage	Steamed	N	*ABTS*	3549	7.10	34.996	0.9861	1419.27	39.99
Cabbage	Steamed	WD	*ABTS*	3549	7.13	35.137	0.9901	1425.01	40.15

Cauliflower	Boiled	N	*ABTS*	3549	12.78	63.014	1.7755	2555.57	72.01
Cauliflower	Boiled	WD	*ABTS*	3549	15.87	78.264	2.2052	3174.04	89.43
Cauliflower	Breaded	N	*ABTS*	3549	15.51	76.504	2.1557	3102.67	87.42
Cauliflower	Breaded	WD	*ABTS*	3549	17.13	84.466	2.3800	3425.56	96.52
Cauliflower	Steamed	N	*ABTS*	3549	7.20	35.512	1.0006	1440.21	40.58
Cauliflower	Steamed	WD	*ABTS*	3549	13.33	65.761	1.8530	2666.99	75.15
Asparagus	Roasted	N	*ABTS*	3549	7.99	15.326	0.4318	1598.28	45.03
Asparagus	Roasted	WD	*ABTS*	3549	18.73	35.917	1.0120	3745.65	105.54
Asparagus	Boiled	N	*ABTS*	3549	12.99	24.915	0.7020	2598.30	73.21
Asparagus	Boiled	WD	*ABTS*	3549	7.93	15.201	0.4283	1585.20	44.67
Asparagus	Grilled	N	*ABTS*	3549	25.96	49.777	1.4026	5191.04	146.27
Asparagus	Grilled	WD	*ABTS*	3549	12.09	23.194	0.6535	2418.84	68.16
Asparagus	Steamed	N	*ABTS*	3549	14.36	27.547	0.7762	2872.76	80.95
Asparagus	Steamed	WD	*ABTS*	3549	12.37	23.726	0.6685	2474.26	69.72
Beans	Boiled	N	*ABTS*	3549	5.68	38.929	1.0969	1136.71	32.03
Beans	Boiled	WD	*ABTS*	3549	9.49	64.998	1.8314	1897.94	53.48

Beans	Steamed	N	*ABTS*	3549	8.07	55.280	1.5576	1614.19	45.48
Beans	Steamed	WD	*ABTS*	3549	9.37	64.205	1.8091	1874.80	52.83
Lettuce	Raw	R	*ABTS*	3549	11.70	144.226	4.0639	2339.67	65.92
Potato	Roasted	N	*ABTS*	3549	8.26	518.430	14.6078	1652.64	46.57
Potato	Roasted	WD	*ABTS*	3549	8.79	551.480	15.5390	1757.99	49.53
Potato	Boiled	N	*ABTS*	3549	8.50	533.379	15.0290	1700.29	47.91
Potato	Boiled	WD	*ABTS*	3549	11.26	706.498	19.9070	2252.16	63.46
Potato	Fried	N	*ABTS*	3549	18.82	1180.581	33.2652	3763.43	106.04
Potato	Fried	WD	*ABTS*	3549	7.11	446.035	12.5679	1421.86	40.06
Potato	Steamed	N	*ABTS*	3549	8.67	543.931	15.3263	1733.93	48.86
Potato	Steamed	WD	*ABTS*	3549	11.55	724.828	20.4235	2310.59	65.11
Cucumber	Raw	R	*ABTS*	3549	7.45	46.934	1.3225	1489.64	41.97
Red pepper	Roasted	N	*ABTS*	3549	12.51	174.834	4.9263	2502.53	70.51
Red pepper	Roasted	WD	*ABTS*	3549	10.72	149.782	4.2204	2143.94	60.41
Red pepper	Raw	R	*ABTS*	3549	9.35	130.646	3.6812	1870.03	52.69
Red pepper	Fried	N	*ABTS*	3549	7.89	110.183	3.1046	1577.14	44.44

Red pepper	Fried	WD	*ABTS*	3549	3.08	43.004	1.2117	615.55	17.34
Red pepper	Grilled	N	*ABTS*	3549	11.40	159.231	4.4866	2279.18	64.22
Red pepper	Grilled	WD	*ABTS*	3549	3.96	55.395	1.5609	792.91	22.34
Tomato	Roasted	N	*ABTS*	3549	7.43	290.939	8.1978	1485.21	41.85
Tomato	Roasted	WD	*ABTS*	3549	11.58	453.807	12.7869	2316.64	65.28
Tomato	Raw	R	*ABTS*	3549	9.19	360.126	10.1473	1838.41	51.80
Tomato	Grilled	N	*ABTS*	3549	11.62	455.176	12.8255	2323.62	65.47
Tomato	Grilled	WD	*ABTS*	3549	7.15	280.114	7.8928	1429.95	40.29
Carrot	Roasted	N	*ABTS*	3549	24.70	243.589	6.8636	4939.45	139.18
Carrot	Roasted	WD	*ABTS*	3549	10.63	104.802	2.9530	2125.15	59.88
Carrot	Boiled	N	*ABTS*	3549	9.45	93.234	2.6270	1890.57	53.27
Carrot	Boiled	WD	*ABTS*	3549	16.28	160.578	4.5246	3256.17	91.75
Carrot	Raw	R	*ABTS*	3549	7.29	71.904	2.0260	1458.06	41.08
Carrot	Grilled	N	*ABTS*	3549	8.54	84.222	2.3731	1707.83	48.12
Carrot	Grilled	WD	*ABTS*	3549	10.52	103.723	2.9226	2103.27	59.26
Carrot	Steamed	N	*ABTS*	3549	6.77	66.781	1.8817	1354.16	38.16

Carrot	Steamed	WD	*ABTS*	3549	6.66	65.706	1.8514	1332.37	37.54
Green leaf vegetables mean	Boiled	N	*FRAP*	6014	1.65	6.333	0.1053	330.24	5.49
(Chard, Spinach)	Boiled	WD	*FRAP*	6014	1.73	6.649	0.1106	346.72	5.77
	Steamed	N	*FRAP*	6014	2.57	9.876	0.1642	514.96	8.56
	Steamed	WD	*FRAP*	6014	3.82	14.633	0.2433	763.03	12.69
Garlic	Roasted	N	*FRAP*	6014	1.78	4.874	0.0811	355.83	5.92
Garlic	Roasted	WD	*FRAP*	6014	2.32	6.350	0.1056	463.57	7.71
Garlic	Raw	R	*FRAP*	6014	3.73	10.220	0.1699	746.07	12.41
Garlic	Fried	N	*FRAP*	6014	1.67	4.566	0.0759	333.32	5.54
Garlic	Fried	WD	*FRAP*	6014	3.08	8.447	0.1404	616.60	10.25
Garlic	Grilled	N	*FRAP*	6014	2.50	6.841	0.1137	499.37	8.30
Garlic	Grilled	WD	*FRAP*	6014	2.18	5.974	0.0993	436.11	7.25
Eggplant	Roasted	N	*FRAP*	6014	3.27	15.224	0.2532	653.76	10.87
Eggplant	Roasted	WD	*FRAP*	6014	1.93	8.987	0.1494	385.91	6.42
Eggplant	Fried	N	*FRAP*	6014	3.79	17.632	0.2932	757.15	12.59

Eggplant	Fried	WD	*FRAP*	6014	3.69	17.167	0.2854	737.16	12.26
Eggplant	Grilled	N	*FRAP*	6014	1.80	8.396	0.1396	360.53	5.99
Eggplant	Grilled	WD	*FRAP*	6014	1.66	7.748	0.1288	332.69	5.53
Eggplant	Breaded	N	*FRAP*	6014	1.70	7.921	0.1317	340.15	5.66
Eggplant	Breaded	WD	*FRAP*	6014	2.23	10.365	0.1723	445.08	7.40
Zucchini	Roasted	N	*FRAP*	6014	1.51	16.097	0.2677	301.31	5.01
Zucchini	Roasted	WD	*FRAP*	6014	2.63	28.092	0.4671	525.83	8.74
Zucchini	Boiled	N	*FRAP*	6014	1.40	14.936	0.2484	279.57	4.65
Zucchini	Boiled	WD	*FRAP*	6014	1.58	16.839	0.2800	315.19	5.24
Zucchini	Grilled	N	*FRAP*	6014	1.90	20.325	0.3380	380.44	6.33
Zucchini	Grilled	WD	*FRAP*	6014	4.18	44.647	0.7424	835.70	13.90
Zucchini	Steamed	N	*FRAP*	6014	1.51	16.184	0.2691	302.94	5.04
Zucchini	Steamed	WD	*FRAP*	6014	1.80	19.285	0.3207	360.98	6.00
Onion	Roasted	N	*FRAP*	6014	2.22	46.172	0.7677	443.50	7.37
Onion	Roasted	WD	*FRAP*	6014	1.53	31.893	0.5303	306.34	5.09
Onion	Raw	R	*FRAP*	6014	1.91	39.725	0.6605	381.56	6.34
Onion	Fried	N	*FRAP*	6014	1.94	40.488	0.6732	388.90	6.47

Onion	Fried	WD	*FRAP*	6014	2.35	48.831	0.8120	469.04	7.80
Onion	Grilled	N	*FRAP*	6014	1.83	38.007	0.6320	365.07	6.07
Onion	Grilled	WD	*FRAP*	6014	2.33	48.581	0.8078	466.63	7.76
Mushroom	Roasted	N	*FRAP*	6014	2.29	8.775	0.1459	457.54	7.61
Mushroom	Roasted	WD	*FRAP*	6014	2.38	9.118	0.1516	475.42	7.91
Mushroom	Grilled	N	*FRAP*	6014	2.39	9.153	0.1522	477.28	7.94
Mushroom	Grilled	WD	*FRAP*	6014	1.88	7.207	0.1198	375.78	6.25
Cabbage	Boiled	N	*FRAP*	6014	1.32	6.511	0.1083	264.04	4.39
Cabbage	Boiled	WD	*FRAP*	6014	2.27	11.198	0.1862	454.16	7.55
Cabbage	Raw	R	*FRAP*	6014	2.86	14.096	0.2344	571.66	9.51
Cabbage	Grilled	N	*FRAP*	6014	2.14	10.552	0.1755	427.93	7.12
Cabbage	Grilled	WD	*FRAP*	6014	3.56	17.574	0.2922	712.72	11.85
Cabbage	Steamed	N	*FRAP*	6014	1.91	9.431	0.1568	382.46	6.36
Cabbage	Steamed	WD	*FRAP*	6014	2.40	11.860	0.1972	480.99	8.00
Cauliflower	Boiled	N	*FRAP*	6014	2.03	9.989	0.1661	405.11	6.74
Cauliflower	Boiled	WD	*FRAP*	6014	1.37	6.771	0.1126	274.58	4.57

Cauliflower	Breaded	N	*FRAP*	6014	3.95	19.479	0.3239	789.98	13.14
Cauliflower	Breaded	WD	*FRAP*	6014	3.45	17.026	0.2831	690.51	11.48
Cauliflower	Steamed	N	*FRAP*	6014	1.77	8.705	0.1447	353.04	5.87
Cauliflower	Steamed	WD	*FRAP*	6014	2.07	10.211	0.1698	414.12	6.89
Asparagus	Roasted	N	*FRAP*	6014	1.95	3.740	0.0622	390.02	6.49
Asparagus	Roasted	WD	*FRAP*	6014	3.76	7.216	0.1200	752.57	12.51
Asparagus	Boiled	N	*FRAP*	6014	1.86	3.571	0.0594	372.38	6.19
Asparagus	Boiled	WD	*FRAP*	6014	2.64	5.071	0.0843	528.84	8.79
Asparagus	Grilled	N	*FRAP*	6014	2.85	5.472	0.0910	570.68	9.49
Asparagus	Grilled	WD	*FRAP*	6014	2.75	5.278	0.0878	550.39	9.15
Asparagus	Steamed	N	*FRAP*	6014	2.46	4.710	0.0783	491.14	8.17
Asparagus	Steamed	WD	*FRAP*	6014	2.48	4.749	0.0790	495.27	8.24
Beans	Boiled	N	*FRAP*	6014	3.47	23.788	0.3955	694.60	11.55
Beans	Boiled	WD	*FRAP*	6014	1.71	11.700	0.1945	341.63	5.68
Beans	Steamed	N	*FRAP*	6014	1.58	10.799	0.1796	315.32	5.24
Beans	Steamed	WD	*FRAP*	6014	1.77	12.094	0.2011	353.14	5.87

Lettuce	Raw	R	*FRAP*	6014	1.55	19.063	0.3170	309.25	5.14
Potato	Roasted	N	*FRAP*	6014	1.76	110.394	1.8356	351.91	5.85
Potato	Roasted	WD	*FRAP*	6014	1.38	86.565	1.4394	275.95	4.59
Potato	Boiled	N	*FRAP*	6014	3.18	199.812	3.3224	636.95	10.59
Potato	Boiled	WD	*FRAP*	6014	1.61	101.248	1.6835	322.75	5.37
Potato	Fried	N	*FRAP*	6014	1.47	91.930	1.5286	293.05	4.87
Potato	Fried	WD	*FRAP*	6014	1.88	118.146	1.9645	376.62	6.26
Potato	Steamed	N	*FRAP*	6014	2.12	133.069	2.2127	424.19	7.05
Potato	Steamed	WD	*FRAP*	6014	1.81	113.605	1.8890	362.15	6.02
Cucumber	Raw	R	*FRAP*	6014	1.69	10.640	0.1769	337.70	5.62
Red pepper	Roasted	N	*FRAP*	6014	2.76	38.584	0.6416	552.28	9.18
Red pepper	Roasted	WD	*FRAP*	6014	4.35	60.721	1.0097	869.15	14.45
Red pepper	Raw	R	*FRAP*	6014	4.93	68.905	1.1457	986.29	16.40
Red pepper	Fried	N	*FRAP*	6014	3.14	43.862	0.7293	627.83	10.44
Red pepper	Fried	WD	*FRAP*	6014	4.46	62.302	1.0359	891.77	14.83
Red pepper	Grilled	N	*FRAP*	6014	3.53	49.284	0.8195	705.43	11.73

Red pepper	Grilled	WD	*FRAP*	6014	2.54	35.473	0.5898	507.75	8.44
Tomato	Roasted	N	*FRAP*	6014	1.50	58.619	0.9747	299.24	4.98
Tomato	Roasted	WD	*FRAP*	6014	1.60	62.646	1.0417	319.80	5.32
Tomato	Raw	R	*FRAP*	6014	2.16	84.808	1.4102	432.94	7.20
Tomato	Grilled	N	*FRAP*	6014	1.46	57.285	0.9525	292.44	4.86
Tomato	Grilled	WD	*FRAP*	6014	2.35	92.222	1.5335	470.78	7.83
Carrot	Roasted	N	*FRAP*	6014	1.61	15.836	0.2633	321.12	5.34
Carrot	Roasted	WD	*FRAP*	6014	2.41	23.783	0.3955	482.26	8.02
Carrot	Boiled	N	*FRAP*	6014	1.86	18.365	0.3054	372.40	6.19
Carrot	Boiled	WD	*FRAP*	6014	1.93	19.046	0.3167	386.22	6.42
Carrot	Raw	R	*FRAP*	6014	1.49	14.680	0.2441	297.69	4.95
Carrot	Grilled	N	*FRAP*	6014	4.01	39.565	0.6579	802.28	13.34
Carrot	Grilled	WD	*FRAP*	6014	2.61	25.720	0.4277	521.55	8.67
Carrot	Steamed	N	*FRAP*	6014	1.50	14.769	0.2456	299.48	4.98
Carrot	Steamed	WD	*FRAP*	6014	2.03	19.989	0.3324	405.33	6.74

Table 45 shows the contribution of vegetables to the daily intake of antioxidant capacity depending on the culinary technique and the degree of intensity. In order to perform the calculations, the mean daily consumption of vegetables (171 g/day/person) was taken into account. Focusing on the type of culinary treatment, in the case of the ABTS method breaded, fried and roasted vegetables (from 66.3 to 67.5%) had the highest contributions to the daily antioxidant intake. For the FRAP method fried and breaded vegetables showed the highest percentages of contribution (from 9.4 to 10.7%). Both situations are related to a higher antioxidant capacity per gram of food, as explained in the previous section. On the other hand, when focusing on the degree of intensity, regarding N vegetables had the highest contribution (for the ABTS method) followed closely by well-done vegetables. However, in the case of FRAP, the higher contribution was obtained for raw vegetables, followed very closely by WD vegetables. These differences could be due to a loss of reducing compounds during cooking. However, Maillard reaction compounds could also participate in antioxidant capacity making it higher in WD than N vegetables. ABTS· trapping ability, however, could increase with cooking but decrease if it is done for a longer time.

Table 45. Contribution of vegetables consumption (depending on their culinary processing) to the daily antioxidant activity (AOX) intake in the Spanish diet.

Culinary treatment	**AOX assay**	**Content** µmol Trolox/ g			**Daily intake** µmol Trolox/day[1]			**Contribution to daily intake** %			**Content per serving intake** µmol Trolox/serving[2]			**Contribution to daily intake** %		
		min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max
Boiled	$TEAC_{ABTS}$	5.7	10.6	18.6	970	1804	317	27.3	50.8	89.4	1137	2114	3717	32.0	59.6	105
Breaded		7.2	11.8	17.1	1231	2008	2924	34.7	56.6	82.4	1442	2353	3426	40.6	66.3	96.5
Fried		3.1	11.8	22.2	525	2021	3781	14.8	57.0	107	616	2367	4431	17.3	66.7	125
Grilled		4.0	10.4	26.0	676	1776	4430	19.1	50.0	125	793	2081	5191	22.3	58.6	146
Raw		5.2	9.3	13.6	892	1581	2323	25.1	44.6	65.5	1045	1853	2722	29.4	52.2	76.7
Roasted		7.4	12.0	24.7	1268	2043	4215	35.7	57.6	119	1485	2394	4939	41.8	67.4	139
Steamed		6.7	10.4	15.8	1137	1778	2696	32.0	50.1	76.0	1332	2083	3159	37.5	58.7	89.0
Boiled	$TEAC_{FRAP}$	0.4	2.1	7.3	75.6	362	1242	1.3	6.0	20.7	88.6	424	1456	1.5	7.1	24.2
Breaded		1.7	2.8	3.9	290	483	674	4.8	8.0	11.2	340	566	790	5.7	9.4	13.1
Fried		1.5	3.2	7.3	250	551	1238	4.2	9.2	20.6	293	646	1450	4.9	10.7	24.1

Grilled		1.4	2.7	5.8	241	458	982	4.0	7.6	16.3	282	537	1150	4.7	8.9	19.1
Raw		1.5	2.6	4.9	254	451	842	4.2	7.5	14.0	298	529	986	4.9	8.8	16.4
Roasted		1.4	2.2	4.3	236	380	742	3.9	6.3	12.3	276	445	869	4.6	7.4	14.5
Steamed		1.3	2.2	4.6	228	383	783	3.8	6.4	13.0	267	449	917	4.4	7.5	15.2
N	$TEAC_{ABTS}$	5.7	11.1	26.0	970	1898	4430	27.3	53.5	125	1137	2224	5191	32.0	62.7	146
R		5.2	9.3	13.6	892	1581	2323	25.1	44.6	65.5	1045	1853	2722	29.4	52.2	76.7
WD		3.1	10.8	20.5	525	1835	3491	14.8	51.7	98.4	616	2150	4090	17.3	60.6	115
N	$TEAC_{FRAP}$	0.4	2.3	7.3	75.6	392	1238	1.3	6.5	20.6	88.6	459	1450	1.5	7.6	24.1
R		1.5	2.6	4.9	254	451	842	4.2	7.5	14.0	298	529	986	4.9	8.8	16.4
WD		1.3	2.6	7.3	228	439	1242	3.8	7.3	20.7	267	514	1456	4.4	8.5	24.2

[1]Considering consumption for a whole year.
[2]Consideing the complete serving ingested a particular day.
N: Normal cooking time; R: Raw (not cooked); WD: Well done.

4. Conclusions

In conclusion, furosine and HMF are useful indicators to control both cooking time and heat intensity of common vegetables. In addition, it has been demonstrated that furosine and HMF correlate with the evolution of antioxidant capacity of vegetables in many different cooking techniques. In this sense, those samples cooked with aggressive techniques (frying, grilling or breading) showed the higher antioxidant values. This could be related with the release of antioxidant compounds both due to degradation of cell structures or the generation of neo-formed Maillard reaction products with high antioxidant capacity. Our results suggests that with raw, steamed, boiled or roasted vegetables the substrates that reach the colon are different at some extent than those that come from fried, breaded or grilled vegetables. This fact, as demonstrated with antioxidant capacity, could promote different microbial communities and therefore have some effect on host health. In addition, another plausible reason could be the use of EVOO on such culinary preparations. Finally, it is noteworthy to mention that the GAR+ (Global Antioxidant Response after gastrointestinal digestion and microbial fermentation) method allows unravelling the contribution to total antioxidant capacity of different fractions obtained after digestion and fermentation of vegetables. Since the liquid and solid fractions obtained after microbial fermentation exert a high antioxidant capacity, future studies should include the GAR+ approach to study the modifications on the bioactivity of food as a first step before animal and human nutritional interventions.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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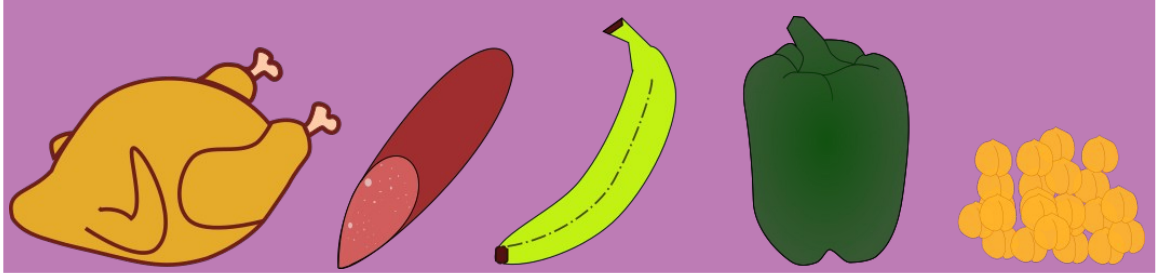
Capítulo IV. Otros alimentos.



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Effect of food thermal processing on the composition of the gut microbiota

En este trabajo se estudió el efecto del procesado térmico (tipo de cocinado) sobre alimentos pertenecientes a los grupos más representativos de la dieta (pollo, plátano, pimiento, garbanzos y pan). Este estudio se realizó tras ser someter al proceso de digestión-fermentación in vitro dichos alimentos, lo que además nos permitió evaluar el efecto que tiene tanto el tipo de alimento como el tipo de cocinado sobre la composición de la microbiota intestinal, así como sobre su funcionalidad. La importancia de este estudio radica en que ofrece datos acerca de qué tipo de cocinado podría ser más saludable, estando por tanto estrechamente relacionado con la presente tesis doctoral.

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Effect of food thermal processing on the composition of the gut microbiota

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ABSTRACT

Cooking modifies food composition due to chemical reactions. Additionally, food composition shapes the human gut microbiota. Thus, the objective of this research was to unravel the effect of different food cooking methods on the structure and functionality of the gut microbiota. Common culinary techniques were applied to five foods, which were submitted to *in vitro* digestion-fermentation. Furosine, HMF (5-hydroxymethyl-furfural) and furfural were used as Maillard reaction indicators to control the heat treatment. Short chain fatty acids production was quantified as indicator of healthy metabolic output. Gut microbial community structure was analyzed through 16S rRNA. Both food composition and cooking methods modified the microbiota composition and release short chain fatty acids. In general, intense cooking technologies (roasting and grilling) increased the abundance of beneficial bacteria like *Ruminococcus spp*. or *Bifidobacterium spp*. compared to milder treatments (boiling). However, for some foods (banana or bread) intense cooking decreased the levels of healthy bacteria.

KEYWORDS: Cooking, food processing, gut microbiota, Maillard reaction, metagenomics.

Introduction

The human intestine is inhabited by a massive number of microorganisms and, although microbes are present throughout the gastrointestinal tract, the distal colon population is the most studied. It has been estimated that around 10^{13}-10^{14} microorganisms are present, most of them belonging to the phyla Firmicutes and Bacteroidetes .[1] It is now very clear that the gut microbiota has important implications in different diseases and is therefore closely related to health status. [2] In this sense, the gut microbiota has been linked to inflammatory bowel disease, obesity, autism spectrum disorders and immune system disorders, [3] among others.

The gut microbiota is influenced by several factors such as age and antibiotics intake, but diet is likely to be one of the most influential factors. [1] Accordingly, several studies have demonstrated important differences in gut microbiota among populations of different regions. [4] However, not only long-term dietary patterns shape the gut microbiota composition and functionality, but also shifts from protein-rich to vegetable-rich diet can rapidly affect the gut microbiota. [5] On the other hand, a significant amount of nutrients escape digestion and absorption at the small intestine and reach the colon, including fibers, resistant starch, some proteins and fats as well as bile acids and some phytochemicals like polyphenols. [1] Such molecules become substrates for microbial transformations, producing molecules with beneficial (e.g. short chain fatty acids, SCFAs) or detrimental effects (e.g. trimethylamine). [4] Therefore, microbial metabolites are some of the main the bioactive molecules that play a role in human health, and thus it is imperative to analyze how their production will be influenced by diet. [2]

Since gut microbiota can be rapidly affected by diet, it is important to unravel the specific effects of foodstuffs from different groups like vegetables, fruits, meat, legumes or cereals, among others. However, the gut microbiota ability to use substrates

could also be influenced by the culinary-heat treatment undergone by the foodstuffs prior to ingestion. [6] Upon cooking, many different compounds will be generated, most of them derived from the Maillard reaction. [7] All these neo-formed compounds could have some effect over the gut microbiota and, in fact, melanoidins are known to behave as fiber, and therefore as substrate for gut microbes. [8] The Maillard reaction (MR), and thus thermal damage, can be monitored along cooking through the formation of furosine (early stage indicator) and 5-hydroxymethylfurfural (HMF) or furfural (intermediate stage indicators). [9]

Only a few studies have been carried out on the effect of cooking conditions on gut microbiota composition, being mostly focused on meats and grain legumes. [6] Therefore, our main objective was to shed light on gut microbiota changes produced after fermentation of meat, fruits, vegetables, cereals and legumes while paying special attention to the influence of cooking techniques and heat damage. In order to achieve this goal, we selected chicken, banana, red pepper, bread and chickpeas and exposed them to the most common culinary techniques for each of them (frying, boiling, grilling, roasting, toasting *Vs.* raw). Then, we linked microbial changes to the type of food, culinary treatment or heat intensity (in terms of thermal damage monitored through furosine and HMF-furfural content).

Materials and methods

Reagents

Furosine was obtained from NeoMPS (Strasbourg, France). Furfural, 5-(Hydroxymethyl)furfural, hydrochloric acid, formic, acetic, propionic and butyric acids, potassium ferrocyanide, zinc acetate, potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, sodium di-hydrogen phosphate, salivary alpha-amylase, pepsin from porcine, bile acids (bile extract porcine), tryptone, cysteine, sodium sulphide, resazurin, hydrochloric acid, methanol and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Pancreatin from porcine pancreas was purchased from Alpha Aesar (Lancashire, United Kingdom).

Samples

Samples were representative foods from 5 different groups of foodstuffs: chicken (meat), chickpeas (legumes), wheat bread (cereals), red pepper (vegetables) and banana (fruits). The main criteria to choose these foods was to have some food representing each of the main consumed groups of foodstuffs. Therefore, we would have high protein food (chicken), starchy foods (banana and bread [fruit and cereal], fiber rich food (pepper), and high carbohydrate and protein food (chickpeas). Each one was submitted to the different types of cooking that are usual for them: chicken was boiled, fried, grilled and roasted; wheat bread was used raw and toasted (low toasting and high toasting degree), red pepper was used raw, fried and roasted; chickpeas were boiled and grilled; banana was used raw, roasted and fried. Boiling was carried out at 100 ^{o}C for 20 minutes (N) with a proportion water:food of 5:1. Extra virgin olive oil

(EVOO) was used as liquid medium for grilling and frying. Grilling was carried out at 220-250 ºC for 3 minutes on each side (N) with a proportion oil:food of 0.5:1. Frying was carried out at 180 ºC for 8 minutes (N) with a proportion oil:food of 5:1. Roasted was carried out at 180 ºC for 8 minutes. Toasted bread was prepared in a toaster at two different degrees: low and high. After cooking, samples were homogenized and stored at -80 ºC until analysis.

In vitro gastrointestinal digestion

All samples were subjected to an *in vitro* digestion process followed by an *in vitro* fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out according to the protocol described by Perez-Burillo et al., (2017). [10] The gastrointestinal *in vitro* digestion was composed of an oral phase (5 minutes at 37 ºC with alpha-amylase 75 U/mL, pH 7.0), a gastric phase (2 hours at 37 ºC with pepsin 2000U/mL at pH 3.0) and an intestinal phase (2 hours at 37 ºC with pancreatin 13.37 mg/mL, bile salts at a concentration of 10 mM and $CaCl_2$ at a concentration of 0.3 mM, at pH 7.0).

In vitro fermentation

The *in vitro* fermentation was carried out according to the protocol described by Perez-Burillo et al., (2017). [10] *In vitro* fermentation was carried out using faecal samples from three healthy donors (not taking antibiotics, people with body mass index within the “normal weight range”, mean Body Mass Index = 21.3). The faecal samples from the donors were pooled together and the pool used as the inoculum. After *in vitro* digestion, the solid residue (fraction not available for absorption) that is left after

removing the supernatant, plus 10% of such digestion supernatant are used as substrate for fermentation. The amount of solid residue used is 500 mg.

After *in vitro* gastrointestinal digestion and *in vitro* fermentation three different fractions were obtained: digestion supernatant (fraction available for absorption at the small intestine), fermentation supernatant (fraction available for absorption at the large intestine) and fermentation solid residue (fraction not available for absorption and excreted with feces).

Furosine assay

Furosine determination was performed following the method described by Rufian-Henares et al. (2013). [11] Briefly, samples were hydrolysed with 7.95 M HCl at 120 °C for 23 h. The hydrolisate was purified with a Sep-pack C_{18} cartridge (Millipore, MA) and the resulting solution was analysed by ion-pair RP-HPLC. The analysis was performed in duplicate and the data are the mean values expressed as μg per g of food.

HMF and furfural assay

HMF determination was performed according to the method described by Rufian-Henares et al. (2008). [9] The ground sample was suspended in deionised water, clarified with Carrez I (potassium ferrocyanide, 15% w/v) and Carrez II (zinc acetate 30% w/v) solutions. The resulting solution was analysed with RP-HPLC. The analysis was performed in duplicate and the data are the mean values expressed as μg per g of food.

Short chain fatty acids determination

SCFAs determination was carried out according to the procedure described in Panzella et al. (2017), [12] with few modifications. SCFAs were determined in fermentation supernatant. After the fermentation process, the supernatant was centrifuged filtered through a 0.22 μm nylon filter and analysed by means of a HPLC system. The analysis was performed in duplicate and the data are the mean values expressed as μmol per g of food.

DNA extraction and sequencing

DNA extraction was performed using a NucliSENS easyMAG platform (Biomérieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/μL in 10 mM Tris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in Klindworth et al. (2013). [13] Primer sequences are: Forward 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA-G3' and Reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA-CHVGGGTATCTAATCC3'. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification, amplicons were multiplexed and 1 ml of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001).

Bioinformatic analysis

Quality assessment of sequencing reads was performed with the prinseq-lite program, [14] applying the following parameters: a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3'-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing where joined using fastq-join from the ea-tools suite. [15] Taxonomic affiliations were assigned using the RDP_classifier from the Ribosomal Database Project (RDP). [16] Reads that had an RDP score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank as unidentified. We assigned 6 taxonomic levels, which were kingdom, phylum, class, order, family and genus.

Statistical analysis

Correlations between bacterial abundance and Maillard reaction products were carried out through multivariate analysis with Statgraphics Centurion XVI.I. Bacterial abundances between groups were compared by analysis of variance (ANOVA) with Statgraphics Centurion XVI.I and R software. Principal Coordinates Analysis (PCoA) with unifrac distance was carried out with R software.

Results and discussion

Furosine content of cooked foods

Furosine (ε-N-(furoylmethyl)-L-lysine) is an artificial amino acid obtained from the acidic hydrolysis of Amadori compounds derived from the MR of heat-processed foods; [7] thus, furosine can be used as an indicator of the early stages of the MR. Furosine was detected in all the food analysed except in chicken (**Table 46**), which could be explained by the very low amount of reducing sugars available for the development of the MR. Furosine content ranged from 809 to 7.61 μg/g in toasted bread and raw pepper, respectively. Results were in accordance with other previously reported. [7, 9, 17] The highest levels were found in bread, followed by pepper, banana, chickpeas and chicken (**Table 46**). There were statistically significant differences ($p < 0.05$) in the furosine content among the different types of cooking procedures within the same type of food. In the case of chickpeas, furosine values were significantly higher for grilling than for boiling (56.9 *Vs.* 12.0 μg/g), most likely due to a higher temperature during cooking. Such was also the case for bread, in which furosine increased significantly with toasting, and further when the degree of toasting was high. Regarding red pepper and banana, furosine values were low in the raw food and increased significantly ($p < 0.05$) in fried and roasted preparations. Furosine is therefore a sensitive indicator of heat damage during cooking in all the assessed foods except for chicken.

Table 46. Furosine, HMF, Furfural and SCFAs Content of Processed Foods.

Sample	Furosine (μg/g of food)	HMF (μg/g of food)	Furfural (μg/g of food)	Acetic acid (μmol/g of food)	Propionic acid (μmol/g of food)	Butyric acid (μmol/g of food)	Total SCFAs (μmol/g of food)
Boiled chickpeas	12.0[a] ± 0.6	0.00[a] ± 0.00	0.79[a] ± 0.03	36.8[a] ± 1.9	54.7[a] ± 0.6	21.3[a] ± 0.4	113[a]
Grilled chickpeas	56.9[b] ± 3.5	2.44[b] ± 0.15	1.13[b] ± 0.06	30.1[a] ± 1.8	49.6[a] ± 2.9	51.6[b] ± 2.1	131[a]
Chickpeas (mean)	*34.4 ± 2.1*	*1.22 ± 0.08*	*0.96 ± 0.04*	*33.5 ± 1.8*	*52.1 ± 1.7*	*36.4 ± 1.2*	*122*
Raw bread	20.5[a] ± 1.1	0.56[a] ± 0.01	0.75[a] ± 0.03	69.6[a] ± 4.8	29.8[a] ± 2.2	13.4[a] ± 0.5	113[a]
Well done toasted bread	809[b] ± 34	57.2[b] ± 4.7	235[b] ± 15	60.4[a] ± 1.6	46.8[a] ± 1.0	5.3[b] ± 0.3	113[a]
Normal toasted bread	537[c] ± 18	6.22[c] ± 0.46	17.1[c] ± 1.2	94.2[a] ± 4.4	33.8[a] ± 2.9	9.5[b] ± 0.5	137[b]
Bread (mean)	*456 ± 14*	*21.3 ± 1.3*	*84.7 ± 4.1*	*74.8 ± 3.2*	*36.8 ± 2.0*	*9.4 ± 0.6*	*121*
Roasted pepper	176[a] ± 15	35.5[a] ± 1.5	0.35[a] ± 0.03	40.8[a] ± 3.8	43.9[a] ± 0.2	29.6[a] ± 1.0	114[a]
Raw pepper	7.61[b] ± 0.53	0.85[b] ± 0.02	0.11[a] ± 0.01	9.5[b] ± 0.8	48.6[a] ± 2.0	35.8[a] ± 1.7	94[a]
Fried pepper	175[a] ± 14	19.2[c] ± 0.2	0.72[c] ± 0.01	15.0[b] ± 0.8	38.9[a] ± 2.5	89.1[b] ± 1.6	143[b]
Pepper (mean)	*120 ± 11*	*18.5 ± 0.8*	*0.39 ± 1.0*	*21.8 ± 2.1*	*43.8 ± 1.7*	*51.5 ± 1.2*	*117*
Roasted banana	73.8[a] ± 3.1	5.71[a] ± 0.31	1.18[a] ± 0.05	71.3[a] ± 7.5	67.6[a] ± 2.8	22.0[a] ± 0.2	161[a]
Raw banana	9.21[b] ± 0.22	0.04[b] ± 0.00	0.41[b] ± 0.02	42.4[b] ± 2.9	26.6[b] ± 1.2	2.5[b] ± 0.0	72[b]
Fried banana	216[c] ± 19	13.1[b] ± 0.5	1.78[c] ± 0.11	68.6[a] ± 4.1	22.1[b] ± 0.5	70.8[c] ± 5.0	162[a]
Banana (mean)	*100 ± 8*	*6.28 ± 0.39*	*1.23 ± 0.30*	*60.8 ± 4.2*	*38.8 ± 1.5*	*31.8 ± 1.6*	*131*
Roasted chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.3[a] ± 0.2	13.9[a] ± 0.4	11.5[a] ± 0.7	33[a]
Fried chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	7.8[a] ± 0.1	13.6[a] ± 0.8	23.6[b] ± 1.0	45[a]
Boiled chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	2.0[b] ± 0.1	10.7[a] ± 0.4	2.5[c] ± 0.1	15[b]
Grilled chicken	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.5[a] ± 0.5	10.6[a] ± 0.1	16.7[a] ± 0.3	34[a]
Chicken (mean)	*0.00 ± 0.00*	*0.00 ± 0.00*	*0.00 ± 0.00*	*5.9 ± 0.2*	*12.2 ± 0.4*	*13.6 ± 0.5*	*32*

Different letters within the same column and type of food indicate statistically significant differences ($p < 0.05$).

HMF and furfural content of cooked foods

HMF and furfural appear during the 1,2-enolisation of Amadori compounds under acidic conditions and sugar degradation at high temperature, known as caramelisation; [9] thus, HMF and furfural are related with intermediate stages of the MR, so that they can be used as indicators of thermal damage. [18] HMF values ranged from 57.2 to 0.04 μg/g in well-done toasted bread and raw banana respectively and from 235 to 0.11 μg/g in well-done toasted bread and raw pepper respectively (**Table 46**). Results were in accordance with other previously reported. [7, 9, 17] These indicators behaved similarly to furosine, in that significant differences in the values of these compounds among the different types of foods were found: bread > pepper > banana > chickpeas > chicken. This situation could be related with food composition, since bread (an excellent medium for the MR due to its content in sugars and proteins) reached the highest HMF and furfural values, whereas chicken (with no available reducing sugars) had no detectable amounts. Moreover, we also observed that HMF and furfural amounts were closely linked to the applied temperature (**Table 46**), with statistically significant differences ($p < 0.05$) among cooking methods within the same food. In this sense, whereas boiled chickpeas had no HMF, grilled chickpeas HMF value was 2.44 μg/g of food. As expected, raw bread showed very low amounts of either HMF or furfural, but when it was submitted to toasting the levels increased, being higher with longer toasting time. Regarding uncooked red pepper, it showed the lowest amounts of furfural, with increasing HMF levels during roasting and furfural content during frying. This could be related with the degradation of HMF to furfural during frying, due to the high temperatures used during this type of coking. Bananas behaved similarly to the other samples, with raw banana showing low amounts of HMF and furfural, whereas either roasted or fried banana showed significantly higher amounts, with the highest amounts

in fried banana. Therefore, according to our results, HMF and furfural were dependent upon composition and type of foodstuff, as well as upon the applied temperature.

Short chain fatty acids generated after microbial fermentation of cooked foods

Several health effects are attributed to SCFAs; decreasing the luminal pH is one of the most obvious, which can impede the growth of pathogenic bacteria. On the other hand, butyrate is used as a substrate by epithelial cells and is important for their functionality, while all three main SCFAs (acetate, propionate and butyrate) are important for the maintenance of the gut barrier. [19] Moreover, while butyric acid is mostly metabolized by colonocytes, acetate and propionate are mainly absorbed and incorporated into different metabolic routes. The participation in different metabolic routes related to energy balance could link these SCFAs to the control of the metabolic syndrome. In accordance, all three have a protective role in diet-induced obesity. [20] On the other hand, butyrate and propionate have been related to production of gut hormones and therefore reduction of food intake. It is thought that SCFAs, and mostly butyrate, could have an important role in colorectal cancer protection, via increasing motility, irrigation, apoptosis, and reducing inflammation. In fact, it has been suggested that the protective effect of dietary fiber over colorectal cancer depends upon the production of butyrate. [21] Propionate has also been associated with the regulation of intestinal inflammation through induction of T-regulatory cell differentiation. [19] Accordingly, it is important to know that the production of SCFAs derives from different foodstuffs that are a main part of the Western diet.

Accordingly, we first studied SCFAs production (**Table 46**) depending on the **type of food** (bread, chickpeas, chicken, red pepper and banana). In this sense, regarding acetic acid, bread produced the highest amount, 74.8 μmol/g, whereas chicken

produced the lowest one, 5.9 μmol/g. All types of foodstuff produced significantly higher amounts of acetic acid than chicken; bread produced significantly higher amounts than banana, red pepper and chickpeas, and the levels of acetic acid after banana fermentation were higher than those of chickpeas and red pepper. In the case of propionic acid, chickpeas where the highest producers with 52.1 μmol/g whereas chicken showed the lowest value, 12.2 μmol/g. Every kind of food produced significantly more propionic acid than chicken, while chickpeas, red pepper and banana produced significantly higher amounts than bread, with chickpeas producing significantly higher amounts than any other foodstuff. Finally, the levels of butyric acid ranged from 51.5 to 9.3 μmol/g for red pepper and bread, respectively. Red pepper produced significantly higher amounts than any other foodstuff, whereas the production by banana and chickpeas was significantly higher than that by chicken and bread. Overall, banana produced the highest amounts of SCFAs followed by chickpeas, whereas chicken yielded the lowest amounts.

Secondly, we studied the possible influence of the **culinary treatment** over SCFAs production **(Table 46)**. Regarding boiled and grilled chickpeas, there were no statistically significant differences in acetic acid or propionic acid production. However, butyric acid production was significantly higher in grilled chickpeas. One possible explanation could be the formation of melanoidins, which can behave as fibre in the gut, [8] therefore increasing butyric acid production. In the case of bread, there were no significant differences between raw or toasted bread regarding acetic or propionic acid production. However, butyric acid production was, in this case, significantly higher in raw bread. On the other hand, red pepper showed a significantly higher production of acetic acid when roasted and of butyric acid after frying; however, no significant differences were observed in propionic acid production. In this sense, products that

appear as a consequence of the thermal treatment, such as melanoidins, could be responsible for the different SCFAs production. In the case of banana, no differences were found between acetic acid production for roasting or frying, but both had a significantly higher production than raw banana. Propionic acid production was also significantly higher in roasted banana, though there were no differences among raw or fried fruit. Regarding butyric acid, both roasted and fried banana yielded significantly higher amounts than raw banana, while the fried fruit had significantly higher values than the roasted one. In this case, it also seems that the culinary treatment has some effect on SCFAs production and that, as in the case of chickpeas and red pepper, cooking favours SCFAs production. Finally, roasted, fried and grilled chicken showed a significantly higher production of acetic and butyric acids than boiled chicken. However, no differences were found in the case of propionic acid. Overall, it seems that cooking has an influence over SCFAs production, which can be positive or occasionally negative, as observed in bread. However, much more research is needed in order to generate conclusive statements.

Finally, we tried to unravel whether or not there were any correlations among SCFAs production and **bacteria** known to be producers of such fatty acids (**Table 47**). Regarding acetic acid, we found positive significant correlations with *Ruminococcus spp.*, *Bifidobacterium spp*. and *Collinsella spp.* In the case of propionic acid, it correlated significantly with the phylum Bacteroides and with *Ruminococcus spp., Butyricimonas spp., Blautia spp., Roseburia spp.,* and *Veillonella spp.* Finally, butyric acid correlated significantly with *Butyricimonas spp., Anaerostipes spp., Intestimonas spp., Roseburia spp., and Faecalibacterium spp.* In this sense, our results are in accordance with existing bibliography about SCFAs producers. [2, 19] Accordingly, bread and banana yielded the highest acetic acid productions and also had significantly higher

abundances of *Bifidobacterium spp., Collinsella spp.,* and *Ruminococcus spp* (**figure 59)**. On the other hand, chicken, which showed the lowest acetic acid production, had also significantly lower abundance of *Bifidobacterium spp.,* and *Collisella spp.* Chickpeas, which had the highest propionic acid production, showed also, along with banana, higher abundances of *Blautia spp.* and *Roseburia spp.,* both related to production of this acid. [2, 19] However, we did not find this kind of correlation with butyric acid.

Table 47. Lineal Correlations (r Values) among Bacteria and SCFAs Content and Maillard Reaction Products.

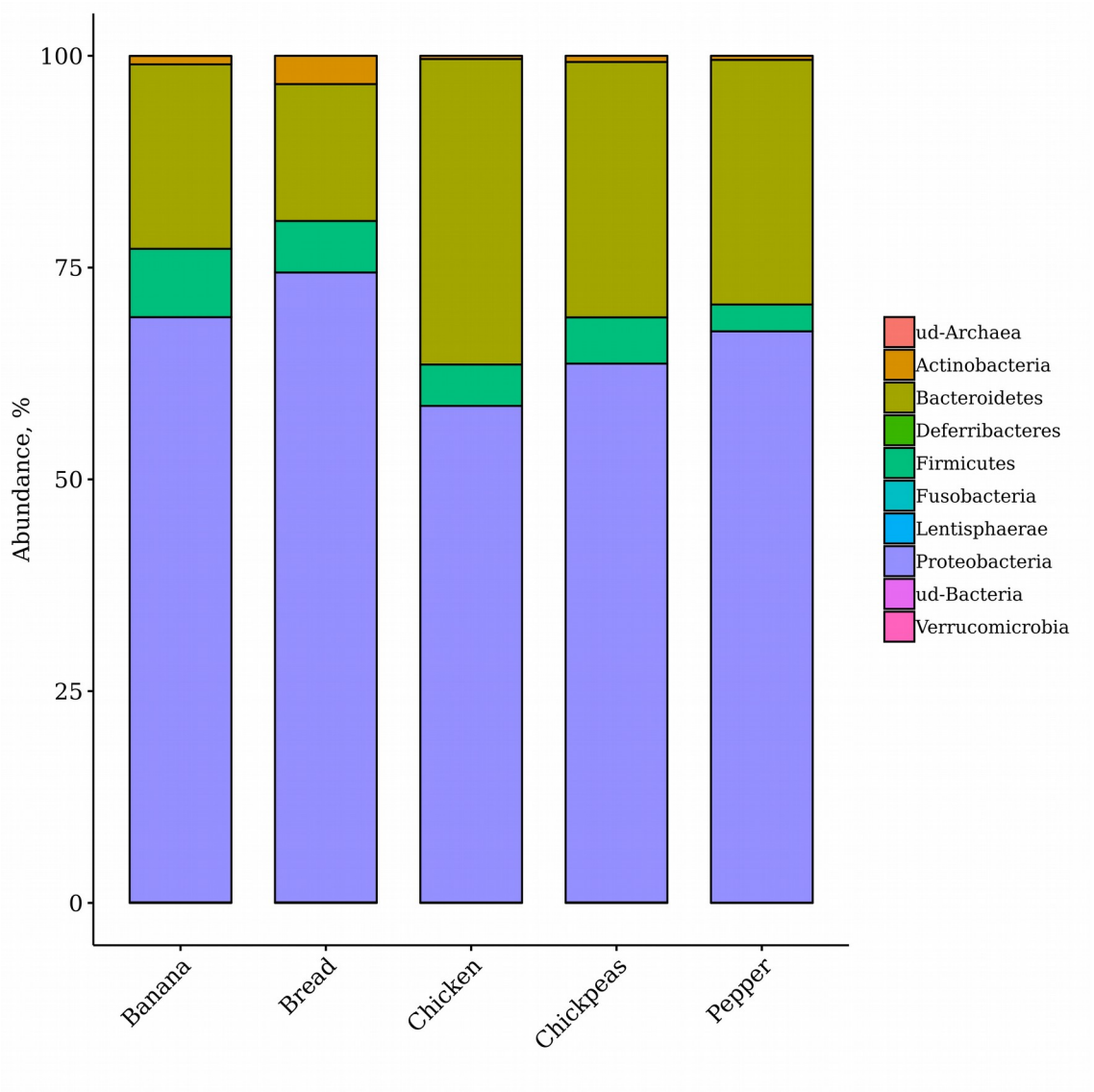
	Acetic acid	Propionic acid	Butyric acid
Phylum Bacteroidetes		0.6586[i]	
Butyricimonas spp.		0.8026[e]	0.8154[a]
Ruminococcus spp.	0.5288[a]	0.9114[a]	
Anaerostipes spp.			0.9282[g]
Clostridium XIVa spp.			
Blautia spp.		0.7550[g]	
Roseburia spp.		0.7801[g]	0.9187[e]
Intestimonas spp.			0.6827[e]
Faecalibacterium spp.			0.7999[a]
Veillonella spp.		0.9512[g]	
Bifidobacterium spp.	0.8162[a]		
Collinsella spp.	0.7589[a]		
	Furosine	**HMF**	**Furfural**
Phylum Actinobacteria	0.8503[a]	0.6954[a]	0.9758[a]
Plylum Verrucomicrobia		0.7541[a]	0.9694[a]
Phylum Firmicutes	0.9173[c]		
Akkermansia spp.		0.7534[a]	0.7366[a]
Butyricicoccus spp.		0.7487[a]	0.8328[a]
Bifidobacterium spp.	0.8272[b]		
Pseudoflavonifractor spp.	0.8462[b]		
Collinsella spp.	0.8629[c]		0.7081[b]
Blautia spp.	-0.8265[e]		
Christensenella spp.			0.9598[d]
Prevotella spp.	0.7638[a]	0.7889[a]	0.9562[a]
Intestinibacter spp.	0.7289[f]	0.7342[f]	0.8084[f]
Eggerthella spp.		0.7130[g]	0.8023[g]
Parasutterella spp.			0.7138[h]
Oscillibacter spp.	0.8954[c]		

[a]Chickpeas, bread, pepper and banana; [b]Chickpeas, bread and pepper; [c]Bread and pepper; [d]Chickpeas and pepper; [e]Bread and banana; [f]Bread, pepper and banana; [g]Chickpeas, bread and banana; [h]Banana and pepper; [i]All

Microbial community composition after fermentation of cooked foods

Cooking is known to modify food composition due to the development of different chemical reactions such as the Maillard reaction. [7] Distinct cooking technologies with different heat transfer media and heating intensities will modify food composition in a different way. Therefore, it is expected that cooking conditions could modify the composition of the gut microbiota. Under our experimental conditions, we observed statistically significant differences in the abundance of Firmicutes and Bacteroidetes between food groups **(Figure 58)** at phylum level. We found the highest abundance of Bacteroidetes in chicken and the lowest in bread and banana. In the case of Firmicutes, the highest abundance was found in bread and banana and a very low abundance was detected in pepper. The Firmicutes/Bacteroidetes ratio was statistically higher ($p < 0.05$) in bread and banana in comparison with chicken and pepper. Moreover, we observed a high abundance of Proteobacterias, most probably due to donors' microbial composition. [22]

Figure 58. Relative bacterial abundance (at Phylum level) after *in vitro* fermentation of the assessed foodstuffs.



At genus level, we found statistically significant ($p < 0.05$) differences among foodstuffs for several beneficial bacteria **(Figure 59)**: *Bifidobacterium spp., Collinsella spp., Gordonibacter spp., Barnesiella spp., Blautia spp., Fusicatenibacter spp., Roseburia spp., Pseudoflavonifractor spp., Alistipes spp., Anaerostipes spp., Coprococcus spp., Butyricimonas spp., Intestinimonas spp., Butyricicoccus spp., Clostridium XIVa spp.,*

Clostridium XIVb spp., Ruminococcus spp., Eggerthella spp., Oscillibacter spp. and Parasutterella spp. All these genera have been related with a decreased risk or severity of different pathologies like inflammatory bowel disease, cancer, diabetes, obesity, etc. (**Table 48**).

Figure 59. Relative abundances (at genus level) of bacteria with significant differences between the assessed foodstuffs. Differences were assessed by ANOVA and compared with the mean relative abundance of the five different foodstuffs. Statistic labels: ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. (+): significantly higher than the mean; (-): significantly lower than the mean.

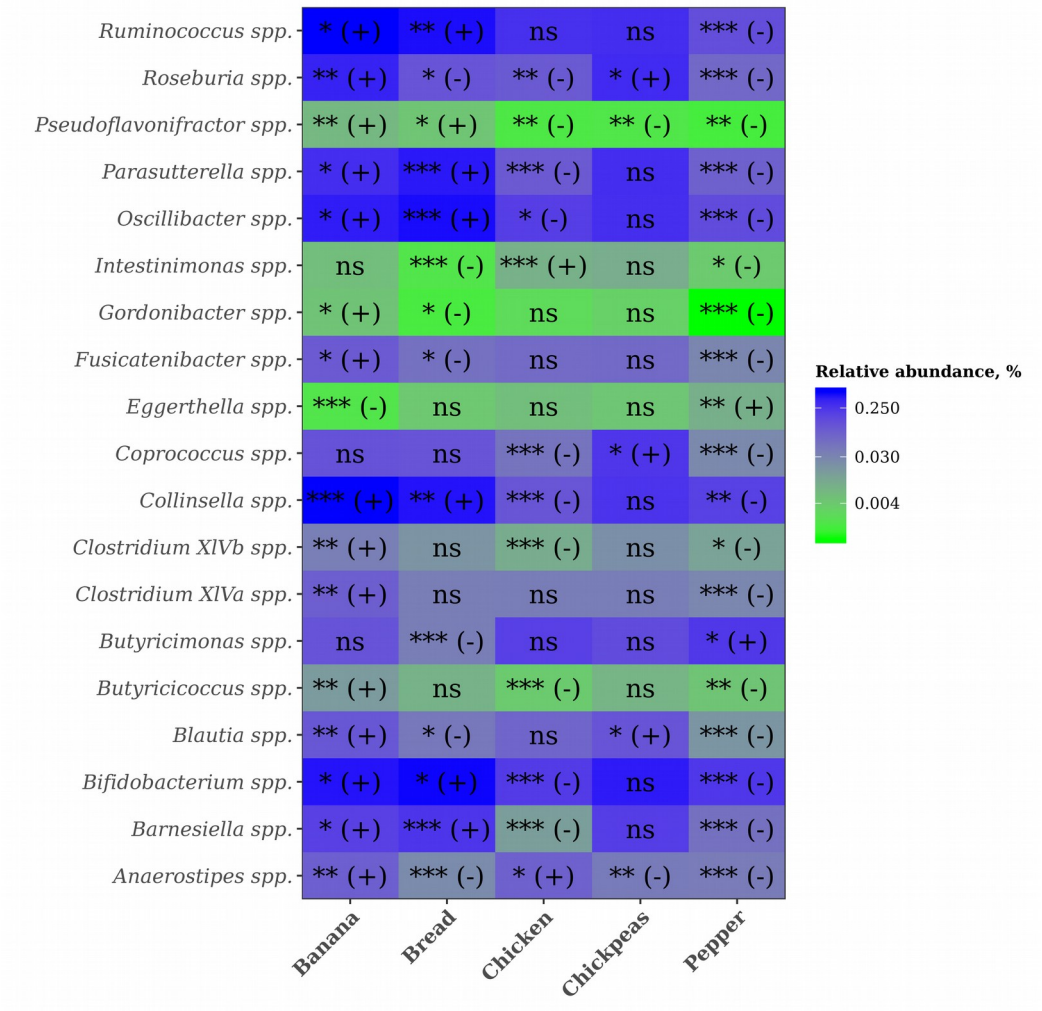


Table 48. Beneficial or Detrimental Effects of Bacteria on Human Health. (+) positive health effect; (-) negative health effect.

Bacteria		Health effect
Akkermansia spp.	+	It helps to control diet induced obesity and associated metabolic disorders. [23]
Christensenella spp.	+	Associated with lower body mass index. [26]
Faecalibacterium spp.	+	Produces butyrate, it helps to regulate the immune system, it could exert a positive role on Chron´s disease. [2]
Veillonella spp.	+	Produces propionate. [2]
Bifidobacterium spp.	+	Reduced in colorectal cancer and in type I diabetes. [27]
Collinsella spp.	+	Reduced in irritable bowel syndrome patients with more severe symptoms. [28]
Gordonibacter spp.	+	Produce anti-inflammatory urolithins from ellagic acid. [29]
Barnesiella spp.	+	May prevent or treat infections by antibiotic resistant bacteria. [30]
Blautia spp.	+	Related to decreased inflammation in cirrhosis and hepatic encephalopathy, reduced in colorectal cancer and type I diabetes. [27], [31]
Fusicatenibacter spp.	+	Reduced in ulcerative colitis patients and probable anti-inflammatory function. [32]
Roseburia spp.	+	Associated with weight loss and decreased glucose intolerance in mice, reduced in ulcerative colitis patients, differs in abundance between type II diabetes patients and non-diabetic people. [33]
Pseudoflavonifractor spp.	+	Related to weight loss along with *Alistipes spp.* [34]
Anaerostipes spp.	+	Produces acetic, lactic and butyric acid. [2], [19]
Coprococcus spp.	+	Produces acetic and butyric acid, and lower amounts of propionic or formic acid. [2], [19]
Butyricimonas spp.	+	Produces butyric acid. [2], [19]
Intestinimonas spp.	+	Produces butyric acid. [2], [19]
Butyricicoccus spp.	+	Produces butyric acid, reduced in ulcerative colitis patients and patients with inflammatory disease in general. [2], [19]
Clostridium XIVa spp.	+	Produces butyric acid. [2], [19]
Ruminococcus spp.	+	Key role in degradation of resistant starch. [35]
Clostridium XIVb spp.	+	Correlated with systemic inflammatory cytokines in patients with HIV-1. [36]
Eggerthella spp.	-	Related to ulcerative colitis, hepatic abscesses and systemic bacteraemia. [37]
Oscillibacter spp.	-	Increased in depression and in high-fat diet. [38]
Parasutterella spp.	-	Related with Crohn´s disease and with dysbiosis in hypertriglyceridemia associated to necrotizing pancreatitis. [39]
Prevotella spp.	-	It outgrows in autoinflammatory disease. [24]
Intestinibacter spp.	-	It has been found to be increased in patients with neurological disorders other than Parkinson´s disease. [25]

Therefore we found that banana had higher abundances of *Anaerostipes spp., Blautia spp., Collinsella spp., Fusicatenibacter spp., Roseburia spp., Ruminococcus spp., Butyricicoccus spp., Clostridium XIVa spp., Gordonibacter spp.,* and *Pseudoflavonifractor spp.* However, it showed lower abundance of *Eggerthella.* Chickpeas showed higher abundance of *Coprococcus spp.* Bread on the other hand, showed higher abundance of *Barnesiella spp., Bifidobacterium spp., Oscillibacter spp.,* and *Parasutterella spp.* However, it showed lower abundance of *Anaerostipes spp., Butyricimonas spp.,* and *Intestinimonas spp.* Red pepper showed higher abundance of *Butyricimonas spp.,* and *Eggerthella spp.* On the other hand, it showed lower abundance of *Blautia spp., Coprococcus spp., Fusicatenibacter spp., Oscillibacter spp., Parasutterella spp., Roseburia spp., Ruminococcus spp.,* and *Gordonibacter spp.* Finally, chicken showed lower abundance of *Barnesiella spp., Bifidobacterium spp., Collinsella spp., Butyricicoccus spp., Clostridium XIVa spp.,* and *Pseudoflavonifractor spp.*

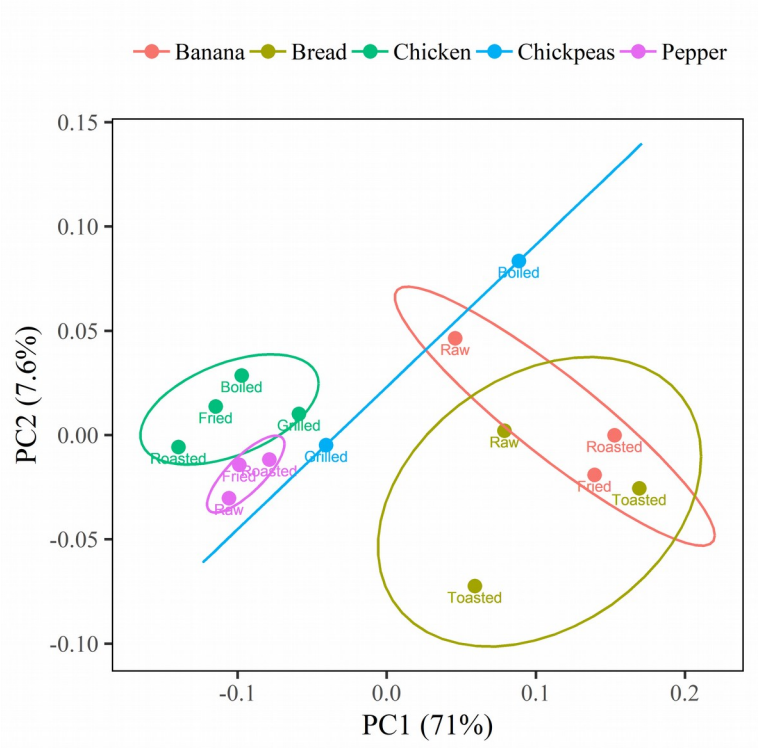
Principal Coordinates Analysis (PCoA) of microbiota composition with phylogenetic weighted UniFrac (**Figure 60**) showed a clear separation among high starch-content foods (banana and bread) and non-starchy foods (chicken and pepper). Permutational multivariate analysis of the variance (PERMANOVA) showed that in fact, different foods produced significantly different ($p < 0.05$) microbial communities. The main difference among them was the higher content of Firmicutes in bread and banana whereas in chicken and pepper Bacteroidetes levels were higher. Therefore, the provision of a specific kind of food (legume, cereal, vegetable, fruit or meat) can change microbial community composition and consequently affect health. Moreover, we observed differences among distinct types of cooking techniques. Accordingly, boiled chickpeas were closer to banana and bread whereas grilled chickpeas were closer to chicken and pepper (**Figure 60**). A possible explanation

could be the participation of starchy carbohydrates in the Maillard reaction during chickpeas grilling, so that they are not available for gut microbiota fermentation, whereas during boiling the degree of the Maillard reaction is quite low, so that starch suffers a low degree of modification and is easily available to bacteria. In the case of bread, toasting also showed some effects, especially when toasting was carried out for a longer time. We observed lower abundance of *Roseburia spp., Coprococcus spp, Blautia spp., Butyricimonas spp., Anaerostipes spp., Clostridium XIVa spp., Clostridium XIVb spp.,* and *Ruminococcus spp* in toasted bread, and even lower levels in the well done form. However, *Collinsella spp.* and *Parasutterella spp.* behaved differently, being higher in toasted bread. Banana displayed higher abundance of *Bifidobacterium spp., Barnesiella spp., and Butyricimonas spp.* in the raw form. However, the abundance of *Roseburia spp., Oscillibacter spp., Coprococcus spp.* and *Parasutterella spp.* was higher in fried and roasted banana. Finally, as **Figure 60** shows, little separation was detected among different types of cooking in chicken or pepper. In this sense, this finding could be important when it comes to choosing the most adequate cooking method.

Moreover, we also found significant correlations among furosine, HMF and furfural and some bacterial taxa (**Table 47**). At the phylum level, Actinobacteria was positively correlated with all three indicators whereas Verrucomicrobia was only correlated with HMF and furfural. At the genus level, we detected several correlations with one or more indicators in different foodstuffs. In chickpeas, bread, pepper and banana, we found positive correlations among *Akkermansia* and HMF-furfural. *Akkermansia muciniphila* (the only known species of the genus) has been found to be reduced in obese and diabetic type II mice and the treatment with such bacteria reversed high-fat-diet related metabolic disorders. [23] Further, we found positive correlations between *Prevotella spp.* and the three indicators in all four foodstuffs.

Prevotella spp. are also quite important for human health since they are susceptible to dietary changes and have been related to inflammatory phenotypes. [24]

Figure 60. PCoA ordination analysis of genus abundance among all profiled samples. Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix.



Additionally, we also detected positive correlations among *Butyricicoccus spp.* and HMF-furfural (**Table 47**). In chickpeas, bread and pepper (not in banana) we found positive correlations among furosine and *Bifidobacterium spp.* and *Pseudoflavonifractor spp.*, and between furfural-*Collinsella spp.* In bread, pepper and banana positive correlations among *Intestinibacter spp.* and all three indicators were also found. This bacterium has been found to

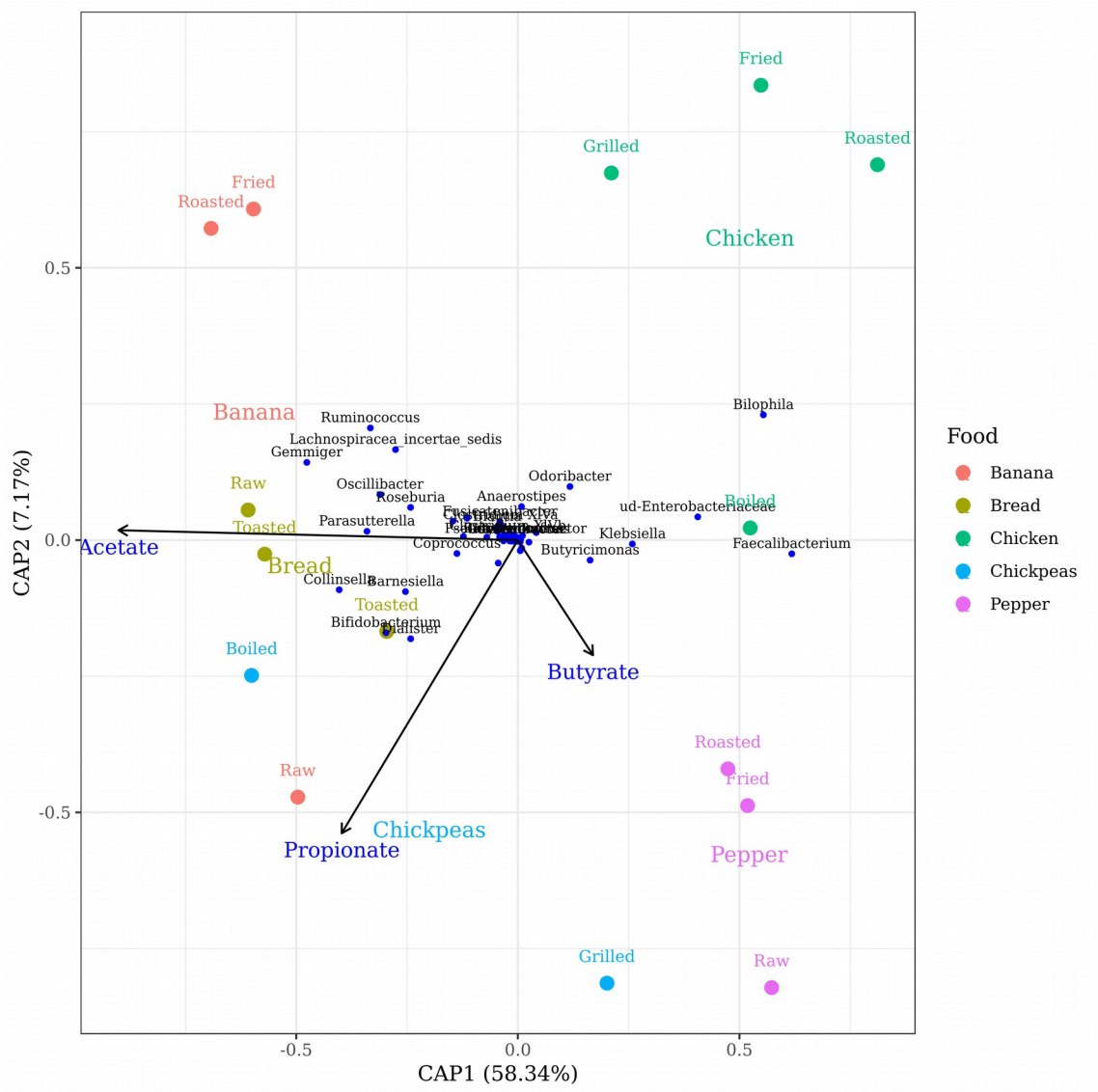
increase in some neurological diseases such as Parkinson´s disease. [25] In chickpeas, bread and banana, *Eggerthella spp.* was positively correlated with HMF and furfural. In bread and pepper, the Firmicutes phylum, *Collinsella spp.* and *Oscillibacter spp.* were positively correlated with furosine. In banana and pepper, *Parasutterela spp.* was positively correlated with furfural. Further, in chickpeas and pepper we detected a correlation between *Christensenella spp.*, which is associated with weight reduction in mice, [26] and furfural. Finally, we also found a single negative correlation between furosine and *Blautia spp.* in bread and banana.

These correlations, most of them positive, could indicate that the Maillard reaction provides substrates for the growth of these bacteria and thus favors their thriving. In fact, it has been already stated that melanoidins, the end-product of the Maillard reaction, are fiber-like products; [8] they escape digestion and absorption, so that they reach the large intestine where they could be substrates for the gut microbiota. However, we also detected one negative correlation, indicating that some of the Maillard reaction products act as inhibitors for certain types of bacteria.

Finally, we performed a distance-based Redundancy Analysis (db-RDA) with Bray-Curtis distance (**Figure 61**). This multivariate analysis allowed us to relate each microbial genus to the samples and therefore to observe in the same plot which foodstuff was richer in which genus. As it can be observed in **figure 61,** there is a clear and significant ($p < 0.05$) separation of the foodstuffs as it happened with UniFrac PcoA. The distance between the foodstuffs and the genus in the plot is indicative of their abundance, the closer they are the higher the abundance in such food. Therefore, we could conclude that bread, raw banana and boiled chickpeas showed higher abundances in beneficial bacteria such as *Roseburia, Ruminococcus, Bifidobacterium, Dialister, Collinsella,* or *Barnesiella.* This would indicate

that such foods would result in a healthier gut microbial community. On the other hand, the arrows of **figure 61** represent SCFA and they point to where higher amounts are found. Thus, bread and banana produced higher amounts of acetate, chickpeas propionate and pepper butyrate.

Figure 61. Distance-based Redundancy Analysis (db-RDA) triplot with Bray-Curtis distance.



As conclusions, Maillard reaction indicators like furosine, HMF and furfural are sensitive indicators to control heat damage during cooking, especially for grilling, roasting and frying. However, their behaviour depends on the composition and type of foodstuff. When cooked foods are submitted to *in vitro* digestion and fermentation, they are readily metabolized by the gut microbiota, increasing the levels of beneficial bacteria like *Ruminococcus spp*. or *Bifidobacterium spp*., among others. In addition, different healthy short chain fatty acids like acetic, propionic and butyric acids are released. Although SCFAs concentration depended on food type, cooking methods and heat intensity had a definitive influence over SCFAs production and microbial composition. The influence of culinary technologies on gut microbiota composition and functionality could be derived from the production of Maillard reaction compounds like melanoidins, which escape digestion and can be fermented by the gut microbiota. According to our results, bread, raw banana and boiled chickpeas produced a healthier gut microbial community characterized by higher abundance of some beneficial bacteria such as *Roseburia, Ruminococcus, Bifidobacterium, Dialister, Collinsella,* or *Barnesiella.* One way or another, it is likely that the gut microbiota can be modulated not only by the type of food but also by the type of cooking and the thermal treatment applied. Therefore, more studies are needed in order to unravel the specific effect of cooking techniques on food composition and their direct effect over the gut microbiota.

Abbreviations: HPLC: High Performance Liquid Chromatography, SCFA: Short Chain Fatty Acids, HMF: 5-hydroxymethyl-furfural, MR: Maillard Reaction,

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Potential probiotic salami with dietary fiber modulates antioxidant capacity, short chain fatty acid production and gut microbiota community structure

El salchichón es un alimento muy consumido en la dieta española. Sin embargo, su elevado contenido en proteínas y grasas saturadas lo hacen un alimento poco recomendable. En este trabajo se estudió la posibilidad de mejorar su formulación en relación a la capacidad antioxidante y efecto sobre la microbiota intestinal. Así, se evaluó la adición de distintos extractos antioxidantes e incorporación de distintas fibras alimentarias, evaluándose los anteriormente comentados efectos, tras ser sometido a digestión-fermentación *in vitro*.

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Potential probiotic salami with dietary fiber modulates antioxidant capacity, short chain fatty acid production and gut microbiota community structure

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Abstract

Dry-fermented sausages are an important and abundant component of the diet of many people. Improving their composition and thus their potential health effects is therefore important. Here we quantified the antioxidant capacity, short chain fatty acids (SCFAs) production, and gut microbiota structure of different salami formulations after in vitro digestion and subsequent fermentation with human gut microbiota. The addition of different types of fiber (citrus fiber, arabinogalactan, and inulin), a probiotic *Lactobacillus rhamnosus*, and an herbal extract to the salami formulation was tested. Incorporating any dietary fiber into salami formulation increased sausage antioxidant capacity and the amount of SCFAs produced during microbiota fermentation. These effects were highest for salami with citrus fiber and citrus fiber with herbal extract. Presence of fiber in salami also led to an altered gut microbiota structure. Citrus fiber and arabinogalactan but not inulin promoted an increase in the abundance of several known polysaccharide degrading genera and resulted in a reduction in the abundance of *Escherichia*, a bacterial genus known to contain many human intestinal pathogens. Overall, the addition of dietary fiber to salami formulation prior to curing improved beneficial health markers of this food product.

Keywords: fermented sausage, prebiotic, antioxidant, short chain fatty acids, gut microbiome.

1. Introduction

Salami is a dry-fermented sausage consisting of mixtures of lean meats and fatty tissues combined with salts, nitrate (curing agent), sugars, spices, and other non-meat ingredients filled into casings. Salamis acquire their properties (flavor, texture, color, etc.) through a fermentation process, in which mainly lactic acid bacteria and coagulase-negative staphylococci are involved (FAO, 2017).

Salami is generally considered as a food with unbalanced nutritional value due to the high fat and salt content and the lack of bioactive molecules, such as phenolic compounds and other phytochemicals (Martínez, Nieto, & Ros, 2014), but they are highly consumed in many countries around the world (Blaiotta, Murru, Cerbo, Romano, & Aponte, 2018). Thus, there is a significant interest to improve nutritional properties of salami, for example, by using probiotic bacteria as a starter for the fermentation process (Giello, La Storia, De Filippis, Ercolini, & Villani, 2018), or by adding different kinds of fibers to the sausage composition (dos Santos, Campagnol, Pacheco, & Pollonio, 2012). Addition of dietary fiber to salami can lead to an increased production of short chain fatty acids (SCFAs) either during salami preparation/curing, or when it is fermented in the colon by resident microbes after ingestion. SCFAs have been associated with many health benefits: they help maintain proper function of the colon (Ríos-Covián et al., 2016), have a protective role on the diet-induced obesity (Lin et al., 2012), protect against colorectal cancer (Ríos-Covián et al., 2016), and regulate intestinal inflammation (Ríos-Covián et al., 2016). Nevertheless, the previous efforts are centered on the SCFAs production during fermentation and ripening process, because SCFAs in the salami influence its taste (Iacumin, Comi, Cantoni, & Cocolin, 2006). Very few *in vitro* studies have focused on SCFAs production by the gut microbiota after meat intake (Shen, Chen, & Tuohy, 2010). To our knowledge, only Thøgersen et al.,

(2018) studied the addition of inulin on Frankfurt sausages feed to rats and their effect on gut microbiota composition and short chain fatty acids production. Therefore, there is a lack of comprehensive studies about the effect of probiotic and fiber addition to salami.

On the contrary, there has also been a rise in the studies aiming to extend the shelf life of salami. Most advancements in this area make use of natural antioxidants or plant extracts to avoid or delay lipid oxidation while preserving taste and smell (Cullere, Hoffman, & Dalle Zotte, 2013). Antioxidants can also reduce oxidative damage that is one of major causes of various chronic diseases including cancer, aging, Alzheimer's disease, inflammation, diabetes, atherosclerosis, and Parkinson's disease (Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011). However, very little has been done to examine the antioxidant capacity that such improved salami products could provide even though there is evidence for the presence of strong antioxidant compounds such as carnosine in meat products (Martínez et al., 2014).

The aims of this paper were to evaluate the antioxidant capacity of salami, to evaluate SCFAs production during salami digestion and fermentation by intestinal microbiota, and to study the alterations in the gut microbial community structure after *in vitro* digestion-fermentation process. To accomplish these aims, antioxidant assays, SCFA measurements, and microbial community structure characterization were performed on traditional salami (control) and several salami samples enriched with different types of fiber and probiotic bacteria.

2. Materials and Methods

2.1. Chemicals

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), carmine indigo, hydrogen peroxyde, gallic acid, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), potassium persulphate, sodium hydroxide, iron (III) chloride hexahydrate, sodium acetate, potassium chloride, potassium di-hydrogen phosphate, sodium mono-hydrogen carbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride dihydrate, sodium di-hydrogen phosphate, tryptone, cysteine, sodium sulphide, resazurin, salivary alpha-amylase, pepsin from porcine, bile acids (porcine bile extract), ethanol, hydrochloric acid, acetonitrile, and acetate, propionate, and butyrate standards were from Sigma-Aldrich (Darmstadt, Germany). Pancreatin from porcine pancreas was purchased from Alpha Aesar (United Kingdom).

2.2. Materials

In the current paper it was tested how the addition of dietary fibers to salami formulation (prior to curing/ripening) can affect the salami's nutritional value. Salami samples were manufactured and provided by a local Spanish company (Elpozo Alimentación, S.A., Alhama de Murcia, Murcia). Samples consisted of a salami control and four salamis with modified formulation. Each formulation contained probiotic *Lactobacillus rhamnosus*, and (i) citrus fiber (commercial name "Citri-Fi") obtained from orange pulp dehydration and composed of 42% pectin, 25% cellulose and hemicellulose, and acquired from Fiberstars (USA), (ii) citrus fiber with additional

herbal extract, (iii) inulin Orafti HPX, which was acquired from Beneo (Belgium), a long-chain chicory inulin product containing 99.5% inulin, or (iv) arabinogalactan (commercial name “Acacia-fiber” acquired from Nexira (France) and characterized in Daguet, Pinheiro, Verhelst, Possemiers, & Marzorati, (2016)). Fibers and herbal extract were chosen from a previous experiment in which we tested antioxidant capacity and SCFA production. These experiments were carried out with fibers and herbal extracts alone, without salami, and in the amounts that they were going to be added to salami. Results are shown in **Tables 49** and **50**.

2.3. Salami preparation and formulation

Each salami sample was formulated according to the following traditional recipe: a mixture of pork meat and fatty tissues was combined with salt, nitrate (curing agent), sugars, black pepper, starch/fiber, and herbal extract.

For the salami control, 2% w/w of starch was added, and a standard starter composed of non-probiotic strains of lactic acid bacteria and catalase-negative streptococci was applied. Fiber-added salamis were supplemented with a probiotic *Lactobacillus rhamnosus* as a starter culture, and appropriate fiber instead of starch was added. Fibers were added in a 2% w/w ratio. An antioxidant herbal extract, composed of equal proportions of lemongrass and rosemary extracts (acquired at a local supermarket), was also incorporated as an ingredient in one salami sample (0.2% w/w), which is the usual proportion for seasoning in sausages and the one use by the company who provided the samples in their other commercial products.

Each formulated salami mix was put into casings and subjected to a ripening-drying process for 40 days. Sampling was done at the end of the ripening process. Each formulation was manufactured in triplicate. Once in the laboratory, and after the

analysis of composition (to make sure the replicates had the same composition), the replicates were mixed together. Samples were homogenized and stored at -80ºC until further analysis. All the analyses were carried out in triplicate.

2.4. In vitro gastrointestinal digestion

All samples were subjected to an *in vitro* digestion process followed by an in vitro fermentation to mimic physiological processes in the human gut. The *in vitro* digestion method was carried out according to the protocol described by Pérez-Burillo, Rufián-Henares, & Pastoriza, (2018b). The solid residue obtained after gastro-intestinal digestion was subjected to *in vitro* fermentation.

2.5. In vitro gut microbial fermentation

The *in vitro* fermentation was carried out according to the protocol described by Pérez-Burillo et al., (2018b). *In vitro* fermentation was carried out using faecal samples from three healthy donors (not taking antibiotics and with mean Body Mass Index = 21.3) that were pooled together to reduced inter-individual variability. As a control, a separate fermentation was performed using only the fecal fermentation mixture without any salami digestions or products (called fermentation fluid or FF). *In vitro* fermentation was carried out at 37ºC for 24 hours (to make a 24 hours process with digestion). The fermentation medium was composed of peptone, cysteine, and resazurin.

After *in vitro* gastrointestinal digestion and *in vitro* fermentation, three different fractions were obtained: digestion supernatant (fraction available for absorption at the

small intestine), fermentation supernatant (fraction available for absorption at the large intestine) and fermentation solid residue (fraction not available for absorption and excreted with feces).

2.6. Antioxidant assays

Antioxidant capacity was measured in three fractions of each salami sample: (i) supernatant obtained after *in vitro* gastrointestinal digestion representing the fraction available for absorption in the small intestine; (ii) soluble phase (supernatant) obtained after *in vitro* microbial fermentation representing the fraction available for absorption in the large intestine; and (iii) solid residue that is left after *in vitro* fermentation representing the non-absorbable fraction that is excreted. The sum of the three fractions represents the total antioxidant capacity of each sample.

Antioxidant capacity was determined using five different methods:

-*$TEAC_{ABTS}$ assay (Trolox equivalent antioxidant capacity against $ABTS^{\cdot+}$ radicals).* It measures scavenging capacity of samples against the artificial radical ABTS. The antioxidant capacity was estimated in terms of radical scavenging activity following the procedure described Re et al., (1999).

-*$TEAC_{FRAP}$ assay (Trolox equivalent antioxidant capacity refered to reducing capacity).* It measures reduction capacity of samples. The ferric reducing ability of each sample solution was estimated according to the procedure described by Benzie & Strain, (1996) and adapted to a microplate reader (FLUOStar Omega, BMG Labtech, Germany).

-*$TEAC_{OH}$ method (Trolox equivalent antioxidant capacity against hydroxyl radicals).* The principle underlying this method is to determine the scavenging activity against hydroxyl (OH·) radicals using carmine indigo as indicator, at physiological pH (7.4) (Pérez-Burillo, Rufián-Henares, & Pastoriza, 2018a).

-*$TEAC_{AAPH}$ method (Trolox equivalent antioxidant capacity against AAPH· radicals).* The method was performed to analyze scavenging activity against AAPH· radicals by using indigo carmine as indicator, at physiological pH (7.24) (Pérez-Burillo et al., 2018a).

-*$GEAC_{RED}$ method (Gallic acid equivalents antioxidant capacity referred to reducing capacity).* This method allowed the analysis of the global reducing capacity of the samples at physiological pH (7.24) (Pérez-Burillo et al., 2018a).

-*Solid residue antioxidant capacity.* Antioxidant capacity of the solid residue was determined following the "QUENCHER" procedure described by Gökmen, Serpen, & Fogliano, (2009). It was carried out using the five antioxidant methods described above.

2.7. Short chain fatty acids determination.

The production of SCFAs as a measure of the gut microbiota functionality was assessed according to the procedure described in Panzella et al., (2017). The analysis of SCFAs was carried out on Accela 600 HPLC (Thermo Scientific).

2.8. High-throughput amplicon sequencing.

Bacterial genomic DNA was isolated from each fermented sample using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research) as we did previously (Rigsbee, Agans, Foy, & Paliy, 2011). Genomic DNA was amplified using two pairs of primers, one targeting 16S rDNA V1-V2 region [forward primer 16S gene complementary sequence AGRGTTYGATYMTGGCTCAG and reverse primer 16S gene complementary sequence GCWGCCWCCCGTAGGWGT], and another targeting V4 region [forward GCCAGCMGCCGCGG and reverse GGACTACHVGGGTWTCTAAT complementary sequences, respectively]. Two different regions were interrogated to reduce biases in community composition estimates associated with the use of any one region of 16S rRNA gene (Liu, DeSantis, Andersen, & Knight, 2008). Forward primers also contained Ion Torrent P1 adapter sequence and 6-nucleotide barcode. PCR amplification was performed with 25ng of starting DNA material and included 10 cycles of linear elongation with only the forward primers used, followed by 25 cycles of traditional exponential PCR (Oleg Paliy & Foy, 2011). Inclusion of linear PCR step decreased the stochasticity of the first few PCR reaction steps and allowed the use of a single PCR amplification reaction per sample (Rigsbee et al., 2011). Purified amplicons were pooled equimolarly and sequencing libraries were prepared with Ion PGM Template OT2 400 kit (Life Technologies, Inc.) according to the manufacturer's protocol. High throughput sequencing was performed on Ion Torrent PGM using Ion PGM Sequencing 400 kit and Ion 316 chip. We obtained an average of 12,889 sequence reads per sample. Sequence reads were processed in QIIME (Caporaso et al., 2010). Sequence read counts for each OTU were adjusted by dividing them by known or predicted number of 16S rRNA gene copies in that organism's genome following a previously described approach (Rigsbee et al., 2011).

Thus derived cell counts were sub-sampled (rarefied) to the lowest value among all samples. The cell counts obtained independently for each sample based on the sequencing of V1-V2 and V4 16S rRNA gene regions were merged together into a single taxon abundance estimate via $A_{CUM}=\sqrt{\left(A_{V1V2}^2+A_{V4}^2\right)/2}$ calculation, where A is an abundance value for each taxon.

To compare microbial community structures, unconstrained principal coordinates analysis (PCoA) utilizing phylogenetic weighted UniFrac distance as a measure of sample dissimilarity was performed on the genus-level microbial abundance dataset (O. Paliy & Shankar, 2016).

2.9. Statistical Analyses

Statistical significance of the data and differences among samples were tested by Student´s t-test at α = 0.05 significance level. Evaluation of the relationship among different assays was carried out by computing the Pearson correlation coefficient. These statistical analyses were performed using Statgraphics Plus software (Statpoint Technologies, Inc., The plains, USA, version 5.1, 2001).

Table 49. Antioxidant capacity of different fibers and plant extracts.

Sample	$TEAC_{FRAP}$ mM	$TEAC_{OH}$ mM	$TEAC_{ABTS}$ mM	$TEAC_{AAPH}$ mM	$GEAC_{RED}$ mM
Green coffee extract	235.14 ± 6.52	423.70 ± 39.47	256.48 ± 19.13	125.15 ± 6.19	332.12 ± 6.87
Green tea extract	261.29 ± 16.56	376.29 ± 23.37	243.74 ± 23.43	132.16 ± 10.31	287.41 ± 23.23
Rosemary	758.83 ± 42.69	929.69 ± 29.08	530.42 ± 33.77	277.21 ± 27.47	824.64 ± 37.24
Lemongrass	906.69 ± 40.45	1198.04 ± 85.90	480.51 ± 21.85	293.33 ± 23.78	816.34 ± 51.15
Rosemary+lemongrass	936.41 ± 39.50	1082.44 ± 37.22	522.91 ± 21.86	254.63 ± 9.49	885.24 ± 44.70
Olive leaf extract	139.11 ± 7.24	271.23 ± 9.20	149.53 ± 8.30	63.24 ± 5.31	198.23 ± 19.43
Inulin	20.24 ± 0.80	86.18 ± 5.02	35.26 ± 1.98	24.04 ± 1.29	145.00 ± 9.92
Acacia-Fiber	21.58 ± 0.34	101.26 ± 7.75	39.14 ± 2.80	23.85 ± 1.77	143.42 ± 13.00
Citrus Fiber	21.51 ± 2.15	153.89 ± 9.69	55.23 ± 2.06	30.56 ± 0.93	199.81 ± 5.12
Carrot Fiber	10.52 ± 0.43	18.41 ± 1.42	23.78 ± 1.93	6.95 ± 0.12	63.00 ± 6.13

Table 50. Short chain fatty acids from different fibers and plant extracts.

Sample	Acetate mM	Propionate mM	Butyrate mM
Green coffee extract	59.53 ± 5.12	54.76 ± 1.88	5.69 ± 0.19
Green tea extract	61.47 ± 3.13	53.02 ± 4.84	4.96 ± 0.25
Rosemary	64.85 ± 5.61	55.57 ± 3.36	7.74 ± 0.64
Lemongrass	69.26 ± 2.18	48.63 ± 5.02	6.23 ± 0.67
Rosemary+lemongrass	67.59 ± 4.45	56.26 ± 2.15	7.06 ± 0.41
Olive leaf extract	55.69 ± 1.56	49.26 ± 1.89	4.21 ± 0.29
Inulin	78.75 ± 2.61	72.44 ± 1.86	10.88 ± 1.06
Acacia-Fiber	81.33 ± 8.77	70.14 ± 5.86	6.56 ± 0.72
Citrus Fiber	85.80 ± 5.52	73.52 ± 4.16	10.24 ± 1.04
Carrot Fiber	69.26 ± 2.15	55.26 ± 1.93	4.56 ± 0.40

3. Results and discussion

3.1. Antioxidant capacity

3.1.1. Antioxidant capacity of the gastrointestinal digestion supernatant.

Antioxidant capacity values are shown in **Table 51**. Adding fiber increased the antioxidant capacity, and addition of citrus fiber plus herbal extract had the highest effect. Overall, antioxidant capacity increased 30% on average due to the addition of fiber. However, this increase was not always statistically significant. Only in the case of citrus fiber plus herbal extract values were, for all five methods, significantly higher than that for the control sample. Citrus fiber salami was also significantly higher than the control in $TEAC_{ABTS}$, $TEAC_{FRAP}$, and $TEAC_{OH}$. Salami with inulin and arabinogalactan were only significantly higher in antioxidant capacity based on the $TEAC_{FRAP}$ method.

Most of the antioxidant capacity generated during gastrointestinal digestion could come from peptides released by protein hydrolysis such as the dipeptides carnosine and anserine, which have been reported as effective hydrophilic antioxidants (Martínez et al., 2014). Gullon et al., (2015) also described an increase in the antioxidant capacity of fiber rich samples after gastrointestinal digestion. As explained by those authors, low pH could prompt structural changes in components such as fiber, but can also help release other compounds trapped in the fiber matrix. Moreover, structural changes during digestion could lead to the increased exposure of functional groups able to scavenge for oxidant species (Pastoriza et al., 2011).

Citrus fiber is mainly composed of pectin, which is a highly branched polysaccharide rich in galacturonic acid and other sugars. Our results are in accordance

with previous literature, in which strong antioxidant capacity has been revealed for polysaccharides containing galacturonic acid (H. Wang et al., 2018; Yao et al., 2018).

Salami with added herbal extract also tended to possess high antioxidant capacity. The herbal extract we used was composed of lemongrass and rosemary. It has previously been reported that lemongrass has a high antioxidant capacity and is rich in phenolic compounds such as gallic acid, quercetin, isoquercetin, rutin or tannic acid (Somparn et al., 2018). Rosemary is also rich in polyphenols such as flavonoids or tannins possessing high antioxidant capacity (Y.-Z. Wang et al., 2018).

Table 51. Antioxidant capacity values obtained for gastrointestinal digestion fraction, gut microbiota fermentation fraction, solid residue fraction, and global antioxidant capacity.

	$TEAC_{FRAP}$	$TEAC_{ABTS}$	$TEAC_{OH}$	$TEAC_{AAPH}$	$GEAC_{RED}$
	mmol Trolox equivalents/kg	**mmol Trolox equivalents/kg**	**mmol Trolox equivalents/kg**	**mmol Trolox equivalents/kg**	**mmol Gallic acid equivalents/kg**
Gastro-intestinal supernatant					
Salami control	10.03 ± 0.65[a]	9.56 ± 0.59[a]	1.23 ± 0.01[a]	2.03 ± 0.48[a]	14.98 ± 1.54[a]
Inulin salami	15.26 ± 0.13[b]	13.43 ± 3.24[b]	1.89 ± 0.09[a]	1.59 ± 0.47[a]	16.23 ± 0.21[a]
Citrus fiber salami	16.23 ± 2.60[b]	14.87 ± 1.01[b]	2.03 ± 0.06[b]	2.56 ± 0.16[a]	15.26 ± 2.21[a]
Citrus fiber + herbal extract salami	18.26 ± 2.06[b]	16.78 ± 3.57[c]	2.13 ± 0.04[b]	4.56 ± 0.31[b]	24.56 ± 2.65[b]
Acacia-fiber salami	17.89 ± 3.31[b]	10.26 ± 2.10[a]	1.56 ± 0.05[a]	2.16 ± 0.12[a]	17.23 ± 1.56[a]
Microbial fermentation supernatant					
Salami control	35.69 ± 2.84[a]	51.56 ± 2.31[a]	24.37 ± 1.36[a]	2.74 ± 0.11[a]	46.78 ± 2.41[a]
Inulin salami	55.36 ± 3.87[c]	44.78 ± 3.04[c]	22.36 ± 2.03[a]	10.29 ± 0.96[c]	53.69 ± 4.14[c]
Citrus fiber salami	81.23 ± 6.06[b]	108.23 ± 6.52[b]	30.68 ± 2.56[b]	30.21 ± 2.90[b]	69.45 ± 4.86[b]
Citrus fiber + herbal extract salami	83.69 ± 7.74[b]	115.45 ± 9.16[b]	55.23 ± 3.55[c]	29.26 ± 1.69[b]	79.23 ± 7.66[b]
Acacia-fiber salami	54.36 ± 2.22[c]	55.12 ± 2.47[a]	26.56 ± 1.02[a]	14.31 ± 1.50[c]	63.24 ± 5.89[b]
Microbial fermentation solid residue					
Salami	2.01 ± 0.13[a]	1.91 ± 0.12[a]	0.25 ± 0.05[a]	0.41 ± 0.10[a]	3.00 ± 0.31[a]

control															
Inulin salami	3.05	±	0.03[b]	2.69	±	0.65[b]	0.38	±	0.02[a]	0.32	±	0.09[a]	3.25	±	0.04[a]
Citrus fiber salami	3.25	±	0.52[b]	2.97	±	0.20[b]	0.41	±	0.09[b]	0.51	±	0.03[a]	3.05	±	0.44[a]
Citrus fiber + herbal extract salami	3.65	±	0.01[b]	3.36	±	0.71[b]	0.43	±	0.04[b]	0.91	±	0.06[b]	4.91	±	0.53[b]
Acacia-fiber salami	3.58	±	0.66[b]	2.05	±	0.42[a]	0.31	±	0.03[a]	0.43	±	0.02[a]	3.45	±	0.31[a]
Total antioxidant capacity															
Salami control	47.73	±	2.55[a]	65.45	±	4.21[a]	25.85	±	1.91[a]	5.18	±	0.20[a]	64.76	±	3.66[a]
Inulin salami	73.67	±	4.39[c]	62.51	±	3.16[a]	24.63	±	3.90[a]	12.20	±	0.49[c]	73.17	±	5.52[c]
Citrus fiber salami	100.71	±	8.91[b]	124.33	±	10.40[b]	33.12	±	2.47[b]	33.28	±	3.69[b]	87.76	±	6.38[b]
Citrus fiber + herbal extract salami	105.60	±	7.96[b]	132.47	±	9.66[b]	57.79	±	6.90[c]	34.73	±	2.68[b]	108.70	±	7.27[d]
Acacia-fiber salami	75.83	±	5.35[c]	69.72	±	5.27[a]	28.43	±	3.56[a]	16.90	±	3.29[c]	83.92	±	4.73[b]

Different letters within the same column and fraction indicate statistical significance ($p < 0.05$).

3.1.2. Antioxidant capacity of the fermentation supernatant.

Antioxidant capacities of fermented samples are summarized in **Table 51**. *In vitro* fermentation also increased antioxidant capacity of salami samples with added fiber. However, in this case, differences with the salami control were noticeably larger than those observed after digestion step. $TEAC_{FRAP}$ method showed significantly higher values in all samples with added fiber in comparison with salami control. The $TEAC_{OH}$ differences were similar to those found for $TEAC_{ABTS}$, with values significantly higher in both citrus fiber salamis. $TEAC_{AAPH}$ method also revealed significantly higher values

in all salamis with fiber in comparison with the control. Finally, $GEAC_{RED}$ showed the same tendency as that revealed by the $TEAC_{FRAP}$ and $TEAC_{AAPH}$ methods (see **Table 51**).

In vitro colonic fermentation seems to have a more marked effect on antioxidant capacity than *in vitro* gastrointestinal digestion due to microbial metabolism of available substrates. The higher values found in salami with added fibers in comparison to the standard formulation are likely the result of the microbial degradation of the added polysaccharides. This has previously been reported for other fiber-rich samples (Zamora-Gasga et al., 2015). Further, a common finding among the five antioxidant testing methods we used in this study was the higher antioxidant capacity found in salami with added citrus fiber. This finding could be due to fiber composition (pectin) yielding a different profile of metabolites with more antioxidant potential during microbial degradation. Another possible explanation is the source of citrus fiber. This fiber is extracted from citrus fruits, and other bioactive compounds could remain trapped inside the fiber structure and thus can be released upon digestion or fermentation (Pérez-Burillo et al., 2018b).

Finally, salami with added herbal extract had the highest antioxidant capacity measured by all five testing methods. Bioactive compounds coming from both lemongrass and rosemary could be the reason behind such high antioxidant capacity. Their metabolization by gut microbiota could yield simpler phenolic acids that contribute to the antioxidant activity (Selma, Espín, & Tomás-Barberán, 2009). According to our results, these new compounds would exert radical scavenging activity and reducing power.

3.1.3. Antioxidant capacity of the solid residue.

The measurement of antioxidant capacity in the solid residue is important for several reasons (Gökmen et al., 2009; Pérez-Burillo et al., 2018b):

- Even after digestion and fermentation, there could be low molecular weight compounds still physically trapped within large macromolecules that resist digestion and fermentation.
- Other compounds that are chemically bound to macromolecules are also resistant to digestion and fermentation processes.
- The solid residue represents the antioxidant capacity exerted by large macromolecules that are not degraded by digestion or fermentation.

The antioxidant capacity values obtained for the solid residues are shown in **Table 51**. As previously stated for the other two fractions, the antioxidant capacity tended to be higher in the samples with added fiber in comparison with the control salami. Therefore, the solid residues retained some antioxidant power, although the values were significantly lower than the antioxidant capacity of the soluble fractions.

3.1.4. Total antioxidant capacity.

Total antioxidant capacity of each salami sample is depicted in **Figure 62**. The total antioxidant capacity was higher in salamis with fiber. In samples with citrus fiber alone or also supplemented with the herbal extract, the antioxidant capacity was significantly higher than in the rest of the samples. Overall, as result of adding fiber to salami formulation, the antioxidant capacity increased by 43% on average. The supernatant fraction obtained after sample fermentation was the main contributor to the global antioxidant capacity for all the samples (**Figure 63**). Interestingly, contribution of the fermentation supernatant was higher in samples with added fiber, which reinforces

the idea that fermentation of fiber by human gut microbiota can yield antioxidant compounds. Therefore, dietary fiber could be used as a good prebiotic supplement to increase the antioxidant capacity of a given food.

Figure 62. Antioxidant capacity in all samples obtained with $TEAC_{FRAP}$, $TEAC_{ABTS}$, $TEAC_{OH}$, $TEAC_{AAPH}$, and $GEAC_{RED}$ assays. Results are expressed in mmol Trolox equivalents/kg of salami for $TEAC_{FRAP}$, $TEAC_{ABTS}$, $TEAC_{OH}$, $TEAC_{AAPH}$, and in mmol gallic acid equivalents/kg of salami for $GEAC_{RED}$. Abbreviations: SC: salami control, CF: salami with citrus fiber, IN: salami with inulin, FG: salami with acacia-fiber, CF-HE: salami with citrus fiber and herbal extract. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. Statistical significance is shown for tests that used salami control as the reference group. Analyses were carried out in triplicate.

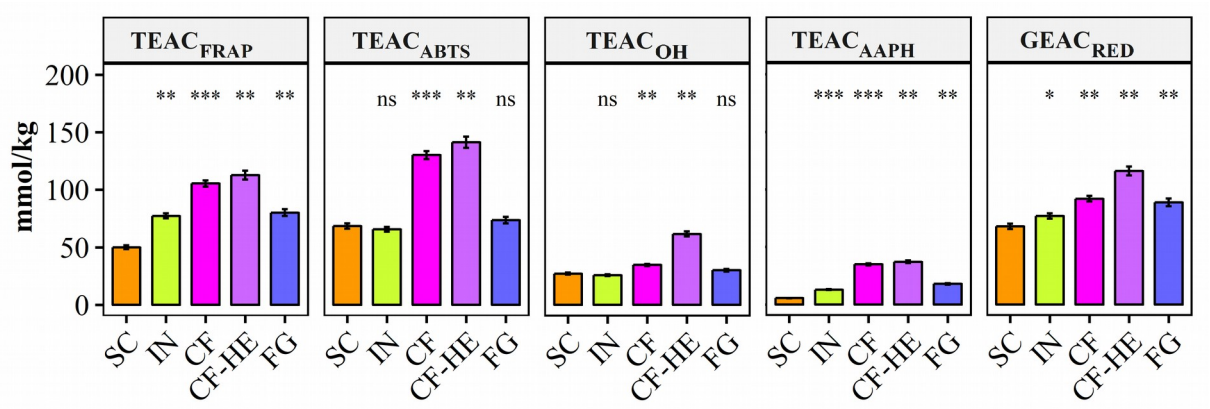
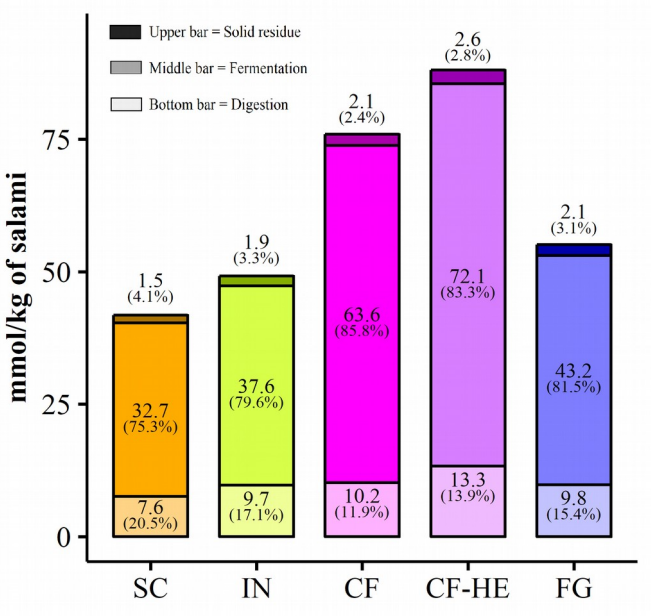


Figure 63. Average contribution of digestion supernatant, fermentation supernatant and solid residue to global antioxidant capacity. For this figure, each antioxidant capacity value for each fraction was calculated as an average of the antioxidant capacity values obtained with all five methods. Abbreviations: SC: salami control, CF: salami with citrus fiber, IN: salami with inulin, FG: salami with acacia-fiber, CF-HE: salami with citrus fiber and herbal extract. Analyses were carried out in triplicate.

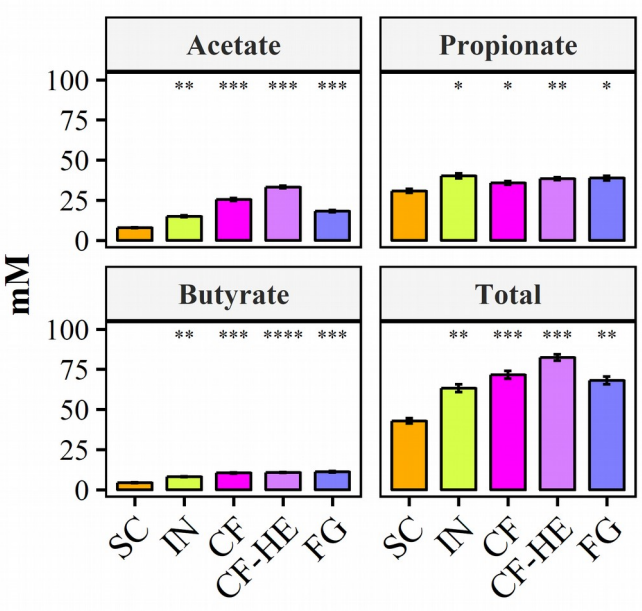


3.2. Analysis of short chain fatty acids

We analyzed SCFAs production after fermentation of salami samples (**Figure 64**). Acetate production was significantly higher in all samples with added fiber. Salami with citrus fiber showed significantly higher acetate production than the other fiber-containing preparations ($p < 0.05$). Propionate production was also significantly higher in salamis containing fiber ($p < 0.05$), though the differences with the control sample were not as drastic as with acetate (see **Figure 64**). Similarly, levels of butyrate were

higher in all samples with added fiber. Overall, adding fiber to salami formulation increased SCFAs production: acetate, propionate and butyrate production increased on average by 66%, 20% and 58%, respectively. Citrus fiber showed higher potential for generating additional acetate compared with inulin and arabinogalactan, whereas propionate and butyrate levels were more similar among different fiber-supplemented salami samples (**Figure 64**). Specifically, salami added with citrus fiber in addition to herbal extract, which showed the highest antioxidant potential, also showed the highest potential for total SCFA production. This sample produced significantly higher amounts of acetate, whereas in the case of propionate and butyrate, produced similar values than acacia-fiber salami.

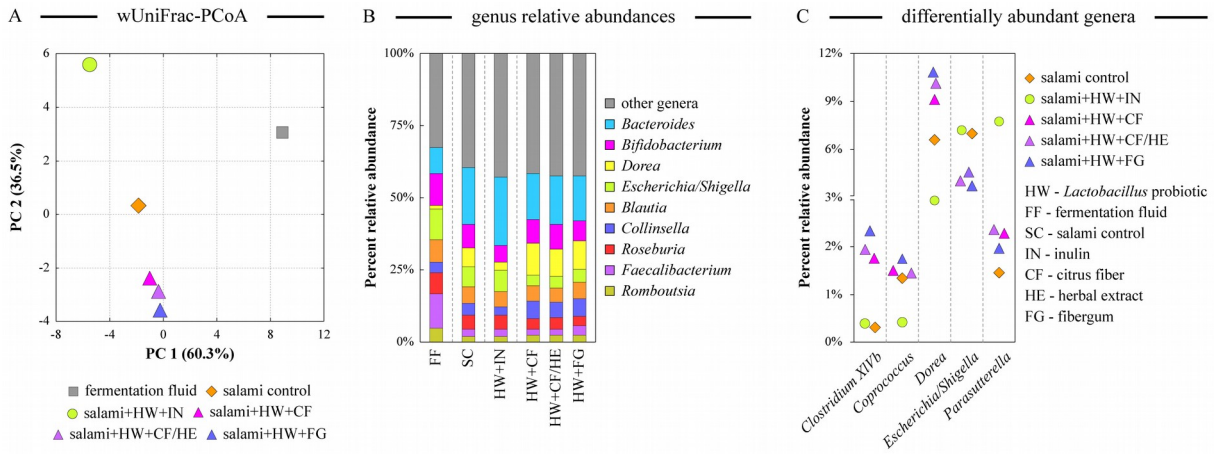
Figure 64. Measurements of short chain fatty acids. Abbreviations: SC: salami control, CF: salami with citrus fiber, IN: salami with inulin, FG: salami with acacia-fiber, CF-HE: salami with citrus fiber and herbal extract. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. Statistical significance is shown for tests that used salami control as the reference group. Analyses were carried out in triplicate.



3.3. Fermentation of salami preparations by human fecal microbiota promotes different community structures.

The microbiota community structure was determined after fermentation of digested salami samples for 24 hours. Overall, the community composition varied among different samples as determined by PCoA ordination analysis (**Figure 65A**). Fermented salami samples containing citrus fiber (pectin is the primary digestible polysaccharide composed of galacturonic acid and other sugars) or Acacia-fiber (composed primarily of arabinogalactan polysaccharide) clustered together, indicating that these prebiotics promoted similar microbial community structure. Inulin, a polymer of fructose and glucose, gave rise to a noticeably different community, and all of these samples produced microbiota community organization different from the fermentation fluid control and unsupplemented salami (**Figure 65A**). However, overall community diversity did not differ significantly among samples (data not shown). In most samples, *Bacteroides*, *Bifidobacterium*, *Dorea*, and *Escherichia/Shigella* were the most abundant genera (**Figure 65B**). Compared to the fermentation fluid control, all salami samples had increased prevalence of *Bacteroides*, which is an efficient protein degrader (Macfarlane, Cummings, & Allison, 1986). Fermentation of inulin promoted *Bacteroides* and *Parasutterella*, consistent with previous findings (Sonnenburg et al., 2016; Zhang et al., 2018), whereas other fibers supported expansion of Clostridia members such as *Dorea* and *Clostridium cluster XI*Vb (see F**igures 65B and 65C**). Adding these fibers to salami also reduced the prevalence of *Escherichia/Shigella* genus (which is known to have many human gut pathogens) in the microbial communities.

Figure 65. Fermentation of salami preparations with fiber by human fecal microbiota promotes different community structures. Panel A displays the output of the unconstrained PCoA ordination analysis of microbial genus abundance dataset among all profiled samples. Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix. The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles. **Panel B** shows relative abundances of the top nine most abundant microbial genera. Abundances of other genera were summed and are represented cumulatively as "other genera". Each column represents a community derived from an independent microbiota-based fermentation of each salami preparation. **Panel C** displays the abundances of five select genera with varied prevalence among fermented samples. These genera were selected numerically as being at least 1.5-fold different between a sample with fiber and the control sample. Color codes and description of sample abbreviations are shown in the legends.



4. Conclusions

As conclusions, in this project we studied the potential benefits of including dietary fiber and probiotic bacteria in salami dry sausage. Adding fiber to the salami increased both its antioxidant capacity and the amount of SCFAs produced during salami fermentation by human gut microbiota. While all tested fibers increased antioxidants and SCFAs production, the effect was highest for citrus fiber. Incorporating herbal extract further elevated the beneficial properties of the salami. Majority of antioxidants were released during salami fermentation by human gut microbiota. In concert with this finding, adding fiber to salami also promoted different microbial structure compared to the traditional salami formulation. Especially noticeable was the ability of citrus fiber and acacia-fiber to reduce the prevalence of members of *Escherichia/Shigella*, a genus with many known pathogenic and toxin-producing species. We conclude that the addition of dietary fiber to dry-fermented meat products can improve their nutritional and health values, and these effects are achieved during fermentation of salami by human gut microbiota in the colon.

Abbreviations: HPLC: High Performance Liquid Chromatography, SCFA: Short Chain Fatty Acids, TEAC: Trolox Equivalent Antioxidant Capacity, GEAC: Gallic Acid Antioxidant Capacity.

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Founding

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Potential probiotic salami with dietary fiber modulates antioxidant capacity, inflammatory markers, short chain fatty acid production and gut microbiota community structure in a human intervention

Tras los buenos resultados obtenidos en el anterior trabajo, en este paper se describen los resultados de la intervención humana realizada con el salchichón reformulado que mejores resultados arrojó en el trabajo anterior. De esta forma se estudió el efecto del consumo del salchichón sobre la capacidad antioxidante plasmática, antropometría, bioquímica sanguínea, marcadores de estado inflamatorio, así como efectos en la microbiota intestinal. De esta forma, se pretende obtener un salchichón mejorado que sea más saludable que el habitual para la dieta de la población general.

***LWT*. Sent for publication.**

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Potential probiotic salami with dietary fiber modulates antioxidant capacity, inflammatory markers, short chain fatty acid production and gut microbiota community structure in a human intervention

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Abstract

A human intervention in healthy volunteers was performed to test the potential health benefits of a fermented salami with a probiotic *Lactobacillus rhamnosus* and added with citrus fiber. Anthropometric measurements and blood biochemistry did not show any significant differences between pre- and post-intervention during 4 weeks with a daily intake of 30g of salami, neither with regular salami (control group) nor with reformulated salami (intervention group). However, inflammatory markers CRP and TNF decreased significantly, suggesting a less inflammatory environment after reformulated salami consumption. Antioxidant plasmatic markers also improved within the intervention group. Regarding short chain fatty acids production, no changes were observed after regular salami consumption. Interestingly, butyrate production was significantly higher after reformulated salami consumption. Gut microbiota community structure, however, was not significantly shaped by neither regular nor reformulated salami. After the intervention with probiotic salami, *L. rhamnosus* was detected (qPCR) in all samples of the intervention group but not in the control group, reinforcing its probiotic effect.

Keywords: salami, probiotic, antioxidant capacity, short chain fatty acids, gut microbiota, human intervention.

1. Introduction

Salami is a dry-fermented sausage consisting of mixtures of lean meats and fatty tissues combined with salts, nitrate (curing agent), sugars, herbs, spices, and other non-meat ingredients filled into casings to follow a microbial fermentation, drying and maturation process. Salamis acquire their properties (flavor, texture, color, etc.) through a fermentation process, in which mainly lactic acid bacteria and coagulase-negative staphylococci are involved (FAO, 2017).

Salami is generally considered as a food with unbalanced nutritional value due to the high fat and salt content and the lack of bioactive molecules, such as phenolic compounds and other phytochemicals (Martínez, Nieto, & Ros, 2014). However, salami is highly consumed in many countries around the world (Blaiotta, Murru, Cerbo, Romano, & Aponte, 2018). This is probably one of the reasons why during the last 10-15 years there has been a great focus of interest on improving meat products quality (Olmedilla-Alonso, Jiménez-Colmenero, & Sánchez-Muniz, 2013). Related studies have gone three different ways: i) modifying meat composition through cattle feeding, like increasing monounsaturated fatty acid (MUFA) content in meat (Gilmore et al., 2011); ii) modifying meat products formulation by adding different functional ingredients such as minerals, vitamins, dietary fiber, plant derived compounds, etc. (Olmedilla-Alonso et al., 2013); iii) by innovations in processing/storage conditions, for example by adding antioxidants to increase shelf life (Cullere, Hoffman, & Dalle Zotte, 2013).

Health benefits of these new products have been often evaluated through human interventions. Since high-meat diets are associated with plasmatic lipids disorders, several of these research projects have focused on achieving a healthier plasmatic lipid

profile to reduce CVD risk. This has been tried by increasing MUFA content (Gilmore et al., 2011), and n-3 PUFA content (Haug, Nyquist, Mosti, Andersen, & Høstmark, 2012; Delgado-Pando, Celada, Sánchez-Muniz, Jiménez-Colmenero, & Olmedilla-Alonso, 2014), or by adding plant sterols (Tikkanen et al., 2001; Tapola, Lyyra, Karvonen, Uusitupa, & Sarkkinen, 2004). Another approach has been the substitution of fats by dietary fiber (Verma & Banerjee, 2010). However, in this case the focus of interest is usually on physico-chemical properties and organoleptic attributes of the product.

On the other hand, it has been proposed that fermented meat products could be a good carrier for probiotics (Vuyst, Falony, & Leroy, 2008), which are expected to play a role in the overall gut microbial biodiversity and hence, the health of the host. However the human studies related are scarce as opposed as in the case of CVD. For instance, Jahreis et al. (2002) studied plasmatic lipids and some immunological markers after consumption of fermented sausages with a probiotic strain. Results were inconclusive, the probiotic bacteria were only identified in feces in some of the volunteers, lipids did not change significantly and only antibodies against oxidized LDL increased. Very recently, Thøgersen et al. (2018) studied the addition of inulin on Frankfurt sausages feed to rats and their effect on gut microbiota composition and short chain fatty acids (SCFAs) production. Therefore, there is a lack of human studies about the effect of probiotic and fiber addition to salami.

Taking all this information into account, in this study we aim to investigate the effects on blood biochemistry, anthropometry, immunological markers, gut microbiota composition, SCFAs production, and antioxidant capacity of plasma and feces, after ingestion by healthy volunteers of 30g/day of salami added with fiber and a probiotic starter during a period of 4 weeks.

2. Materials and methods

2.1. Subjects and Study design

Twenty-four healthy subjects were recruited in Granada. Subjects were 20-30 years old and had a body mass index (BMI) within normal range (18.5-25). The study consisted on longitudinal nutritional intervention, doubled blinded, with placebo and two parallel groups: control and intervention group. Subjects were asked to not consume probiotics during a week before starting the study. Participants were randomly divided into control and intervention group. Control group was given regular salami and instructed to intake 30g/day (serving size) for 4 weeks. Intervention group was given the reformulated salami and instructed to intake 30g/day for the same period time. The day before starting the intervention, fecal and blood samples were taken and anthropometric measurements were also performed. At the end of the follow-up period, the same samples were obtained.

The study complied with the principles of the declaration of Helsinki. The Ethics Committee of the University of Granada approved the study protocol, and informed consent was obtained from all participants.

2.2. Salami preparation

Salami samples were manufactured and provided by a local Spanish company (Elpozo Alimentación, S.A., Alhama de Murcia, Murcia). Each salami sample was formulated according to the following traditional recipe: a mixture of pork meat and fatty tissues was combined with salt, curing salt agent, black pepper, starch/fiber, and typical Mediterranean herbal extract.

For the control salami, 2% w/w of starch was added, and a standard starter composed of non-probiotic strains of lactic acid bacteria and catalase-negative streptococci was applied. Fiber-added salamis were supplemented with a probiotic *Lactobacillus rhamnosus* as a starter culture, and citrus fiber (obtained from orange pulp dehydration and composed of 42% pectin, 25% cellulose and hemicellulose, and acquired from Fiberstars, USA). Fiber was added in a 1.5% w/w ratio. A mix of antioxidant herbal extract, composed of olive, coffee and tea extracts, was also incorporated as an ingredient in the improved salami sample (0.3% w/w), which is the usual proportion for seasoning in sausages and the one use by the company who provided the samples in their other commercial products (**Table 52**). Fiber and herbal extract were chosen after an *in vitro* test previously published (Pérez-Burillo et al., 2019).

Salamis were put into casings to exclude oxygen and subjected to a ripening-drying process for 40 days. After curing, salamis were sliced and packaged as they are for commercial purposes.

2.3. Anthropometric Measurements.

Guidelines of the International Society for the Advancement of Kinanthropometry (ISAK) were followed for the anthropometric analysis (Stewart, Marfell-Jones, Olds, & Ridder, 2011). All anthropometric measurements were carried out at the same place by an ISAK-certified level II anthropometry researcher. The following instruments were used: GPM Stadiometer (± 1 mm accuracy); Tefal scale (± 50 g accuracy); Holtain skinfold compass (± 1 mm accuracy); Holtain caliper (± 1 mm accuracy); Holtain flexible metallic metric belt (± 1 mm accuracy). The following measurements were taken: height, weight, skinfolds (triceps, biceps, subscapular,

suprailiac, supraspinal, abdominal, thigh, and calf), perimeters (waist, hip, relaxed biceps, flexed and contracted biceps, anckle, forearm, chest, thigh and calf, and waist/hip index), and diameters (bicromial, biliocrestal, bicondylar humerus, bistiloid and bicondylar femur). The body mass index (BMI) was calculated from height and weight. Then, the percentages of fat mass, non-fat mass and water were calculated. Bone density, and visceral fat was also calculated along with the basal metabolism.

2.4. Blood biochemistry

Venous blood was used to determine health-related biochemical markers. The analysis was performed in the morning after a 12-hour fasting period. The samples were stored in the dark in containers with ice and processed within the hour after extraction. Plasma was separated by centrifugation of the blood samples at 1500 rpm for 20 min at 18 to 25°C. The following parameters were measured with an automatized blood biochemistry system (DiaSys Diagnostric Systems GmbH, Germany)): glycaemia (GL; mg/dl), total cholesterol (TC; mg/dl), HDL cholesterol (cHDL; mg/dl), LDL cholesterol (cLDL; mg/dl) and triglycerides (TG; mg/dl), urea (mg/dL), creatinin (mg/dL), uric acid (mg/dL), total proteins (g/dL), transaminases ALT and AST (U/L), GGT (gamma glutamil transpeptidase, U/L), alkaline phosphatase (U/L), total billirrubine (mg/dL) and iron (µg/dL). Oxidized LDL was measured through ELISA kit (OxiSelect™ Human Oxidized LDL ELISA Kit, Cell Biolabs, Inc. USA).

Haematology was assessed through the following parameters: leukocytes (x103/µL), red blood cells (x106/µL), haemoglobine (g/dL), haematocrit (%), MCV (Mean Corpuscular Volume, fL), MCH (Mean Corpuscular Haemoglobin, pg) and MCHC (MCH Concentration, g/dL). Leukocyte formula was also assessed: platelets (x103/µL), neutrophils, eosinophils, basophils, lymphocytes and monocytes (%).

2.5. Inflammatory and immunological markers

TNF alpha, IL-6 and IL-10 were measured through the TNF alpha Human ELISA kit from Thermo Fisher scientific (USA). C-Reactive Protein (CRP) was measured through the Human C-Reactive Protein ELISA Kit (CLIA) from Biomatik (USA). Phagocytic activity was measured as described in Gill & Rutherfurd (2001).

2.6. Fecal antioxidant capacity and plasma antioxidant status

- *FRAP assay* was used to measure the antioxidant capacity of feces. The FRAP assay was carried out according to the procedure described in Benzie & Strain (1996) adapted to a microplate reader. Results were expressed as mmol Trolox/g of sample.

I) Catalase (CAT) activity in plasma was determined following the method described by Pastoriza, Roncero-Ramos, Rufián-Henares & Delgado-Andrade (2014) based on monitoring H_2O_2 decomposition by spectrophotometric measurement at 240 nm. Results were expressed as μmol/min/mL.

II) Glutathione peroxidase (GPX) activity in plasma was determined as described in Pastoriza et al. (2014). This method is based on the instantaneous formation of oxidized glutathione during the reaction catalyzed by GPX. Results were expressed as μmol/min/ mL.

III) Malondialdehyde (MDA) analysis in plasma was carried out as stated in Olusi (2002). MDA produced by hydrolysis of lipid hydroperoxides when heated under acid conditions reacts with thiobarbituric acid (TBA) to form a red complex, which absorbs light at 532 nm. Results were expressed as μmol/L.

2.7. SCFAs analysis

The production of SCFAs as a measure of the gut microbiota functionality was assessed according to the procedure described in Panzella et al. (2017). The analysis of SCFAs was carried out on Accela 600 HPLC (Thermo Scientific). Results were expressed as mM.

2.8. DNA extraction and sequencing

DNA extraction was performed using a NucliSENS easyMAG platform (Biomérieux) following the standard protocol. Microbial genomic DNA was used at a concentration of 5 ng/µl in 10 mM Tris (pH 8.5) for the Illumina protocol for 16S rRNA gene Metagenomic Sequencing Library Preparation (Cod. 15044223 Rev. A). PCR primers targeting the 16S rRNA gene V3 and V4 regions were designed as in (Klindworth et al., 2013). Primer sequences are: Forward 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA-G3' and Reverse 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA-CHVGGGTATCTAATCC3'. Primers contained adapter overhang sequences added to the gene-specific sequences, making them compatible with the Illumina Nextera XT Index kit (FC-131-1096). After 16S rRNA gene amplification, amplicons were multiplexed and 1 ml of amplicon pool was run on a Bioanalyzer DNA 1000 chip to verify amplicon size (~550 bp). After size verification, libraries were sequenced in an Illumina MiSeq sequencer according to manufacturer's instructions in a 2x300 cycles paired-end run (MiSeq Reagent kit v3 MS-102-3001).

2.9. Bioinformatic analysis

Quality assessment of sequencing reads was performed with the prinseq-lite program (Schmieder & Edwards, 2011) applying the following parameters: a minimal length (min_length) of 50 nt and a quality score threshold of 30 from the 3'-end (trim_qual_right), using a mean quality score (trim_qual_type) calculated with a sliding window of 10 nucleotides (trim_qual_window). Read 1 and read 2 from Illumina sequencing where joined using fastq-join from the ea-tools suite (Aronesty, 2011). Taxonomic affiliations were assigned using the RDP_classifier (Cole et al., 2009) from the Ribosomal Database Project (RDP). Reads that had an RDP score below 0.8 were assigned to the next higher taxonomic rank, leaving the last rank as unidentified. We assigned 6 taxonomic levels, which were kingdom, phylum, class, order, family and genus.

2.10. *Lactobacillus rhamnosus* qPCR

The HN001 primer sequences used were Forward 5′-CGCTTAGGACTCAGGATACA-3′ and Reverse 5′-GCTTGCGTCAGATTTTCAGTA-3′, according to published sequences (GenBank acc no. NZ_ABWJ00000000). PCR conditions used to amplify fecal DNA templates were pre-incubation at 95°C for 10 min; followed by 45 cycles of denaturation at 95°C for 10 s, annealing (69°C) for 10 s and extension at 72°C for 30 s; with a final cooling at 40°C for 30 s. DNA amplification products were analysed with the QIAxcel System (QIAGEN, Hilden, Germany).

2.10. Statistical analysis

The homogeneity of variance was assessed using the Levene test and the normal distribution of the samples with the Shapiro-Wilk test. The Student's t-test was used to analyze parametric data. The significance level was set at 5% ($p < 0.05$) in all tests. SPSS 22.0 for Windows (IBM SPSS Inc., New York, USA) was used for data analyses.

Table 52. Composition of Each Salami Formulation.

	Salami Control	Fiber added salami
Nutrient	**Content, g/100 g**	**Content, g/100 g**
Energy, kcal	384	382
Water, g	37.9	37.9
Protein, g	20.6	20.6
Lipids, g	32.1	32.0
Saturated, g	12.9	12.8
Monounsaturated, g	14.5	14.4
Polyunsaturated, g	4.8	4.8
Carbohydrates, g	3.2	3.3
Starch, g	2.0	0.0
Fiber, g	0.0	2.0
Herbal extract, g	0.0	0.2
Ash, g	4.2	4.4
L. rhamnosus	0.0	2^{10}

3. Results and discussion

3.1. Anthropometric analysis

No significant differences in anthropometric parameters were detected in the control group after 4 weeks consuming the salami control (**Figure 66A**). In this case, results are throwing some interesting conclusions since regular salami consumption did not modified BMI index, weight, body fat percentage, or any skin-fold thickness measurement. Therefore, at least in relation with anthropometric measurements, daily salami intake does not translate in weight gain or increase of body fat percentage (**Figure 66A**).

Similar results were observed for the intervention group, no significant differences were detected in any of the studied parameters after 4 weeks consuming the improved salami (**Figure 66B**). In addition, no statistically significant differences were found between the control and intervention groups at the post-intervention time ($p > 0.05$) for all the assessed parameters. From these results it can concluded that the improved formulation (fiber, probiotic) does not have any effect on body composition, at least at the dose administered. Still, salami consumption with either of the two formulations could be part of the diet without affecting anthropometric parameters, contrary to the general knowledge about the unhealthy effects of salami (Martínez, Nieto, & Ros, 2014).

3.2. Blood biochemistry

Salami is usually left out of diets due to its unbalanced composition in macronutrients, with high levels of saturated fats and proteins. However, salami consumption by control group (**Figure 67A**) with 30% less lipids than a other typical salamis, did not increase the levels of plasmatic lipids (total cholesterol, LDLc or TG). This could be a bit surprising since though cholesterol levels are more difficult to be affected by dietary patterns, TG are not. Since

volunteers showed previously to have plasmatic lipids levels within the normal range, it could be expected some increase. Not surprisingly, HDL levels did not change either (**Figure 67A**). These results suggest that regular salami can be part of a varied diet without influencing on plasmatic lipids. However, since this study was carried out with healthy volunteers, these results cannot be extrapolated to people in risk of CVD and more experiments should be done in this population. Regarding the rest of the biochemical parameters (i.e. hematology and leukocyte formula), it wasn't found either significant differences. Therefore, it can be concluded here that regular salami consumption does not affect blood biochemistry, at least in healthy subjects during a 4 weeks period intake.

Regarding the intervention group, the results were in the same line as in control group (**Figure 67B**). Plasmatic lipids did not change significantly although it was found a tendency to increase HDL levels ($p = 0.069$) and to decrease LDL concentration ($p = 0.058$). Jahreis et al. (2002) also found no modification is plasmatic lipid profile after consumption of sausages fermented with a probiotic strain. However, in our case the tendencies found could be derived from the presence of fiber, which is known to decrease blood LDL (Olmedilla-Alonso et al., 2013). Therefore, although the new ingredients added, at least at such dose, were not able to improve blood parameters, they didn't worse and tended to improve the lipid profile, so that future experiments with a larger population are needed to test this hypothesis.

Figure 66A. Anthropometric measurements in control group.

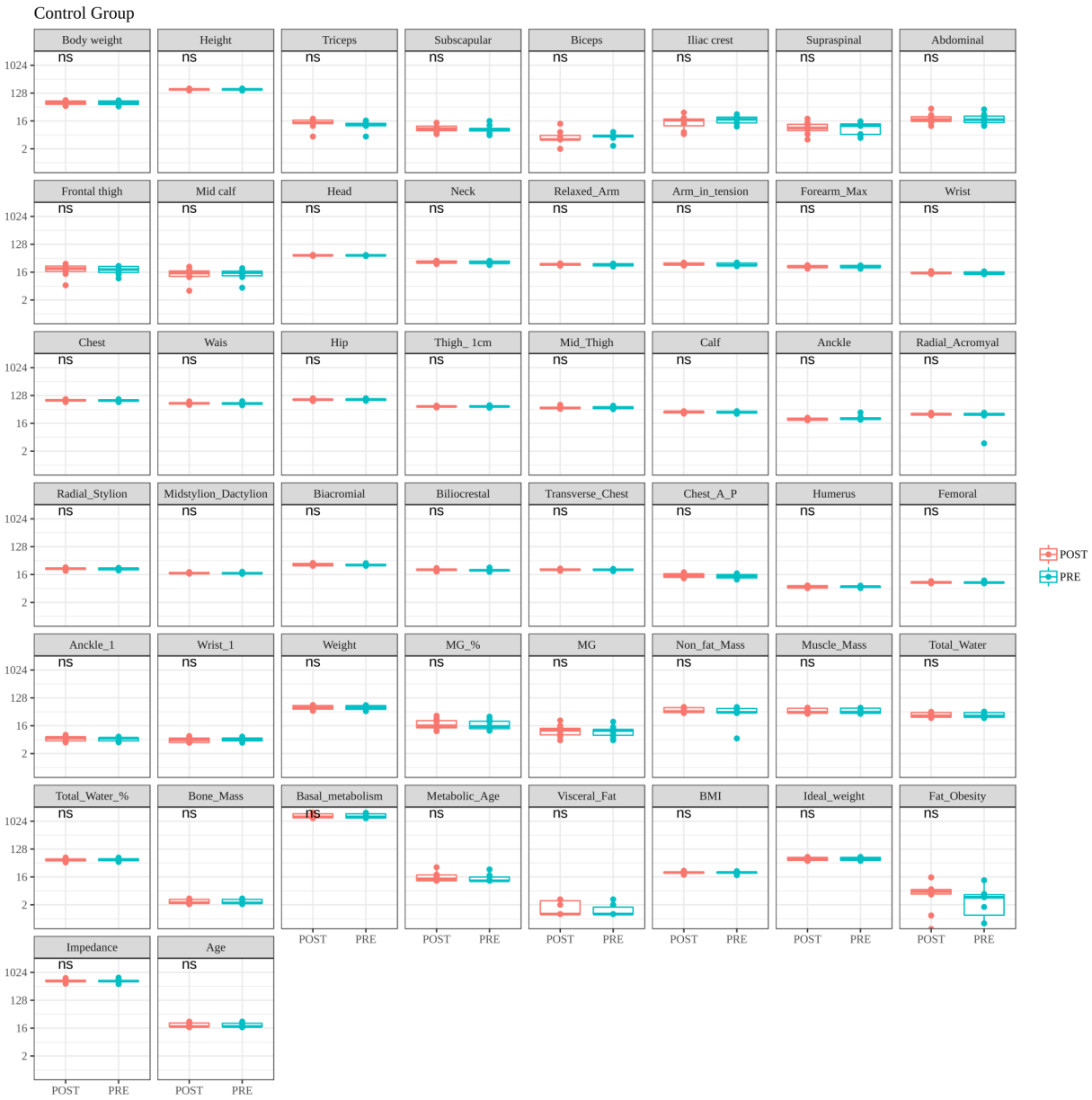


Figure 66B. Anthropometric measurements in intervention group.

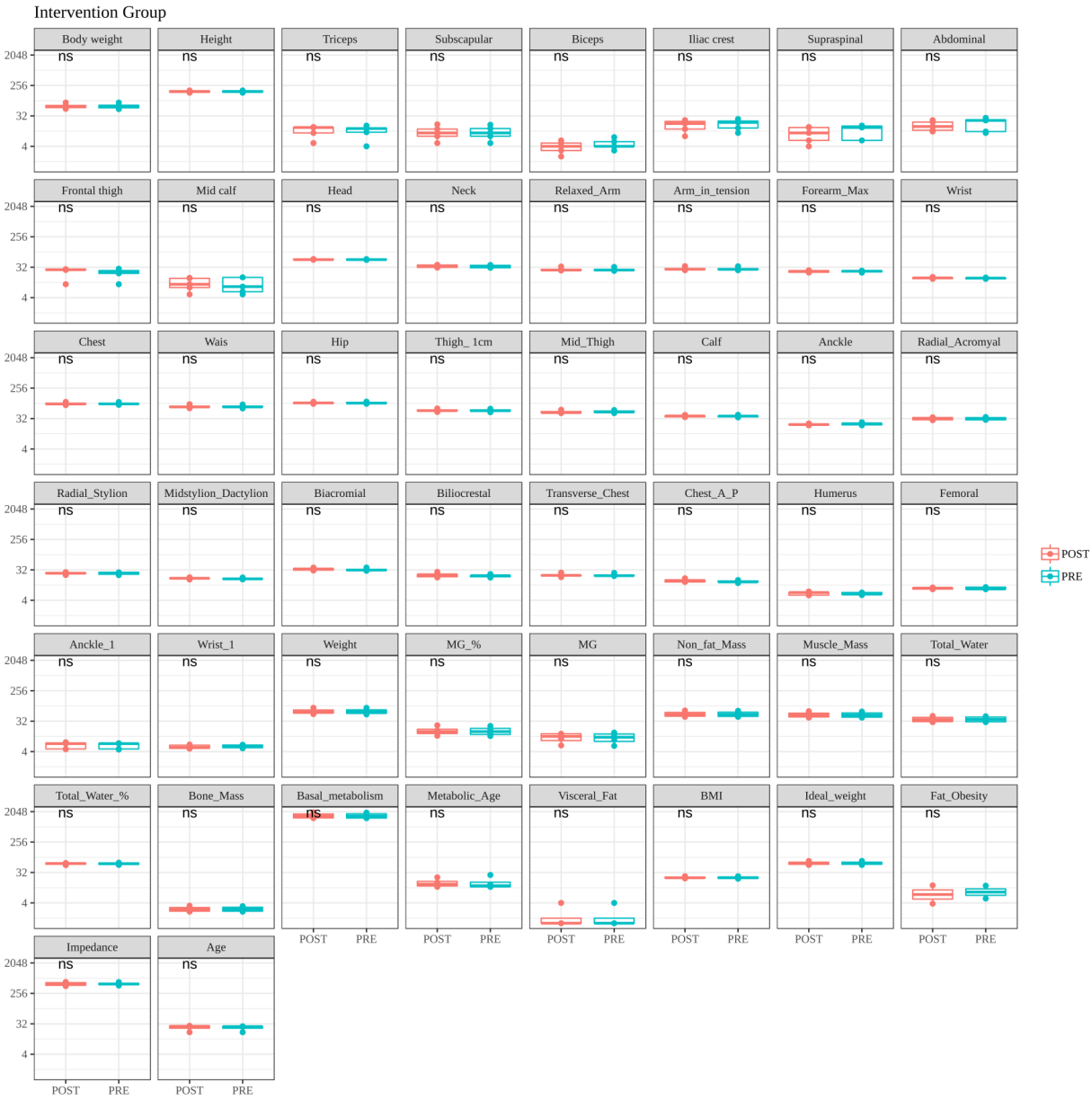


Figure 67A. Blood biochemical biomarkers in control group.

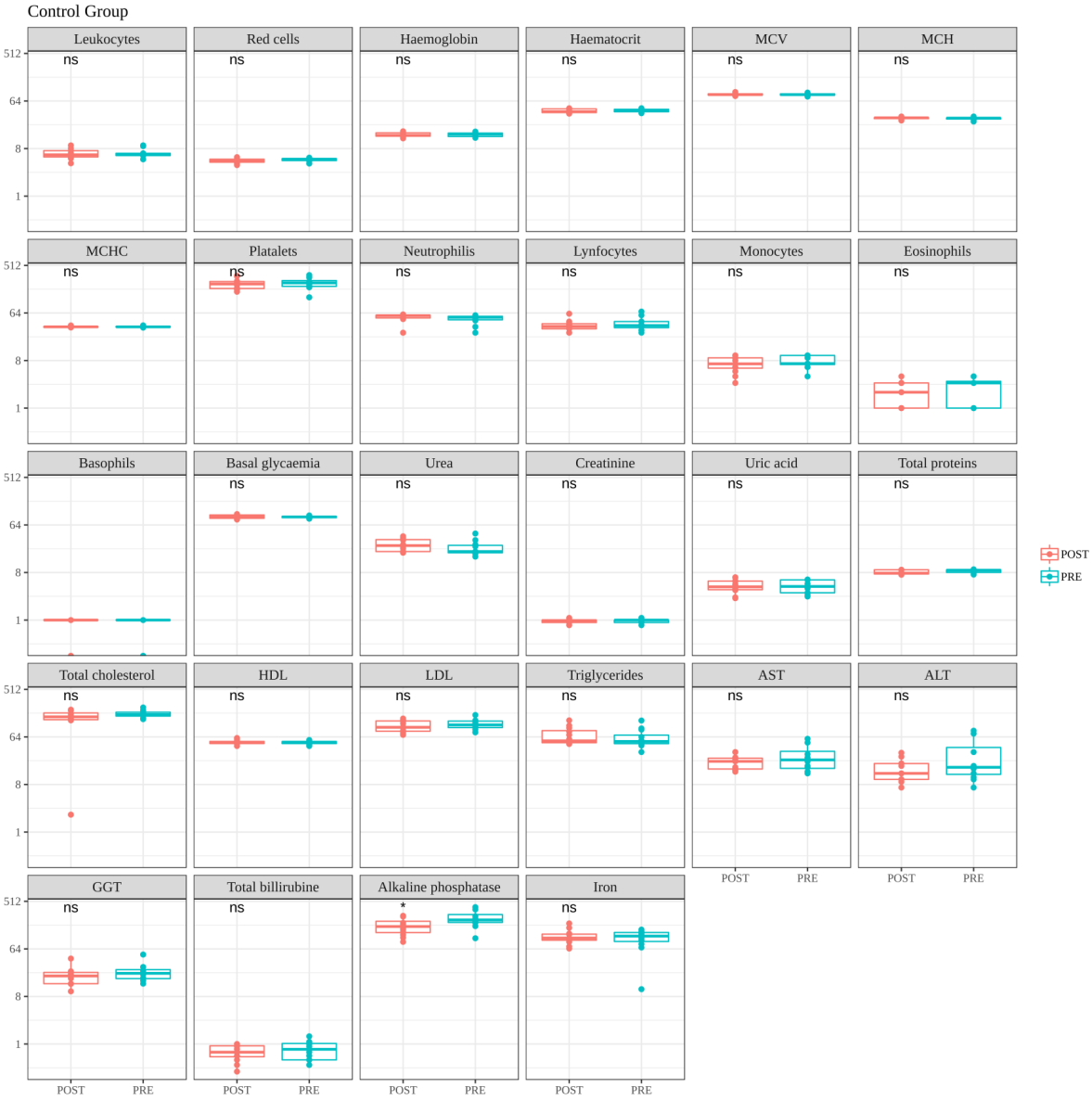


Figure 67B. Blood biochemical biomarkers in intervention group.

3.3. Antioxidant capacity

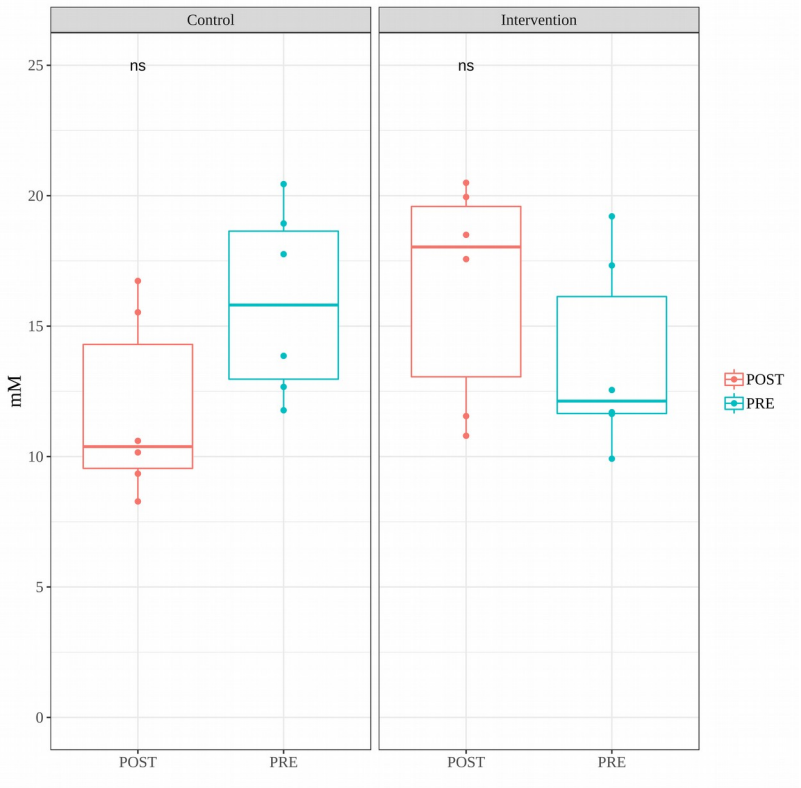
To study the influence of salami intake, either control or intervention salami, on body antioxidant status we analyzed the fecal antioxidant capacity with the FRAP assay (**Figure 68A**), and the plasma antioxidant capacity through plasmatic GPX, catalase, Ox-LDL and MDA (**Figures 68B** and **68C**). Within the control group, no significant differences in any of the parameters measured we found, suggesting that incorporating salami to a varied diet neither increase nor decrease the redox status of the subject. The levels of MDA, catalase and GPX were in the same range than those reported for adult population of the same body mass index (Olusi, 2002).

On the other hand, regarding the intervention group, the antioxidant capacity of feces did not show any changes before and after the intervention period, although there was a tendency ($p = 0.066$) to increase (**Figure 68A**). However, plasmatic markers improved after the 4 weeks intervention period. In fact, GPX and catalase activity increased significantly ($p < 0.05$) whereas Ox-LDL and MDA decreased significantly ($p < 0.05$), suggesting a positive modulation of the redox status (**Figure 68B** and **figure 68C**). In fact, MDA levels were below the usual range reported for individuals of the same body mass index (Olusi, 2002). This beneficial modulation could be due to the antioxidant extract incorporated to salami, since it has been demonstrated before that complementing diet with antioxidants increase the expression of plasmatic antioxidant enzymes (Alshammari, Balakrishnan, & Al-Khalifa, 2017). Other possible explanation is a role of the added fiber. As our previous research showed *in vitro* (Pérez-Burillo et al., 2019) salami added with this fiber showed a great potential as an antioxidant, probably due to fiber composition as well as to fiber metabolization by gut microbes. Thus, the antioxidant species released, either from the fiber matrix or from microbes'

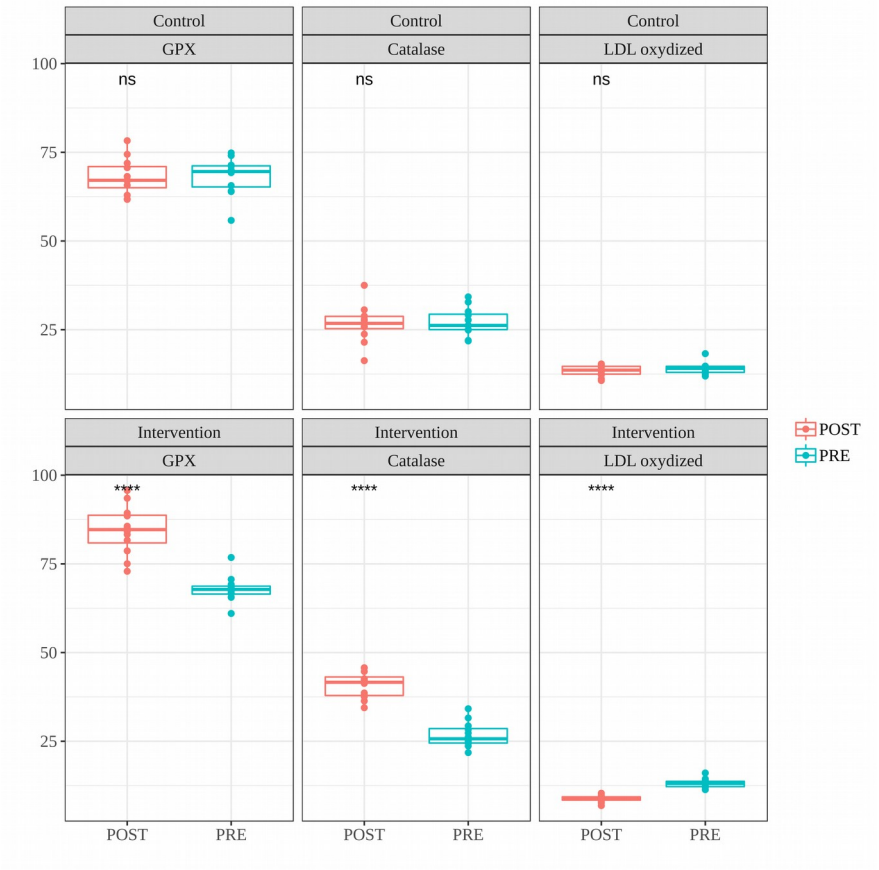
metabolic activity, could be absorbed at the large intestine and thus modulate the antioxidant status of plasma.

Figure 68. Panel A shows antioxidant capacity of volunteers feces' measured by FRAP method. Panel B shows plasmatic antioxidant markers GPx (µmol/min/ml), Catalase (µmol/min/ml), and LDL oxydized (mU/L). Panel C shows MDA plasmatic levels. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE situation as the reference group. Analyses were carried out in triplicate.

A



B


Control
GPX
Control
Catalase
Control
LDL oxydized
Intervention
GPX
Intervention
Catalase
Intervention
LDL oxydized
ns
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100
75
50
25
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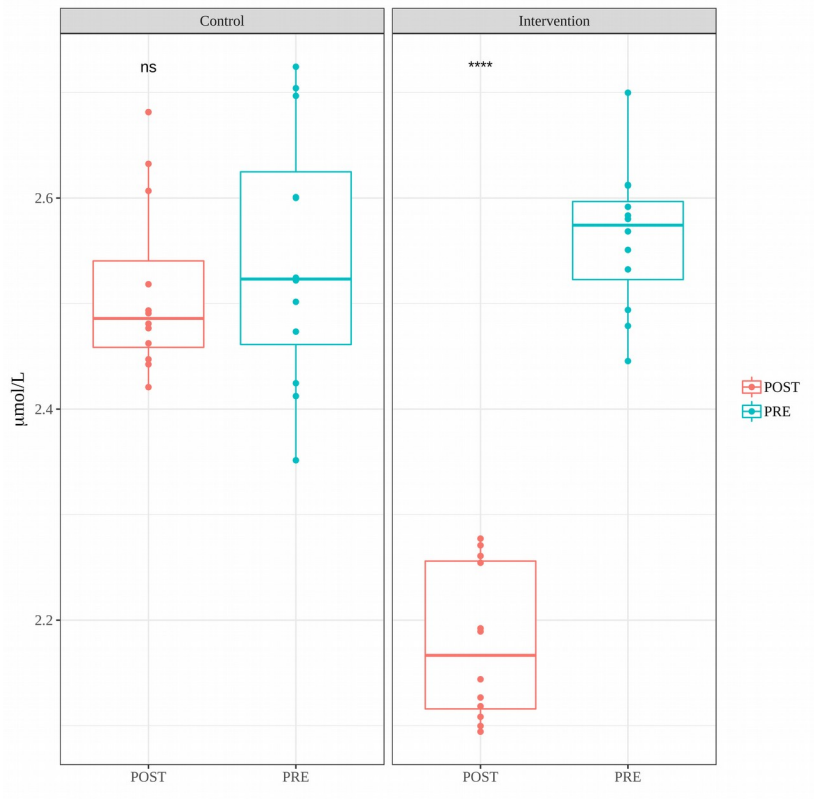
PRE

C

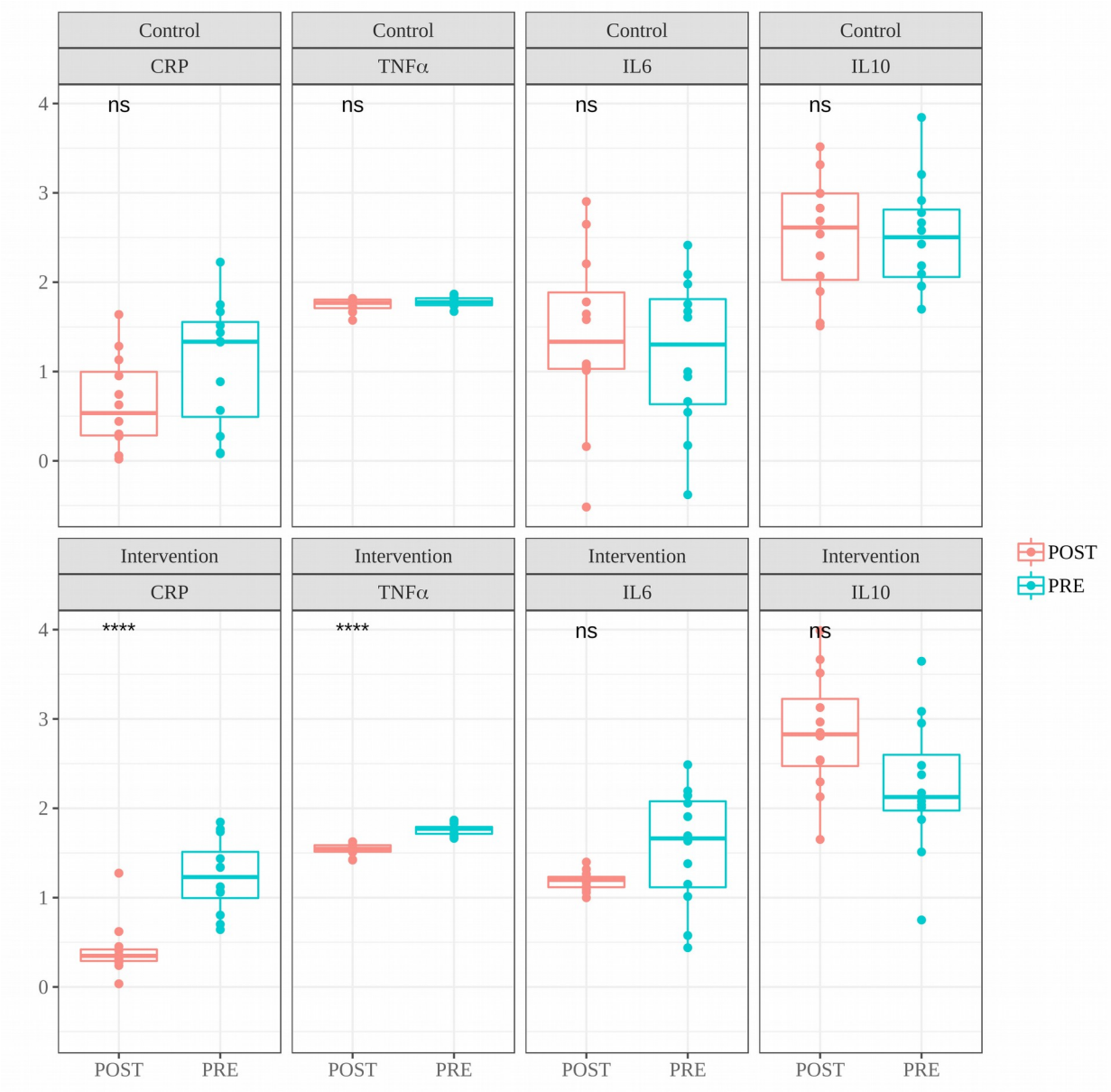

Control
Intervention
ns
****
µmol/L
2.6
2.4
2.2
POST
PRE

3.4. Inflammatory and immunological markers

Several markers involved in inflammation immunological status were measured: CRP, IL-6, IL-10, TNF-α and fagocitic activity. PCR is an unspecific inflammation marker that increases during inflammatory processes, IL-6 and TNF-α behave as pro-inflammatory species while IL-10 is an anti-inflammatory interleukine. Within the control group, there were no significant differences before and after the 4 weeks period of salami intake, since the levels of all parameters did not change (**Figure 69)**. Therefore, regular salami intake did not affect inflammatory status.

On the other hand, the intervention group showed an improvement in their inflammatory status after the intake period. A significant decrease ($p < 0.05$) was detected in the levels of CRP and TNF-α, suggesting a less inflammatory environment (**Figure 69)**. The rest of the parameters did not change significantly. These results could be due to the improved formulation, including the probiotic bacteria and the fiber component. For example, an improvement of the inflammatory and immunological status has been demonstrated previously in elderly supplemented with the immunostimulatory probiotic strain *L. rhamnosus* HN001 (Gill & Rutherfurd, 2001). However, it is know that modifications in fagocitic activity are only found in elderly population and not in adult population after exposure to a probiotic strain (Gill, Rutherfurd & Cross, 2001). In addition, as it will be explained below, butyrate levels were significantly increased in the intervention group after the intake period. This SCFA has been previously related to anti-inflammatory processes (Donohoe et al., 2014), being therefore another possible reason for the improved inflammatory status of volunteers, apart of the probiotic strain used for the fermentation process.

Figure 69. Plasmatic levels of inflammatory markers CRP (mg/L), TNFα (pg/mL), IL-6 (pg/mL), and IL-10 (pg/mL). Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE situation as the reference group. Analyses were carried out in triplicate.



3.5. Short chain fatty acids

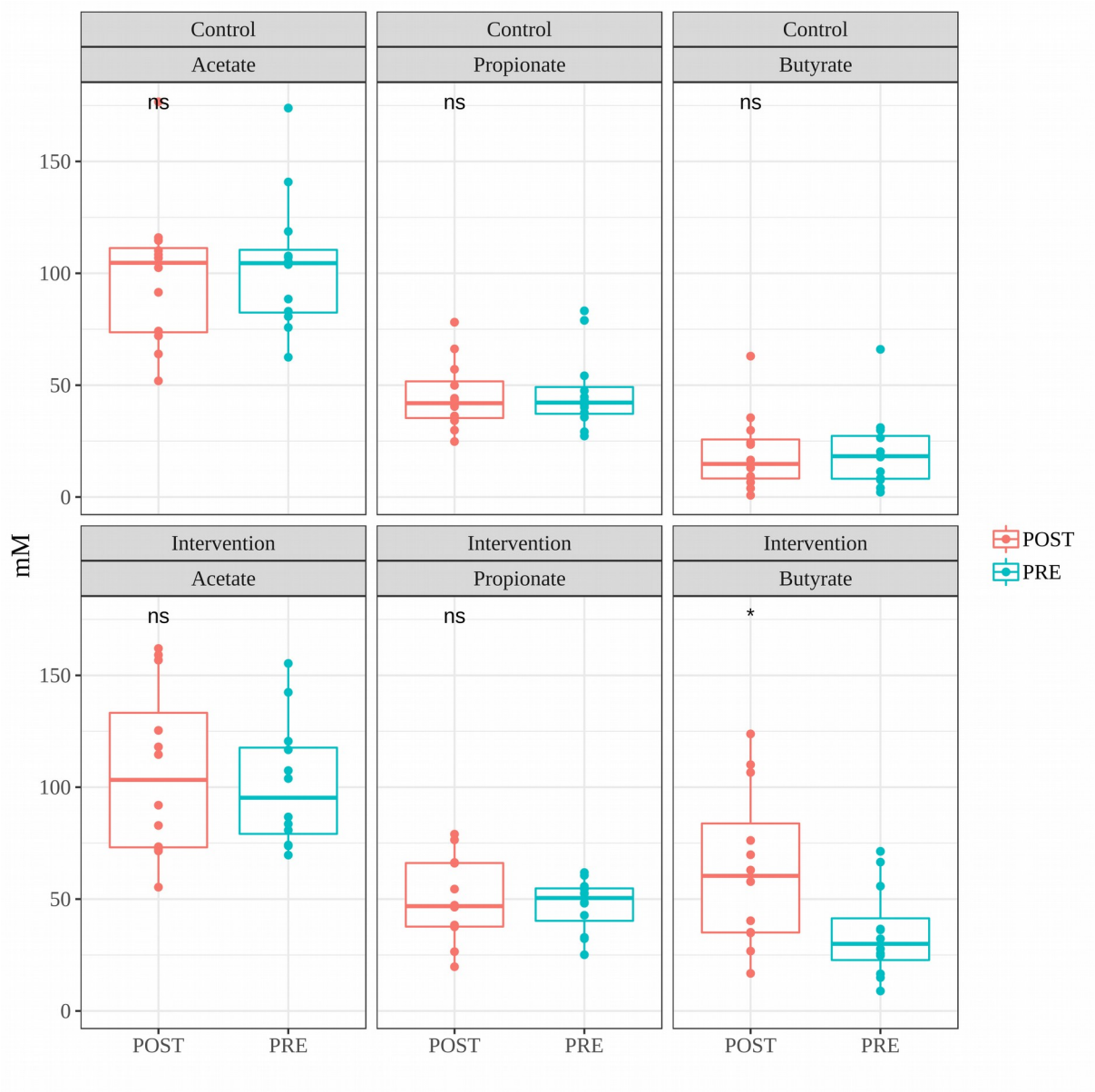
Several health effects are attributed to SCFAs. Decreasing the luminal pH is the obvious effect. The three main SCFAs (acetate, propionate and butyrate) are all important for the maintenance of the gut barrier (Ríos-Covián et al., 2016). Moreover, while butyric acid is mostly metabolized by colonocytes as source of energy and carbon, acetate and propionate are mainly absorbed and incorporated into different metabolic routes, which in turn could link the control of metabolic syndrome and diet-induced obesity (Lin et al., 2012). SCFAs also have an important role in colorectal cancer protection (Donohoe et al., 2014). In fact, it has been suggested that the protective effect of dietary fiber against colorectal cancer depends upon the production of butyrate and other SCFAs.

Regarding the control group, no significant changes were detected after the intake period, in fact, SCFAs levels were almost the same before and after (**Figure 70**). Acetate, propionate and butyrate production barely changed. On the other hand, within the intervention group, acetate production tended to increase ($p = 0.063$) after the intake period. In addition, there was a statistically significant increase ($p < 0.05$) on butyrate levels after intervention (**Figure 70**). This result could explain, at least partially, why some inflammatory markers improved after consumption of the re-formulated salami. This higher butyrate production could be attributed to the fiber component of the new formulation. As it has been previously demonstrated (Flint, Duncan, Scott, & Louis, 2015), polysaccharides degradation lead to butyrate production, being *Faecalibacterium* and *Eubacterium* the most abundant butyrate producers in human gut. As stated above, this new salami formulation is based on a previous *in vitro* study in which we investigated the potential of different fibers to produce SCFAs (Pérez-Burillo et al., 2019). As citrus fiber was the most promising agent, we kept using it for the present

formulation. As our previous research showed (Pérez-Burillo et al., 2019), results after intervention suggests that citrus fiber is also able to favor butyrate production. Higher butyrate production could be due to larger abundance in those genus known to be butyrate producers, being *Faecalibacterium, Eubacterium, Roseburia, Anaerostipes, Coprococcus, Butyricimonas, Clostridium XIVa, Intestinimonas* and *Butyricicoccus* their major representatives (Flint et al., 2015*;* Ríos-Covián et al., 2016).

After analyzing gut microbial community structure by 16S rRNA, we checked these genuses to study whether their abundance was higher after intervention, and to compare the control *Vs.* intervention group. No significant differences were found before and after salami intake, neither in control nor in intervention group. However, in intervention group some not statistically significant increases in the presence of these genus abundance were found after salami intake: *Faecalibacterium, Eubacterium, Anaerostipes, Coprococcus, Butyricimonas, Clostridium XIVa,* and *Intestinimonas.* Even though these increases were not significant, their synergic action could have lead to a significantly higher butyrate production. Moreover, after checking for spearman correlations between butyrate concentrations and butyrate producers abundance, it was found a positive significant correlation between butyrate and *Clostridium XIVa* (r^2 = 0.57) and *Faecalibacterium* (r^2 = 0.46). These correlations were not found in the control group, reinforcing the idea that the improved salami slightly modifies the functionality of the gut microbiota so that it produces higher concentrations of healthy SCFAs.

Figure 70. Short chain fatty acids levels measured in volunteers feces'. Statistical significance: *: p < 0.05, **: p < 0.01, ***: p < 0.001, ns: not significant. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE situation as the reference group. Analyses were carried out in triplicate.



3.6. Gut microbial community structure

At phylum level, no significant differences between pre- and post-intervention were found, neither in control or intervention groups (**Figure 71**). After studying the community dissimilarity through Unifrac phylogenetic distance PCoA before and after salami intake (Lozupone & Knight, 2005), no significant differences between both groups were found, showing no clear separation in PCoA plot (**Figure 72A-B)**. At genus level, after applying OPLS-DA to investigate discriminant bacteria in both groups before and after salami intake, it was not found any genus significantly associated to one or the other group. LefSE analysis (Segata et al., 2011) was also applied, but again no significant differences were found. However, as stated before, some tendencies where discover when investigating butyrate producers, and some of these bacteria were found to be higher (not significantly) after improved salami intake. These results are in line with those reported by other authors (Tannock, Munro, Harmsen, Welling Smart & Gopal, 2000; Lahtinen et al., 2012) who found that the administration of different foods enriched in different *L. ramhnosus* strains don't modify the overall fecal microbiota, with slight modifications in the lactobacilli populations.

We also investigated gut microbial community alpha diversity through Shannon and Simpson indexes. As our previous analysis suggested, no significant differences were found, showing a very similar diversity before and after salami intake. Therefore, salami intakedoes not have any significant influence on gut microbiota community structure, at least in healthy people. However, though not significant, improved salami intake did result in a significantly higher butyrate production, probably due to some increase in certain butyrate producers.

Figure 71. Barplot of gut microbial community structure at *phylum* level. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE situation as the reference group.

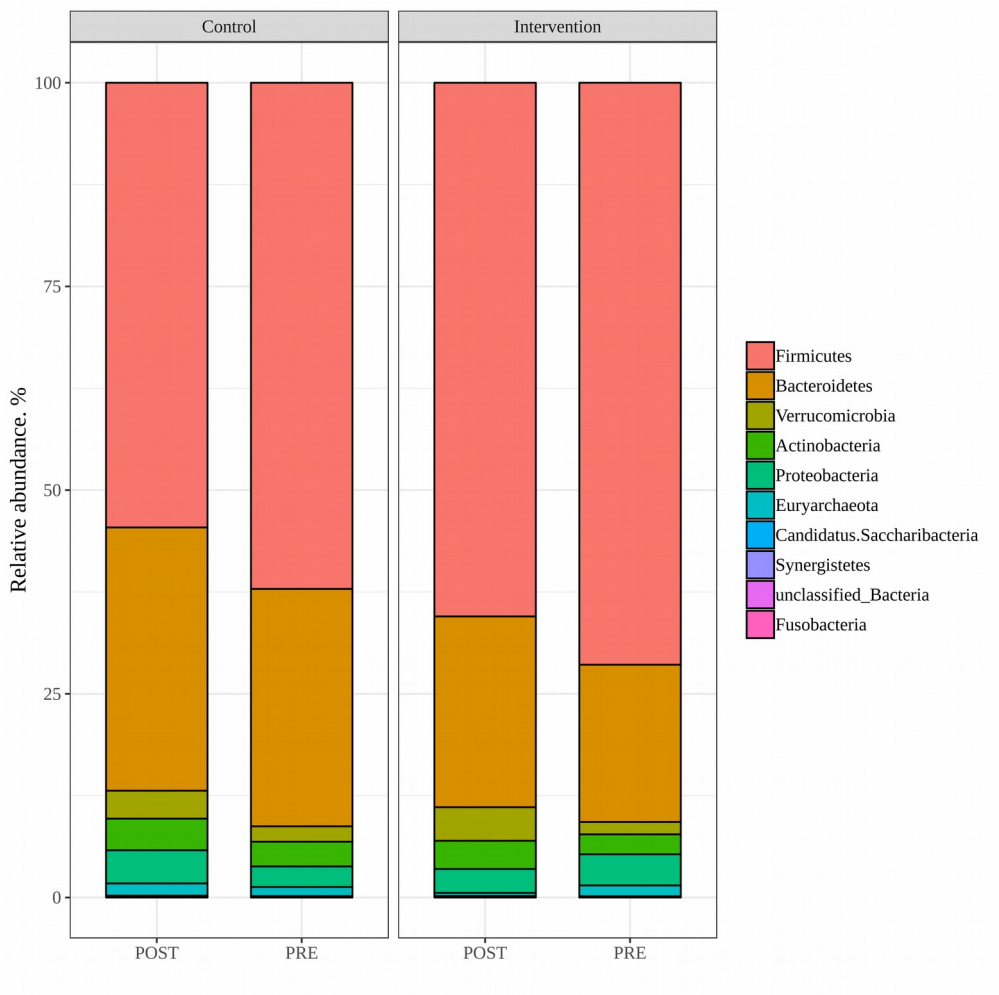
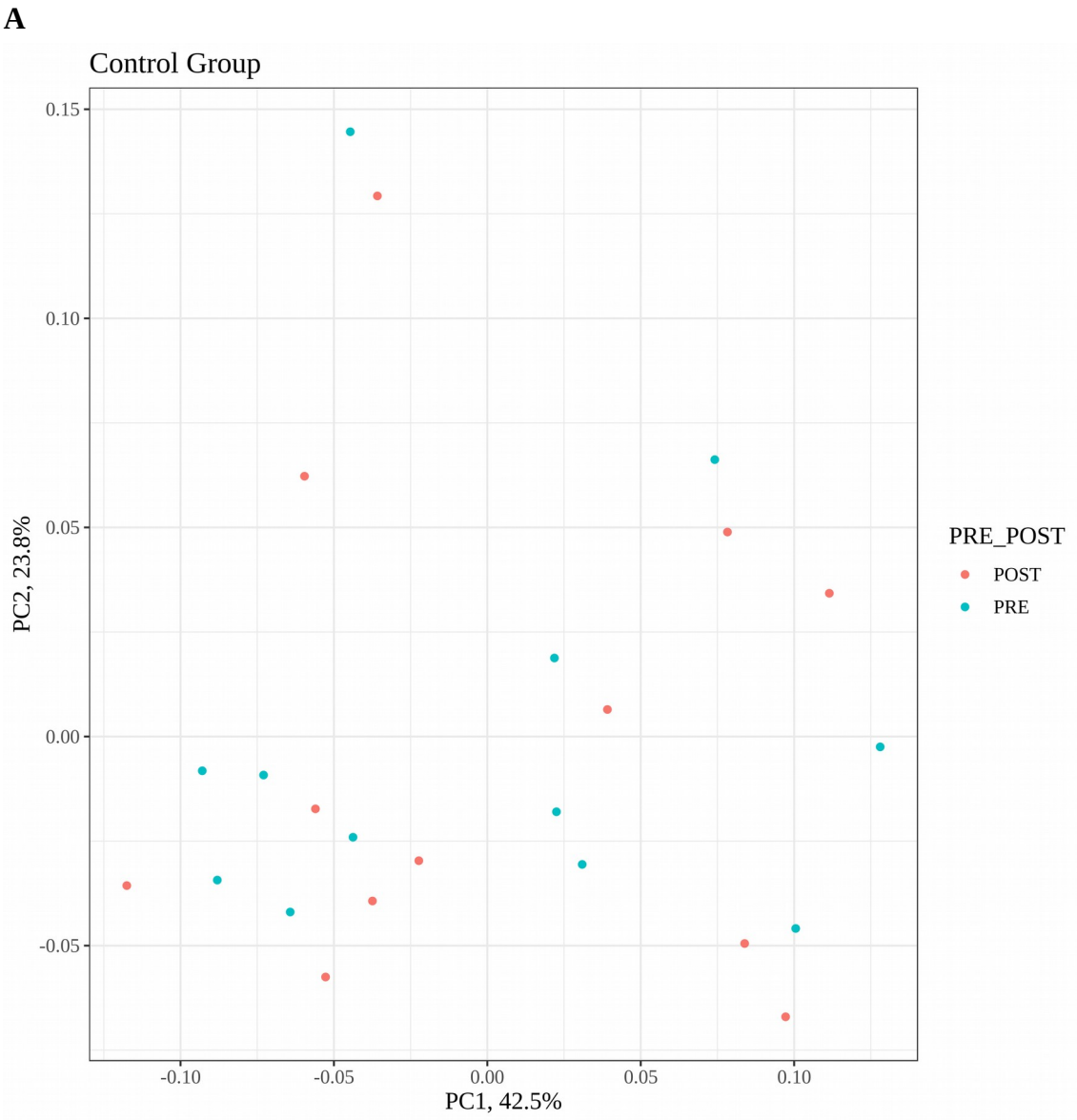
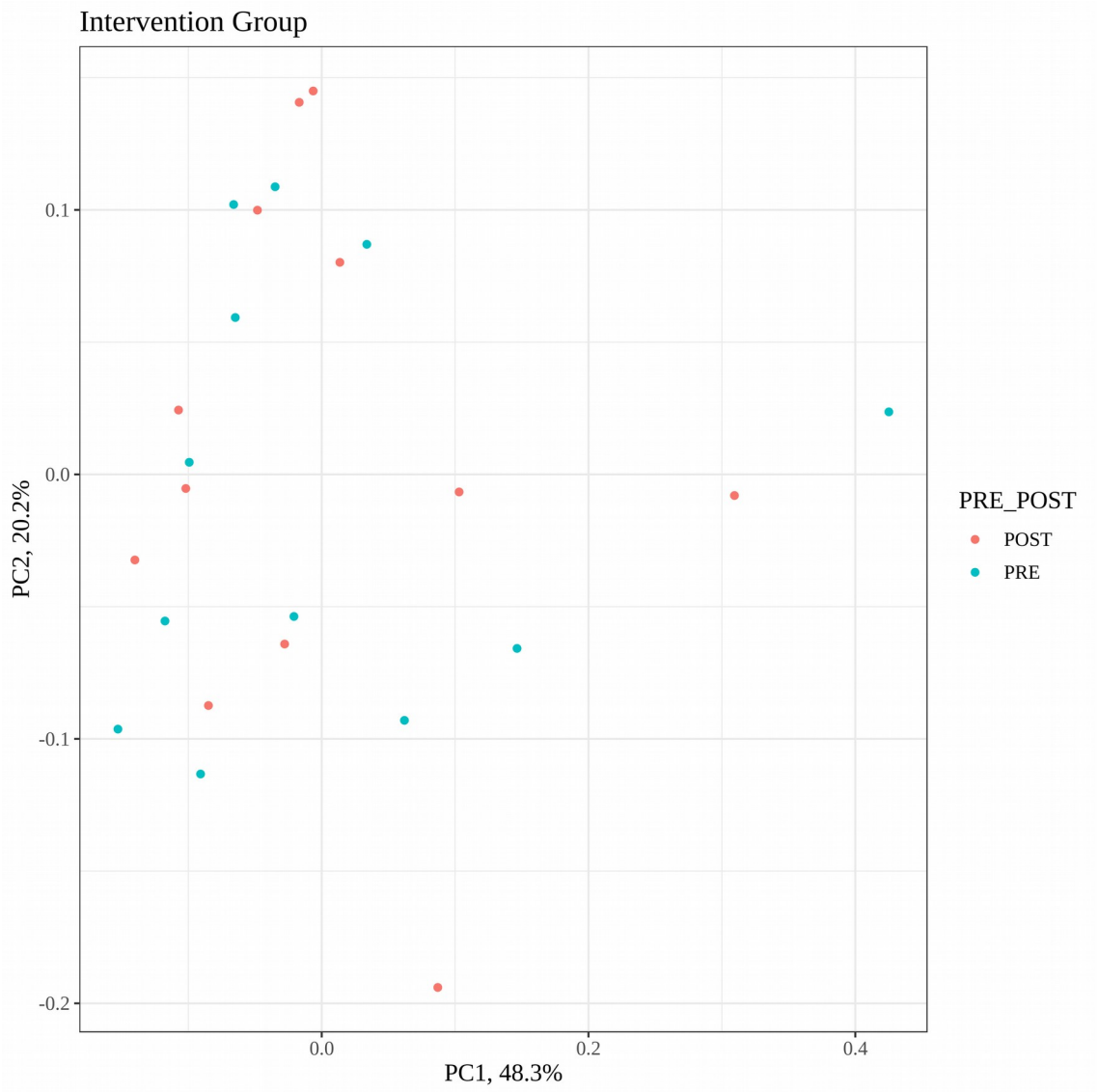


Figure 72. Principal Coordinates Analysis with UniFrac Phylogenetic distance. Panel A shows control group, and Panel B shows intervention group. PRE: before salami consumption, POST: after salami consumption. Statistical significance is shown for tests that used PRE situation as the reference group.



B


Intervention Group
0.1
0.0
-0.1
-0.2
PC2, 20.2%
0.0
0.2
0.4
PC1, 48.3%
PRE_POST
POST
PRE

3.7. qPCR *Lactobacillus rhamnosus*

In order to classify a bacterial as a probiotic species, one of the first conditions is to survive in the gastrointestinal tract in enough number to exert a positive health effect in the host (Saxelin et al., 2010). In general, $10^{8–10}$ bacterial cells/day has been recommended as the minimum number of a probiotics to provide a beneficial effect in human beings (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011). In the case of the probiotic salami, about 4-5^{8} bacterial cells/g were obtained (**Table 52**), so that the consumption of 30g/day potentially provides enough number of *L. rhamnosus* cells to obtain a health effect.

A probiotic strain not only should be viable in a food product but it also must survive the passage through the gastrointestinal tract (reach in a viable state the large and short intestine) in order to exert a positive effect on the host health (Dommels et al., 2009). Therefore, it is necessary to analyze the presence of the probiotic strain in human fecal samples. In this sense, *L. rhamnosus* was absent in all volunteers (both control and intervention groups) during the run-in phase. Then, after the intervention with probiotic salami, *L. rhamnosus* was detected in all samples of the intervention group (but not in the control group), with an average level of this species of log 6.78 cells/g. This level is similar to those reported by other authors of *L. rhamnosus* in probiotic fermented sausages (Rubio, Martín, Aymerich, & Garriga, 2014) and cheese (Lahtinen et al., 2012).

4. Conclusions

A human intervention in healthy volunteers was performed to test the potential health benefits of a salami fermented with a probiotic *Lactobacillus rhamnosus* and added with citrus fiber. Whereas no changes were observed in anthropometric measurements or blood biochemistry, reformulated salami improved some inflammatory and immunological markers (CRP and TNF-α), antioxidant plasmatic markers and butyrate production. Moreover, *L. rhamnosus* was found by qPCR in all volunteers from intervention group, suggesting that this probiotic was able to establish itself in the gut and, as consequence, could be related to the improved parameters observed. Accordingly, this reformulated salami could be used as a substitute of regular salami in usual diets, or even included if not present, since it not only have no negative effects, but also have some interesting potential health benefits.

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Founding

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Adaptation of the human gut microbiota metabolic network during the first year after birth

Los niños, desde su nacimiento hasta su primer año de vida, realizan unos grandes cambios en su alimentación, pasando de nutrirse exclusivamente con leche a alimentarse con una fuente muy variada de alimentos, que tiene un efecto tanto sobre su capacidad antioxidante como sobre la microbiota intestinal. De esta forma, en este trabajo se estudió la modulación de la microbiota intestinal mediada por su alimentación así como la capacidad antioxidante de las heces de niños de entre 1 semana y 1 año de edad. Se concluyó que la introducción de la alimentación complementaria (vegetales, fruta y cereales) es el principal modulador de ambos. Además, mediante la elaboración de redes metabólicas se ha demostrado la posibilidad de predecir los metabolitos liberados por la microbiota intestinal en función de la alimentación del niño. Esta información es muy importante ya que puede utilizarse por tanto para influir en la alimentación del niño, de manera que se favorezca la producción de metabolitos saludables como agentes antioxidantes o, como se demuestra en el trabajo, de ácido ferúlico, un compuesto fenólico que participa en el desarrollo neurológico del niño.

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Adaptation of the human gut microbiota metabolic network during the first year after birth

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Keywords: personalized nutrition, metabolic networks, human gut microbiome, metagenomics, metabolomics.

Abstract

Predicting the metabolic behavior of the human gut microbiota in different contexts is one of the most promising areas of constraint-based modeling. Recently, we presented a supra-organismal approach to build context-specific metabolic networks of bacterial communities using functional and taxonomic assignments of meta-omics data. In this work, this algorithm is applied to elucidate the metabolic changes induced over the first year after birth in the gut microbiota of a cohort of Spanish infants. We used metagenomics data of fecal samples and nutritional data of 13 infants at five time points. The resulting networks for each time point were analyzed, finding significant alterations once solid food is introduced in the diet. Our work shows that solid food leads to a different pattern of output metabolites that can be potentially released from the gut microbiota to the host. Experimental validation is presented for ferulate, a neuroprotective metabolite involved in the gut-brain axis. Additionally, antioxidant capacity of infants' feces was measure and correlated with ferulate and dietary intake of antioxidants.

1. Introduction

The study of nutrition has become increasingly concerned with human metabolism and the individualized human metabolic responses to diet. This approach was defined as personalized nutrition or nutrigenetics (Mutch et al., 2005). However, although personalized nutrition is frequently considered in the context of diet–gene interactions, individual human physiology depends not only on human genes, but also on the gut microbiota (Sonnenburg and Bäckhed, 2016). The gut harbors a densely populated microbial ecosystem containing a number of bacterial cells larger than the number of eukaryotic cells in the entire human body. The colon is the major site for the gut microbiota´s 'co-metabolic' activity, which enhances the efficiency of energy harvest from foods and influences the synthesis, bioavailability and function of nutrients (Tremaroli and Bäckhed, 2012). This activity produces different beneficial compounds that regulate host health, such as short chain fatty acids (SCFAs), polyphenol metabolites, neuroactive chemical species, etc. In this context, one of the major challenges in nutrition and health is to elucidate the interaction between diet and the metabolism of the gut microbiota (Besten et al., 2013).

Systems Biology and metabolic networks are an elegant approach to predict the overall functionality of the gut microbiota as well as the biosynthesis of specific health-related metabolites in response to diet. Current network-based methods to analyze gut microbiota metabolism are divided in two different strategies. On the one hand, some studies have used a supra-organism approach, which ignores boundaries for species and models community-level metabolism, based on graph-theory, by integrating metagenomics (Greenblum et al., 2012) or taxonomic data (Sridharan et al., 2014) with metabolic reaction repositories, such as KEGG (Kanehisa et al., 2015) or SEED (Henry et al., 2010). A more evolved approach than graph-based methods is constraint-based

modeling (CBM), which includes mass-balance and thermodynamic constraints (Price et al., 2004). Current CBM approaches focus on inter-species models, requiring the genome-scale metabolic reconstruction of each organism as input data. A remarkable work was recently presented in Magnúsdóttir et al. (2017), which released the first large-scale human gut microbiota reconstruction involving 773 different species resident in the human gut. These methods typically integrate 16S rRNA sequence data of bacterial species contained in the samples. Despite these relevant advances, multi-species CBM is still in its infancy and key technical challenges must be addressed (Magnúsdóttir and Thiele, 2018).

In a previous work (Tobalina et al., 2015), we presented a mixed approach that builds on CBM but, at the same time, uses a supra-organismal strategy. In particular, our approach was constructed to identify metabolic networks that capture the differences between two scenarios of interest based on the functional and taxonomic assignments of available meta-omics data. In this work, this algorithm is extended and applied to elucidate the metabolic changes induced over the first year after birth in the gut microbiota of a birth cohort of Spanish infants. To that end, we used metagenomics data of fecal samples and nutritional data for 13 infants at five time points during the first year after birth. Our aim is to analyze the resulting context-specific metabolic networks for each time point considered and establish metabolic differences.

2. Materials and Methods

a. Reference metabolic network

We obtained the list of reactions and metabolites from the Model Seed (Henry et al., 2010), a freely available resource to reconstruct, compare and analyze genome-scale metabolic networks. We introduced the following changes: first, in order to model compounds that can be potentially released by the gut microbiota to the human host, we added an irreversible output exchange reaction for each metabolite defined in the extracellular compartment of the Model Seed database. Second, we extracted from the metabolic model presented in Heinken and Thiele (2015) the subset of output exchange reactions not considered in the previous step. Overall, we have 717 output metabolites in our reference metabolic network. Third, we defined an irreversible input exchange reaction for each input metabolite identified in our nutritional assessment of infants involved in the study (see below). In total, we have 135 input nutrients, including minerals, carbohydrates, amino acids, vitamins, lipids, fiber and flavonoids. Fourth, as in Tobalina et al. (2015), we included a biomass reaction in our network, which represents a consensus equation for the metabolic requirements of different members of the human gut microbiota to support growth. In total, we have 17664 metabolites and 14124 reactions, which are stored in the stoichiometric matrix, S.

Our objective is to contextualize this reference metabolic network for each condition in our study based on available metagenomics and nutritional data. In other words, for each condition, we aim to select a particular subset of active metabolites and reactions. To that end, the algorithm presented in Tobalina et al. (2015) was applied. As noted above, the algorithm presented in Tobalina et al. (2015) uses a supra-organismal strategy to select active reactions; however, unlike existing graph-based methods, the resulting context-specific metabolic networks satisfy mass-balance constraints and

biomass production, as typically done in CBM. On the other hand, although this algorithm was first tested with metaproteomics data, it can be similarly used in cases where metagenomics or metatranscriptomics data are available. Clearly, metaproteomics data are more reliable to infer active enzymes in a microbial community; however, metagenomics is more common in the literature and widely used to infer metabolic capabilities (Greenblum et al., 2012; Sridharan et al., 2014), as it is done here. In addition, although the correlation between gene abundances and mRNA or protein levels has not been sufficiently explored, it is considerably high in some cases reported in the literature (Franzosa et al, 2014; Zhao et al, 2015), which supports the type of analysis conducted here. We describe below how metagenomic and nutritional data were integrated into the algorithm in Tobalina et al. (2015).

b. Metagenomic data

From (Vallès et al., 2014), we collected 454 pyrosequencing metagenomic data of the gut microbiota of 13 Spanish infants at 5 different time points during the first year after birth (1 week, 1 month, 3 months, 7 months and 1 year). For the second time point considered (1 month), we only have data for 9 out of 13 infants and, therefore, we have 61 samples overall. In brief, all infants were born at term (>37 weeks of gestation), ten of them by vaginal delivery and three by C-section. Their mothers had not taken antibiotics in at least three months before the onset of labor. Six women received antibiotics during delivery. Nine infants were exclusively breastfed during at least three months, three received a few formula feedings during the first days of life and one was partially breastfed during the first month and formula-fed thereafter. All infants remained healthy throughout most of the sampling period and solid foods were introduced into their diets between 4 and 6 months after birth, following patterns typical of Spanish Mediterranean infant diets. Previous statistical analyses have established that

metagenomic variation in these samples is mainly driven by the infants' age, as differences among infants within a sampling time point (including those that may result from variation in mode of birth, feeding regime or antibiotic use) are smaller than those present among infants of different ages (Vallès et al., 2014). This justifies the comparisons among metabolic networks at different time points presented in the Results section.

The functional annotation of sequenced reads was conducted using HMMER2 (Finn et al., 2011) against TIGRFAMs database 9.0 (Haft et al., 2003). As a result, for each of the 61 samples available, we obtained the read count assigned to 2703 proteins annotated in TIGRFAMS. We denote $a_{ijt}$the read count for protein i ($i=1,\ldots,N$) in infant j ($j=1,\ldots,J$) at time point t ($t=1,\ldots,T$). N, J and T are the total number of TIGRFAMS proteins, infants and time points, respectively.

On the other hand, the taxonomic assignment of sequenced reads was carried out with BLASTX (Altschul et al., 1990), obtaining for each analyzed sample the read count for 632 taxa. We denote $b_{wjt}$the read count for taxonomy w ($i=1,\ldots,W$) in infant j ($j=1,\ldots,J$) at time point t ($t=1,\ldots,T$). W is the total number of taxonomies considered.

i. Absolute classification of TIGRFAMs proteins

Functional metagenomics data were first summarized per time period, namely $a_{it}=\sum_{j=1}^{J} a_{ijt}$, which substantially reduces the variability in sequencing depth for different samples and increases the read count data for the cases analyzed. For each time point considered, we identified the subset of highly (H_t) and lowly (L_t) abundant (TIGRFAMs) proteins based on summarized read counts. To that end, we take as a null

hypothesis that all proteins are equally abundant and, therefore, assume that the read count for each protein follows a Poisson distribution $\chi_{it}\left(\hat{\lambda}_t\right)$, where the mean value is normalized by time point: $\hat{\lambda}_t = \sum_{i=1}^{N} a_{it} / N$. We consider as lowly abundant those proteins with an observed read count significantly less abundant than expected under the above hypothesis (significant threshold: p-value ≤ 0.05; p-value= $p\left(\chi \| it\left(\hat{\lambda}_t\right) \leq a_{it}\right)$). If the opposite occurs, we consider such protein as highly abundant.

ii. Differential classification of TIGRFAMs proteins

In order to avoid the selection of lowly abundant proteins, we first filtered proteins i) that were classified as lowly expressed in all time points considered or ii) for which 50% of infants had no reads assigned in all time points considered. We then conducted differential abundance analysis for the rest of proteins in the TIGRFAMs database between each successive time point. This analysis was done with *edgeR* (Robinson et al., 2010), using the trimmed mean of *M*-values (TMM) normalization, which blocks different sources of variability associated with read count data. We selected as differentially abundant proteins between two successive time points ($K_{t,t+1}$) those proteins with p-value ≤ 0.05. Again, we removed from $K_{t,t+1}$ those proteins classified as lowly expressed in time points *t* and *t+1* or proteins for which 50% of infants had no reads assigned in time points *t* and *t+1*.

iii. Taxonomic Analysis

Again, in order to reduce the sequencing depth variability among samples, we first summarized the taxonomic assignment per time period, namely $b_{wt} = \sum_{j=1}^{J} b_{wjt}$.

Second, for each time period, we selected those taxa with an abundance (x_t) higher than 1%: $x_t = \{w | (b||wt / \sum_{w=1}^{W} b_{wt}) \geq 0.01\}$, as done in Vallès et al. (2014). For these taxa, we obtained the set of related genomes from the KEGG website (Kanehisa et al., 2015). Enzymes from these genome annotations that were neither included in proteins in H_t nor L_t were included in M_t.

iv. Summary

Based on metagenomic data, for each time period considered, we have a different set of highly abundant (H_t) and lowly abundant (L_t) TIGRFAMs proteins, as well as a different set of enzymes annotated from relevant taxonomies (M_t). We denote the set of enzymes from the reference metabolic network not included in H_t, L_t, or M_t as D_t. Namely, D_t involves the subset of non-identified enzymes that are currently annotated for organisms not present in the community. Note here that we used Enzyme Commission (EC) number to code for enzymes. Metabolic proteins annotated in TIGRFAMS have at least one EC number assigned. By linking enzymes to reactions via EC numbers, sets H_t, L_t, M_t and D_t can be transformed to the reaction level for each time step. The same can be done for $K_{t,t+1}$, the list of differentially abundant TIGRFAMs proteins between two consecutive time-steps.

c. Nutritional data

In order to assess the daily intake of food and nutrients for each infant, we used a semi-quantitative food frequency questionnaire based on the validated questionnaire by Vioque et al. (2013). The infants' food consumption was specified by their mothers. Food frequency consumption of different infants was recorded 1 week and 1, 3, 7, and 12 months after birth, similarly to metagenomic data, taking into account lactation, the

formulas used and the regular food for supplementing lactation. Nutrient intake was calculated using the online software i-Diet (https://i-diet.es/), which was developed for the use of professionals in the field of nutrition and dietetics. As a result, daily consumption of 135 nutrients was obtained.

For each time point, we identified the active input metabolites and added their associated exchanges to H_t. Instead, the exchange reactions associated with inactive input metabolites (zero abundance) were excluded from the reference network. On the other hand, the relative abundance of identified metabolites between each successive time point was compared using a paired t-test. We used the following threshold cutoff for differentially abundant metabolites: p-value ≤ 0.05 and increase/decrease by fold-change ≥ 1.5. Exchange reactions that are associated with differentially abundant metabolites were included in the set $K_{t,t+1}$.

d. Data integration and metabolic reconstruction

As detailed in Tobalina et al. (2015), we seek a functional network that includes the maximum number of highly likely reactions (H_t) and the minimum number of lowly likely reactions (L_t). We complete the network using the reactions in the reference network, preferably those annotated in taxonomic groups present in the community (M_t). Note here that, in order to capture the metabolic differences between time points considered, we particularly force the inclusion of the maximum number of over-abundant TIGRFAMs enzymes and input metabolites in each situation.

As typically done in CBM, the selected reactions must satisfy the mass balance equation, the growth medium and thermodynamic constraints and the biomass production:

$$S \cdot v = 0 \quad \textbf{(Eq. 1)}$$

$$v^{min} \leq v \leq v^{max} \textbf{(Eq. 2)}$$

$$v_{bio} \geq \varepsilon \quad \textbf{(Eq. 3)}$$

where v represent reaction fluxes, v^{min} and v^{max} the lower and upper bounds for reaction fluxes, respectively, v_{bio} the flux through the biomass reaction and ε the minimum required flux through the biomass reaction. Note here that, aside from input and output reaction exchanges, the rest of reactions are potentially reversible and they are split into two different steps (forward and backward reactions) with non-negative fluxes (v^{min}=0). In addition, we fixed v_j^{max}=α=1000, except for exchange reactions associated with inactive input metabolites, whose upper bound is zero (equivalent to deletion). Finally, we set ε=1. In Tobalina et al. (2015), it was shown that the results are robust to the value of ε and α.

In order to guide the search of a reaction network that satisfies Eqs. (1)-(3) and takes into account metagenomics and nutritional data, we used the algorithm presented in Tobalina et al. (2015), which consists of a 3-step iterative procedure based on linear optimization and a reaction scoring based on the classification of reactions described above. In the first two steps (Steps 1-2), steady-state central metabolic pathways for biomass production are established based on single reaction knockout perturbations. Here, we also included double reaction knockout perturbations to have more complete networks. The resulting networks are then expanded to include over-abundant nutrients and enzymes and emphasize metabolic differences at each scenario (Step 3). In order to have a more complete view of the output metabolites obtained from over-abundant nutrients and enzymes, we implemented a single reaction knockout perturbation strategy

for output metabolites obtained in Step 3. The algorithm was implemented in MATLAB, using IBM Ilog Cplex to solve optimization problems.

e. Metabolomic validation

To validate our approach (see Results section), we measured the levels of ferulate (ferulic acid) in different time steps using a targeted metabolomics approach. Note here that we used aliquots of the same samples from which metagenomic data was obtained. Details are presented below.

i. Extraction of ferulic acid

Fecal samples frozen upon collection were processed by resuspension of approximately 200 mg of sample per mL of phosphate buffered saline. Samples were sonicated for 15 minutes and centrifuged at 13000 rpm for 10 minutes, and the supernatant was set aside. One mL of the supernatant was mixed with 1 mL of diethyl ether in a 2 mL tube and was kept in the dark for 24 hours. Afterwards, the supernatant (containing diethyl ether along with phenolic compounds) was separated into a clean 10 mL tube. Thereafter, 1 mL of diethyl ether was added to the 2 mL tube, mixed by inversion and the supernatant (diethyl ether) separated into the 10 mL tube. This step was repeated one more time, so that 3 mL of diethyl ether were collected into the 10 mL tube. Afterwards, anhydrous sodium sulphate was added to eliminate humidity. Diethyl ether was then evaporated with vacuum at 30^{o}C. Phenolic compounds were resuspended in 1 mL of a water:methanol mix (50:50) and transferred to a High-Performance Liquid Chromatography (HPLC) vial right after filtering them through a 0.22 µ filter.

ii. HPLC measurement of ferulic acid

Ferulic acid identification was carried out by HPLC following the method described in Moreno-Montoro et al. (2015). The HPLC system was a Thermo Fisher-Scientific Accela 600 equipped with a quaternary pump, an autosampler, a column oven and a variable wavelength UV–vis detector (PDA) set at 280 nm. The analytical column was a reverse phase C18 column thermostatized at 25 °C. Mobile phase A was water with 0.1% of formic acid and phase B was acetonitrile with 0.1% of formic acid. The method was carried out with a flow rate of 0.7 mL/min with the following gradient: 0% of B for 15 minutes, 100% of B at minute 110, 100% of B for 10 minutes and 0% of B for 5 minutes. Twenty µL were injected and the ferulic acid peak was identified by comparison with a reference standard. A calibration curve was performed with a reference standard with concentrations ranging from 5 ppm to 0.0001 ppm.

iii. Cell count

To ensure that measurements were obtained for similar amounts of bacterial cells, we evaluated the number of cells per mL in each sample suspension to determine the volume required for the ferulic acid assay. Cell count was performed with a Neubauer Haemocytometry chamber, which is the standard procedure for cell counting. Cells were resuspended in 1 mL of water and diluted accordingly to obtain around 100 cells per large square in the hemocytometer. Trypan blue was added to this suspension to dye cells and facilitate counting. Ten µL of such suspension were placed in the hemocytometer and cells were counted in the 4 corners of the 5x5 grid, obtaining afterwards an average value for the four corners.

f. Antioxidant capacity of infant feces'

i. $TEAC_{OH}$ method.

In this method, performed to unravel the scavenging activity against OH· radicals, carmin indigo was used as the redox indicator, following the method of Pérez-Burillo, Rufián-Henares & Pastoriza (2018). It is carried out at physiological pH (7.24). The results obtained are expressed as mmol Trolox equivalents per kg of sample (mM).

ii. $TEAC_{ABTS}$ assay.

This method measures the activity of the samples against ABTS· radicals. The ABTS assay was conducted as described by Re et al., (1999) with slight modifications. Results are expressed as mmol equivalents of Trolox per kg of sample (mM).

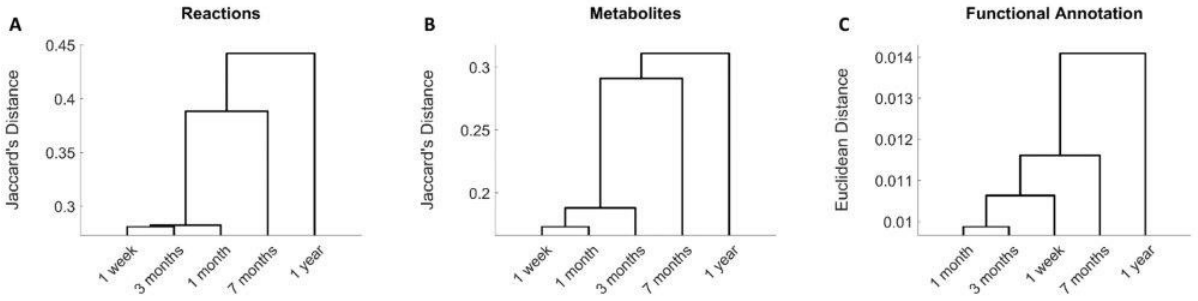
iii. $TEAC_{FRAP}$ assay.

The ferric reducing ability of the extract of each sample was estimated following the procedure described by Benzie and Strain (1996) with minor modifications. Results are expressed as mmol equivalents of Trolox per kg of sample (mM).

3. Results

Based on Tobalina et al. (2015) and data presented above, we calculated a consensus metabolic network for the gut microbiota of infants of 1 week and 1, 3, 7 and 12 months of age. It is important to note that these networks are not fully comprehensive but they emphasize the main metabolic differences across two consecutive conditions.

Figure 73. Hierarchical clustering analysis of reconstructed metabolic networks of gut microbiota of infants at 1 week, 1 month, 3 months, 7 months and 1 year of age. Distances based on active reactions (A), metabolites (B) and functional metagenomic annotations (C). In (A), for each time point, we defined a binary vector that stores active reactions in its reconstruction. We compared these binary vectors for the different time points using Jaccard's distance. A similar analysis was done for metabolites in (B). For functional metagenomic annotation data in (C), we used Euclidean distance.



Based on Jaccard's distance, we evaluated the similarity at the reaction and metabolite levels between the different computed networks (**Table 54**) and conducted hierarchical clustering analysis (**Figure 73a-b**). Networks associated with data collected after 7 months and 1 year of birth are clearly separated from data taken after 1 week, 1 month and 3 months. This significant change is related with the introduction of solid food, between 4-6 months after birth, which modifies nutritional patterns and, thus, the input exchange reactions (active nutrients) in the reconstructed networks. The effect of solid diet is more clearly observed after one year, where we found more significant

differences at both taxonomic and functional level, as discussed in detail in Vallès et al. (2014). This analysis shows that we were able to capture the main metabolic network adaptation during the first year after birth. Note here that the computed metabolic networks capture more clearly the effect of solid foods than functionally annotated metagenomic data, as observed in the dendrogram of **Figure 73c**, which reinforces the usefulness of the integrative approach presented here.

For every pair of successive time points, we compared the metabolic pathways involved in their resulting networks through KEGG maps (**Tables 53-57**). However, in order to summarize the functional changes associated with the introduction of solid die, following the results in **Figure 73**, we merged the metabolic networks before (1 week, 1 month and 3 months) and after (7 months and 1 year) the solid diet introduction and analyzed KEGG maps. For comparing both scenarios, we used a dissimilarity score (J_p), based on Jaccard's distance, which was introduced in Tobalina et al. (2015). We ranked the KEGG pathways according to this measure.

Table 53: Jaccard's distance between reconstructed networks at different time points

	1 week	**1 month**	**3 months**	**7 months**	**1 year**
1 week	0.000	0.264	0.312	0.348	0.450
1 month	0.264	0.000	0.315	0.391	0.455
3 months	0.312	0.315	0.000	0.325	0.447
7 months	0.348	0.391	0.325	0.000	0.427
1 year	0.450	0.455	0.447	0.427	0.000

Table 54: Ten most dissimilar KEGG pathways between metabolic networks at 1 week and 1 month.

KEGGID	Name	1 week	1 month	Dissimilarity score
map00630	Glyoxylate and dicarboxylate metabolism	10	19	6.81
map00240	Pyrimidine metabolism	40	41	6.13
map00330	Arginine and proline metabolism	17	24	5.89
map00360	Phenylalanine metabolism	3	11	5.81
map00565	Ether lipid metabolism	0	4	4
map00010	Glycolysis / Gluconeogenesis	9	16	3.06
map00627	Aminobenzoate degradation	3	6	2.86
map00910	Nitrogen metabolism	9	14	2.8
map00643	Styrene degradation	2	6	2.66
map00030	Pentose phosphate pathway	18	15	2.57

Table 55: Ten most dissimilar KEGG pathways between metabolic networks at 1 month and 3 months.

KEGGID	Name	1 month	3 months	Dissimilarity score
map00240	Pyrimidine metabolism	41	47	7.30
map00630	Glyoxylate and dicarboxylate metabolism	19	15	6.40
map00340	Histidine metabolism	12	9	6.12
map00052	Galactose metabolism	4	11	4.45
map00750	Vitamin B6 metabolism	0	4	4.00
map00360	Phenylalanine metabolism	11	5	3.27
map00900	Terpenoid backbone biosynthesis	15	8	3.27
map00643	Styrene degradation	6	2	2.67
map00350	Tyrosine metabolism	4	4	2.57
map00860	Porphyrin and chlorophyll metabolism	12	17	2.33

Table 56: Ten most dissimilar KEGG pathways between metabolic networks at 3 months and 7 months.

KEGGID	Name	3 months	7 months	Dissimilarity score
map00941	Flavonoid biosynthesis	0	17	17.00
map00340	Histidine metabolism	9	13	8.42
map00564	Glycerophospholipid metabolism	25	14	6.00
map00622	Xylene degradation	1	7	5.14
map00523	Polyketide sugar unit biosynthesis	0	5	5.00
map00600	Sphingolipid metabolism	8	2	4.50
map00750	Vitamin B6 metabolism	4	0	4.00
map00940	Phenylpropanoid biosynthesis	1	4	4.00
map00630	Glyoxylate and dicarboxylate metabolism	15	14	3.30
map00052	Galactose metabolism	11	5	3.27

Table 57: Ten most dissimilar KEGG pathways between metabolic networks at 7 months and 1 year.

KEGGID	Name	7 months	1 year	Dissimilarity score
map00071	Fatty acid metabolism	29	3	25.20
map00062	Fatty acid elongation	18	0	18.00
map00281	Geraniol degradation	12	0	12.00
map00941	Flavonoid biosynthesis	17	4	11.67
map00340	Histidine metabolism	13	10	7.11
map00730	Thiamine metabolism	6	0	6.00
map00630	Glyoxylate and dicarboxylate metabolism	14	13	5.71
map00232	Caffeine metabolism	12	4	5.33
map00622	Xylene degradation	7	1	5.14
map00523	Polyketide sugar unit biosynthesis	5	0	5.00

Table 58. Ten most dissimilar KEGG pathways between metabolic networks before and after solid food introduction. For each KEGG pathway in Table 55, the entries in "Before solid diet" and "After solid diet" columns represent the number of its annotated reactions that are involved in the merged reconstructions before and after solid diet introduction, respectively.

KEGGID	Name	Before solid diet	After solid diet	Dissimilarity score
map00941	Flavonoid biosynthesis	0	18	18.00
map00630	Glyoxylate and dicarboxylate metabolism	27	21	7.65
map00130	Ubiquinone and other terpenoid-quinone biosynthesis	6	16	6.25
map00940	Phenylpropanoid biosynthesis	1	8	6.13
map00622	Xylene degradation	1	7	5.14
map00523	Polyketide sugar unit biosynthesis	0	5	5.00
map00750	Vitamin B6 metabolism	4	1	4.00
map00360	Phenylalanine metabolism	11	7	3.69
map00500	Starch and sucrose metabolism	14	15	3.30
map00440	Phosphonate and phosphinate metabolism	0	3	3.00

Table 58 shows the top 10 most dissimilar KEGG pathways between metabolic networks before and after solid food introduction. The importance of the metabolism of phenolic compounds after the introduction of solid food is clearly reflected in **Table 58** with the activation of "Flavonoid biosynthesis" and "Phenylpropanoid biosynthesis" maps. These changes are linked to the intake of fruits and vegetables. In addition, infants before solid diet introduction seem more dependent

on vitamin B6 metabolism, which is in line with previous reports suggesting the need for supplementation during breast-feeding (Falsaperla et al., 2017).

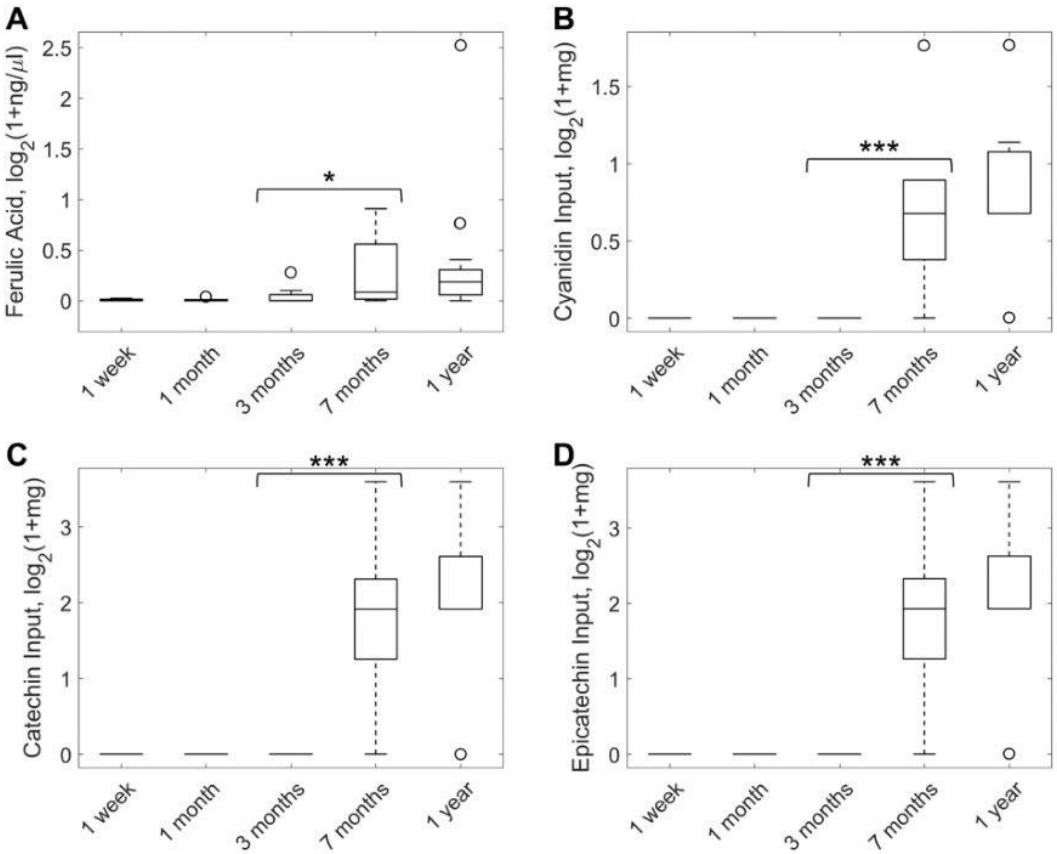
Importantly, the modulation of the gut microbiota of infants after the introduction of solid diet leads to different output metabolites. These output metabolites may be released to human cells and fluids and, thus, regulate host health. Among the predicted output metabolites that differentiate the networks before and after solid food introduction, we focused on ferulate (ferulic acid), which is a phenolic compound involved in the "Phenylpropanoid biosynthesis" KEGG map. Ferulate is a neuroprotective metabolite (Cheng et al., 2008), involved in the gut-brain axis (Westfall et al., 2017), which has been previously associated with cognitive development in embryonic rats (Yabe et al., 2010).

In order to evaluate the statistical significance of ferulate, we first conducted 50 bootstrap random permutations of metagenomic and nutritional and applied our network reconstruction pipeline to each of them. The output exchange reaction of ferulate was active in less than 5% of these random reconstructions, which provides additional evidence of the result presented here. **Figure 74a** shows the targeted metabolomic analysis of ferulate in fecal samples during the first year after birth of the infants considered. It can be observed that the levels of ferulate significantly increase after 7 months (one-tailed paired Wilcoxon test, p-value=0.0116), maintaining a similar value after 1 year (non-significant differences between 7 months and 1 year and significant differences between 1 year and the rest of time points). Therefore, the levels of ferulate seem to be linked to solid diet introduction, as predicted by our algorithm.

Based on our metabolic reconstructions, we calculated input nutrients that are degraded to form ferulate. For this analysis, we adapted the K-shortest Elementary Flux

Modes algorithm (De Figueiredo et al., 2009) and enumerated minimal combinations of nutrients that produce ferulate. Three input nutrients were identified: cyanidin, catechin and epicatechin. **Figure 74b** shows the consumption of cyanidin of infants in our study along the first year after birth. These data were taken from the available nutritional data described above. It can be observed that the consumption of cyanidin is solid diet-specific, mainly associated with the intake of fruits. A similar result was found for catechin and epicatechin (**Figure 74c-d**).

Figure 74. Analysis of ferulate production in faeces samples taken from infants over the first year after birth. Metabolomic analysis of ferulate (A) and consumption of cyanidin (B), catechin (C) and epicatechin (D) (precursors of ferulate) based on nutritional data. Note that * and *** indicate p-value < 0.05 and 0.001, respectively.



Our hypothesis is that, once solid diet is introduced, ferulate starts being synthesized by the gut microbiota of infants from available cyanidin, catechin and epicatechin. Of course, it may happen that the ferulate found in many plant-based foods (not accounted in our nutritional data) explains the differences observed in **Figure 74a**. However, we have found extensive literature supporting that the biosynthesis of ferulate from cyanidin, catechin and epicatechin is carried out by the gut microbiota (Ferrars et al., 2014; Yang et al., 2014).

Additionally, we measured antioxidant capacity in infants' feces with three different methods: $TEAC_{FRAP}$, $TEAC_{ABTS}$, and $TEAC_{OH}$ (**Figure 75**). The three of them showed similar results, being the highest antioxidant values found in 7 months and 1 year old infant feces. Antioxidant values from 7 months and 1 year were always significantly (one-tailed paired Wilcoxon test, p-value < 0.05) higher than those obtained for 1 week, 1 month or 3 months. Moreover, antioxidant capacity of 1 week, 1 month and 3 months were significantly lower than the mean except in the case of $TEAC_{FRAP}$ in which 3 month was not significantly different. In addition, not significant differences were found between 1 week, 1 month and 3 months antioxidant values. On the other hand, significantly positive Spearman correlations were found between 7 months and 1 year antioxidant values and ferulate levels, as well as with cyanidin, catechin and epicatechin intake (**table 59**). These correlations are really showing that a higher consumption of vegetables or plant derived foods would lead to a higher antioxidant capacity in feces, most probably not only due to ferulate, or cyanidin, catechin, and epicatechin.

Figure 75. Antioxidant capacity found in faeces samples taken from infants over the first year after birth. Note that *, **, ***, and *** indicate p-value < 0.05, 0.01, 0.001, and 0.0001 respectively.

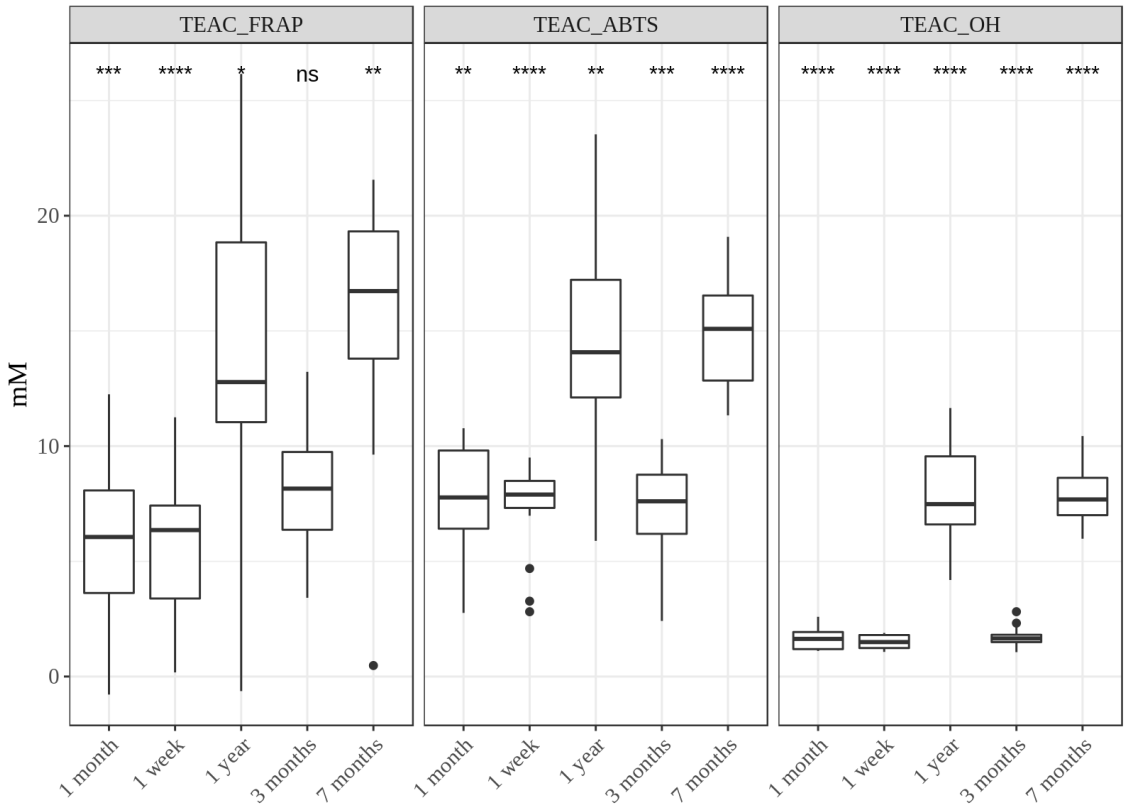


Table 59. Spearman correlations (r values). * denotes which ones are significant (p < 0.05)

	Ferulate	**Cyanidin**	**Catechin**	**Epicatechin**
Antioxidant capacity- 7 months	* 0.635	* 0.703	0.523	*0.621
Antioxidant capacity- 1 year	*0.711	*0.689	0.514	*0.603

4. Discussion

Constraint-based modeling is a promising tool to analyze the interaction of diet, gut microbiota and host. While inter-species metabolic models are currently under development, in this work we apply a supra-organism CBM approach, previously presented in Tobalina et al. (2015), in order to elucidate metabolic changes induced in the gut microbiota of infants during the first year of life based on functional and taxonomic assignment of metagenomics and on nutritional data. Our approach was successful in predicting clear metabolic patterns before (*e.g.* vitamin B6 metabolism) and after solid foods were introduced (*e.g.* metabolism of phenolic compounds).

The main application of our approach is to predict active gut microbiota metabolites that could regulate host health, as illustrated in the case of ferulate. In particular, we predict that ferulate starts getting produced in the gut microbiota once solid food is introduced in the infant diet, which is supported by the metabolomic analysis provided and previous literature reporting its biosynthesis from predicted nutrients (cyanidin, catechin and epicatechin). This result is of interest, since ferulate has been associated with neuroprotection and cognitive development, which reinforces the need and importance of solid food for the infant's growth. Moreover, our results show how antioxidant capacity increases 2-fold after complementary feeding highlighting again the importance of this stage of life and the inclusion of vegetables. Since ferulate depends on vegetable intake, Spearman correlations found could indicate a positive relation between antioxidant capacity and plant derived food consumption.

According to the World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO), complementary feeding should start at the age of 6 months, a time at which the brain and the gut are still developing and maturing. The transition from exclusive breastfeeding to family foods should cover the period

from 6 to 18-24 months of age. Both FAO and WHO agree that this period of life is especially important since it is a time of vulnerability and therefore the choice of complementary foods is crucial for the proper physical and neurological development of children. Much work will be needed to understand the impact of solid food introduction patterns on gut microbiota metabolism and infant development and health, but our work demonstrates that the analysis of supra-organismal metabolic networks via CBM methods can help in this endeavor.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CONCLUSIONES

Los resultados presentados en la presente tesis doctoral muestran cómo la capacidad antioxidante de los alimentos y su efecto sobre la microbiota intestinal, tras ser ingeridos, dependen principalmente del tipo de alimento (carne, vegetal, cereal, etc.). Sin embargo, el tratamiento térmico, ya sea industrial o casero, puede tener también un impacto importante tanto en la capacidad antioxidante del alimento como sobre la microbiota intestinal humana. Esta conclusión general deriva de una serie de **conclusiones específicas**, obtenidas a partir de los artículos recogidos en la presente tesis doctoral:

1. Los nuevos métodos desarrollados de determinación de capacidad antioxidante nos permiten evitar el empleo de disolventes orgánicos y pHs distintos al fisiológico, y por tanto, determinar la capacidad antioxidante de los alimentos en unas condiciones más cercanas a las fisiológicas.

2. La digestión gastrointestinal in vitro, junto con la fermentación colónica in vitro, nos permiten simular la extracción fisiológica de nutrientes y otros compuestos bioactivos de los alimentos, tanto a nivel de intestino delgado como de intestino grueso. Además, esta metodología in vitro es útil para llevar a cabo estudios preliminares a cerca del efecto de los alimentos sobre las comunidades microbianas del intestino y su funcionalidad.

3. En cuanto a los alimentos analizados, el té es un alimento con un gran poder antioxidante. La composición del té está afectada por un gran número de variables como el tipo de cultivo, origen, madurez de las hojas, temperatura de infusión, tiempo de infusión, almacenamiento, etc. Especialmente importantes son el tiempo y la temperatura de infusión, así como el tiempo y temperatura de almacenamiento de las

hojas de té. Los dos primeros parámetros van a determinar la extracción de compuestos antioxidantes, así como las propiedades sensoriales. Por otro lado, el almacenamiento es muy importante, ya que las hojas de té secas pueden sufrir oxidaciones y pardeamiento químico, modificandose en ambos casos los compuestos bioactivos responsables de sus propiedades.

4. Respecto al café, es una bebida altamente antioxidante y con un alto contenido en distintos compuestos fenólicos. El perfil fenólico del café verde y tostado es distinto, y dicho perfil se modifica durante el paso del café a través del tracto digestivo. Aunque dichos compuestos no se ven especialmente afectados por la digestión gastrointestinal, sí que son profundamente metabolizados por la microbiota intestinal, resultando en un perfil fenólico completamente diferente.

5. La composición del café verde y tostado es diferente y por ello la microbiota intestinal se ve afectada de distinta manera por cada uno de ellos. Algunos de los principales compuestos del café implicados en la modulación de la microbiota intestinal son los polisacáridos, más concretamente los manooligosacaridos. Estos pueden actuar como agentes prebióticos promoviendo, entre otras actividades, el crecimiento de bacterias beneficiosas. De esta forma, los posos de café, tras la hidrólisis del manano que contienen, pueden usarse como fuente de prebióticos. Sin embargo, el tostado implica la formación de distintos compuestos derivados del pardeamiento químico, compuestos furánicos como HMF y furfural, los cuales pueden actuar como agentes inhibidores del crecimiento bacteriano. Por ello, se hace necesario un paso previo de lavado de los posos para eliminar dichos compuestos.

6. Las frutas poseen un elevado potencial antioxidante, el cual es útil frente a distintas enfermedades crónicas como las cáncer, síndrome metabólico o patologías

cardiovasculares, entre otras. Los zumos de frutas conservan parte de esa capacidad antioxidante así como compuestos bioactivos procedentes de la fruta de origen. Sin embargo, su procesado industrial y almacenamiento juegan un papel importante, ya que durante ambos se desarrolla el pardeamiento químico, apareciendo nuevos compuestos y modificando otros que ya estaban presentes, lo que modifica sus propiedades beneficiosas.

7. El cocinado casero tiene una gran influencia en la capacidad antioxidante de los vegetales, ya que ayuda a romper las estructuras celulares y favorece la liberación de compuestos bioactivos. También aparecen nuevos compuestos derivados del pardeamiento químico. Los vegetales crudos, hervidos, y cocinados al vapor aportan al colon un perfil de nutrientes distinto al de vegetales cocinados mediante técnicas más agresivas como fritura, a la plancha o asado. De forma general, los indicadores de Maillard, furosina y HMF son adecuados para monitorizar el daño térmico y la capacidad antioxidante de los vegetales durante el cocinado.

8. La modificación de la microbiota intestinal a través de la dieta depende principalmente del tipo de alimento. Sin embargo, el tratamiento térmico o tipo de cocinado al que estos se someten también ejerce una cierta influencia, lo que se habrá que de tener en cuenta cuando se pretendan alcanzar ciertas modificaciones de la funcionalidad microbiana.

9. La formulación del salchichón puede ser modificada para mejorar su capacidad antioxidante así como para mejorar su efecto sobre la microbiota intestinal.

10. La introducción de la alimentación complementaría (frutas, vegetales y cereales) es el principal factor que determina la modulación de la microbiota intestinal y capacidad antioxidante de las heces de los niños de una semana a un año de edad. Con

la aplicación de redes metabólicas podemos predecir qué metabolitos producirá la microbiota intestinal y así adecuar la ingesta de alimentos a las necesidades específicas del niño.

General conclusion

The results presented in this doctoral thesis show how antioxidant capacity and gut microbiota are mainly dependent on the type of food (meat, vegetal, cereal, etc.). However, thermal treatment, either industrial or home-performed, can also have an important impact on both antioxidant capacity and gut microbes. We have also show how one of the most produced food derived wastes, spent coffee grounds, can be reused to improve both, antioxidant capacity and gut health, as a functional ingredient. This general conclusion arise from the specific conclusions drawn from each of the papers presented:

Specific conclusions

1. Newly developed antioxidant capacity methods carried out at physiological pH allow us to avoid the use of organic solvents and not physiological pH, ant thus, measure antioxidant capacity at closer conditions to physiological.

2. *In vitro* gastrointestinal digestion coupled with *in vitro* fermentation, allow us to mimic physiological extraction of nutrients from food, at both small intestine and large intestine levels. Moreover, this *in vitro* methodology, is useful carry out preliminary studies about gut microbiota community structure as well as about their functionality.

3. Tea has a great antioxidant potential. Its comoposition is affected by a large number of variables: crop, origin, maturity of leaves, temperature of infusion, time of infusion, storage. Specially important are time and temperature of infusion along with the storage. Time and temperature will affect directly the extraction of antioxidant

compounds, as well as sensory properties. On the other hand, storage is crucial as dry tea leaves can suffer from oxidation modifying bioactive compounds such as catequins. Moreover, Maillard reaction can advance during storage with the associated loss of nutritional value.

4. Coffee is a very antioxidant brew with high content in different phenolic compounds. Phenolic profile of green and roasted coffee are different, and such profile changes also during their transit along the gastrointestinal tract. Although they are not specially affected by gastrointestinal digestion, phenolics are deeply metabolized by gut microbiota, resulting in an very different profile from the original.

5. Composition of green and roasted coffee are different, and thus, gut microbiota is affected differently by them. Some of the key coffee compounds implicated in shaping gut microbiota community are polysaccharides, more specifically mannooligosacchides. They alone are able to act as prebiotics and promote bacterial growth. Spent coffee ground, which still have high amount of MOS in their composition, can be used as source of prebiotics. However, roasting involves the appearance of some furanic compounds that can inhibit bacterial growth, such as HMF and furfural. Therefore, a previous cleaning of the spent coffee grounds is needed to fully exert their ability as promoters of beneficial bacteria.

6. Fruits have a huge antioxidant potential which is useful against several diseases such as cardiovascular, cancer, or metabolic syndrome. Juices still preserve some of the antioxidant capacity and bioactive compounds from the original fruit. However, industrial processing and storage play an important role since Maillard reaction also takes place proving some modification during storage and processing.

7. Cooking have a great influence on vegetables' antioxidant capacity, since it helps breaking cells structure making easier to release bioactive compounds and also due to newly formed compounds from chemical browning. Raw, boiled, and steamed vegetables provide a different profile of nutrients to the colon which will probably influence gut microbiota. Overall, Maillard thermal indicators (furosine and HMF) are valid markers to monitor thermal damage and antioxidant capacity during vegetable cookings.

8. Gut microbiota shaping by diet depends mostly on the type of food, however thermal processing has also some influence, proving that the type of cooking will be important when targeting some microbial functions.

9. Salami formulation can be improved by including antioxidant extracts and a fiber component, to increase its antioxidant capacity and gut microbial functionality.

10. Complementary feeding (vegetables, fruits, and cereals) is the main factor that influeces gut microbiota and antioxidant capacity of feces modulation in infants from 1 week to 1 year old. Metabolic networks allow us to predict the output of metabolites from gut microbiota which can be used to modify infant diets according to their specific needs.

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