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EFECTO DE LOS ALIMENTOS Y SU PROCESADO TÉRMICO SOBRE LA FUNCIONALIDAD DE LA MICROBIOTA INTESTINAL: RELACIÓN CON LA CAPACIDAD ANTIOXIDANTE

Tesis Doctoral

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A mis padres, Asun y Antonio
y a mi hermana, Marta

“The movement you need is on your shoulder”

Hey Jude, The Beatles

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ÍNDICE

Índice de figuras

Índice de tablas

Abreviaturas

ANOVA One-way Analysis of Variance

AOX Antioxidant Capacity

db-RDA Distance Based Redundancy Analysis

EC European Legislation

EVOO Extra Virgin Olive Oil

FC Folin-Ciocalteu

GAE Gallic Acid Equivalents

HMF 5-Hidroximetilfurfural

HPLC Cromatografía Líquida de Alta Resolución / High Performance Liquid Chromatography

IOC International Olive Council

MA Maillard Reaction

MUFA Monosaturated Fatty Acids

OMS Organización Mundial de la Salud

OPLS Orthogonal Projections to Latent Structures

OPLS-DA Orthogonal Projections to Latent Structures Discriminant Analysis

PCA Principal Component Analysis

PUFA Polyunsaturated Fatty Acids

ROS Especies Reactivas de Oxígeno

TEAC Capacidad Antioxidante Equivalente de Trolox/ Trolox Equivalent Antioxidant Capacity

$TEAC_{ABTS}$ Trolox Equivalent Antioxidant Capacity against $ABTS^{\cdot+}$ radicals

$TEAC_{DPPH}$ Trolox Equivalent Antioxidant Capacity against DPPH radicals

$TEAC_{FRAP}$ Trolox Equivalent Antioxidant Capacity refereed to reducing capacity

UHPLC Cromatografía Líquida de Ultra Alta Resolución / Ultra High Performance Liquid Chromatography

Resumen

La microbiota intestinal humana se compone de billones de células microbianas y miles de especies bacterianas. Las características compositivas específicas difieren entre los individuos, y aunque la microbiota madura es bastante resiliente, puede ser alterada por distintos factores. La dieta es uno de los más importantes. Además, en los últimos años, se ha demostrado que la microbiota intestinal juega un papel principal en el desarrollo, evolución o aparición de ciertas enfermedades crónicas y relacionadas con el sistema inmune como obesidad, celiaquía, o alergias alimentarias. Una de las principales funciones de la microbiota intestinal en el organismo es la de metabolizar aquellos nutrientes que no se han digerido en el tracto gastrointestinal, liberando una serie de compuestos a nivel colónico. Muchos de estos compuestos son altamente antioxidantes, lo cual es beneficioso para el organismo, ya que los estos se relacionan de forma estrecha y negativa, con enfermedades no transmisibles. En la presente tesis doctoral se lleva a cabo el estudio de la capacidad antioxidante de diferentes alimentos tras su cocinado, digestión y fermentación *in vitro*. La fermentación se lleva a cabo con diferentes tipos de microbiota intestinal (adultos sanos, niños sanos, niños obesos, niños celíacos y niños con alergia a las proteínas de la leche de vaca), lo que permite ver la diferencia en la funcionalidad entre ellas. Además, se tiene en cuenta el tipo de cocción aplicada al alimento, ya que también este factor influye en la capacidad antioxidante total liberada.

Abstract

The human gut microbiota is composed of trillions of microbial cells and thousands of bacterial species. The specific compositional characteristics differ between individuals, and although the mature microbiota is quite resilient, it can be altered by a number of factors. Diet is one of the most important. Moreover, in recent years, the gut microbiota has been shown to play a major role in the development, progression or onset of certain chronic and immune-related diseases such as obesity, celiac disease, or food allergies. One of the main functions of the intestinal microbiota in the body is to metabolize nutrients that have not been digested in the gastrointestinal tract, releasing a series of compounds at the colonic level. Many of these compounds are highly antioxidant, which is beneficial for the body, as they are closely and negatively related to non-communicable diseases. This doctoral thesis studies the antioxidant capacity of different foods after cooking, *in vitro* digestion and *in vitro* fermentation. Fermentation is carried out with different types of intestinal microbiota (healthy adults, healthy children, obese children, celiac children, and children with cow's milk protein allergy), which allows us to see the difference in functionality between them. In addition, the type, of cooking applied to the food is taken into account, as this also influences the total antioxidant capacity released.

INTRODUCCIÓN

1. Alimentación en el ser humano

La evolución humana está conectada a continuos cambios en la dieta (**Figura 1**). A lo largo de la historia se ha producido un cambio de forma gradual hacia el consumo de alimentos con alta densidad energética, mayor calidad nutricional y más fácilmente digeribles. Este cambio está directamente relacionado con el desarrollo de nuevas tecnologías [1]. La primera transición nutricional ocurrió con la llegada del fuego como instrumento culinario, hace unos 2.5 millones de años. El ser humano pasó de seguir una dieta vegetariana a una dieta omnívora. La segunda transición nutricional llegó con la aparición de la agricultura, hace 10000 años. Esto permitió una mayor disponibilidad de alimentos a lo largo del año, así como la producción de alimentos ricos en carbohidratos. En el siglo XIX se dio la tercera transición nutricional, gracias al desarrollo industrial con la aparición de la electricidad y el vapor. En este momento se incrementó el consumo de azúcares refinados y grasas, disminuyendo el consumo de alimentos frescos y fibra alimentaria. Este cambio se produjo muy rápido, evolutivamente hablando. El genoma humano no tuvo tiempo de adaptarse [2] provocando esto una pérdida en la diversidad en la microbiota intestinal heredada durante generaciones [3]. Esta "mala adaptación" a la dieta moderna ha sido propuesta como una de las posibles causas de la aparición de enfermedades modernas como cáncer, obesidad, enfermedades cardiovasculares, diabetes, entra otras [4].

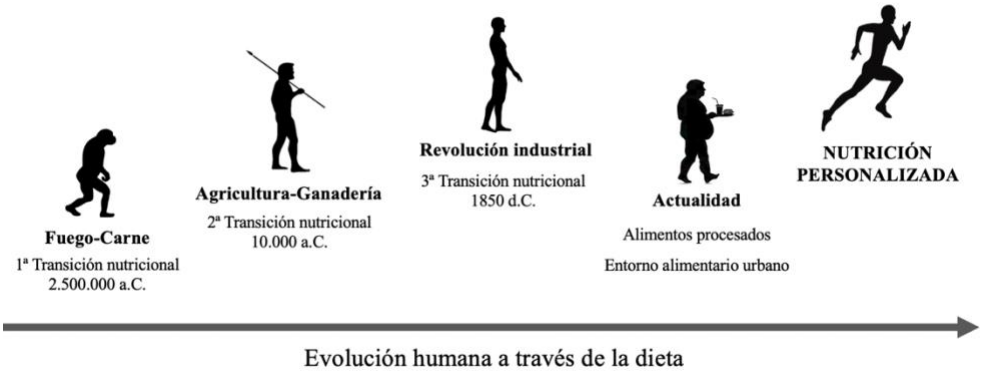


Figura 1. Cambios en la dieta a lo largo de la historia.

España es el segundo país de Europa con más prevalencia de sobrepeso y obesidad [5]. En las últimas décadas, el sobrepeso y la obesidad han crecido considerablemente [6] afectando a la población adulta, pero también a población infantil. Esto es especialmente preocupante en aquel grupo de población que no tiene adherencia a la dieta mediterránea, como son los adultos jóvenes, adolescentes y niños [7]. El número de lactantes y niños de entre 0 y 5 años con sobrepeso u obesidad aumentó de 32 millones en 1990 a 41 millones en 2016 según la Organización Mundial de la Salud (OMS). Por su parte, en el estudio ALADINO [8] se vio que más del 20% de los niños entre 6 y 9 años tenían sobrepeso, y más de un 18%, obesidad. Esto es motivo de preocupación debido a las consecuencias que tiene la obesidad sobre la salud, mental y física. Algunas de estas pueden ser baja calidad de vida [9], problemas de tipo psicosocial (discriminación social y burlas), problemas emocionales (ansiedad, depresión o baja autoestima), restricciones (tanto escolares como funcionales debido a problemas con articulaciones, dificultad respiratoria, entre otras), y otros problemas fisiológicos (como hiperandrogenismo, problemas ortopédicos, desórdenes del sueño, etc.). Entre los posibles factores que influyen en el desarrollo de la obesidad, se pueden encontrar el bajo estatus socioeconómico, nivel de educación,

predisposición genética, peso elevado en el nacimiento, bajos niveles de actividad física y conductas sedentarias, restricción y control alimentario, pero el más importante siempre será la dieta [10].

Progresivamente se ha ido adoptando en España un tipo de dieta occidental, en la que predomina la ingesta de alimentos ultraprocesados, bebidas azucaradas y un consumo de energía excesivo. Además, esto va acompañado de una alimentación escasa de alimentos ricos en fibra, como son los cereales integrales, frutas u hortalizas [11]. Entre los factores que condicionan a la alimentación, se encuentran las preferencias alimentarias. Estas se ven influidas por la disponibilidad de alimentos que normalmente hay en casa, publicidad, cultura y otras normas sociales en torno a los alimentos en general. Algunas son innatas pero otras preferencias se adquieren con el tiempo y comienzan en las primeras etapas de la vida, normalmente perdurando en el tiempo. También están muy relacionadas con las preferencias del entorno, como es el ejemplo de padres o cuidadores y niños. La exposición repetida a las condiciones en las que se consume un cierto alimento hace también que algo se convierta en preferencia por defecto [12].

La capacidad de llevar a cabo una alimentación saludable se ve comprometida en ocasiones. Uno de los obstáculos es pertenecer a un grupo socioeconómico bajo, ya que los alimentos saludables por lo general son más costosos que aquellas alternativas no tan saludables [13], así como aquellas dietas saludables y aceptables culturalmente a menudo no están al alcance de familias con ingresos bajos [14]. Los alimentos que tienen mayor densidad de nutrientes (como son frutas, hortalizas y alimentos de origen animal) suelen tener un precio más elevado que aquellos con una densidad calórica elevada. Esto supone un problema para aquellas personas con bajos ingresos,

ya que tienden a consumir aquellos con menor coste [15]. Otra de las posibles barreras para llevar una alimentación saludable es el lugar en el que se vive. Los países en desarrollo experimentan una rápida urbanización en la que se lleva a cabo una transición nutricional hacia el consumo de alimentos procesados, azúcares simples, grasas animales, y en general un consumo de energía excesivo [16]. Las personas que habitan en zonas urbanas tienen una alimentación diferente a la de aquellas personas que viven en zonas rurales. La dieta de las personas que viven en zonas urbanas se caracteriza por un aumento de la densidad energética, mayor consumo de azúcar y grasa, así como de alimentos de origen animal, alimentos preparados fuera del hogar y procesados [17]. El contraste entre los patrones alimentarios urbanos y rurales están más acentuados en los países de ingresos más bajos. En aquellos países con ingresos más altos es común que el mercado entre en las zonas rurales, ya que existen sistemas nacionales integrados de distribución de alimentos [18]. También influye el acceso a los distintos alimentos, es decir, en las zonas urbanas tienen más fácil acceso a distintos tipos de comercios como supermercados e hipermercados o pequeños comercios. Estos tipos de comercios se trasladan a las zonas rurales, pero a menor nivel [19]. El marketing es otro factor que influye en el deseo de consumir ciertos alimentos. Aquellas empresas de snacks, bebidas azucaradas, y este tipo de productos, hacen una fuerte inversión para que sus productos se encuentren en aquellos lugares donde la gente se reúne como pueden ser colegios, universidades, bibliotecas, etc.

Los cambios hacia una dieta occidental son el principal factor causante de esta epidemia de obesidad y otras enfermedades no transmisibles que también van ligadas a la alimentación [20]. Las más comunes son cáncer, enfermedades respiratorias crónicas, diabetes y enfermedades cardiovasculares, y causan en torno a 38 millones de muertes anuales según

la OMS. Adicionalmente hay otras patologías relacionadas con la alimentación que en los últimos años han sido muy destacadas. Una de ellas es la alergia alimentaria, o alergias alimentarias, las cuales influyen negativamente sobre el bienestar y la calidad de vida de niños y sus familias [21]. Son definidas como un efecto adverso para el organismo debido a una respuesta inmunitaria específica que se da tras la exposición a algún alimento, y abarca síntomas inmediatos que afectan a la piel y a los sistemas respiratorio, cardiovascular y gastrointestinal [22]. Otra patología que se encuentra en auge hoy en día es la enfermedad celíaca o celiaquía. Se trata de una enfermedad autoinmune crónica que provoca daños en el epitelio intestinal, provocando sintomatología en el tracto gastrointestinal [23]. Los síntomas se hacen notables con el consumo de gluten, formado por proteínas (prolaminas y glutelinas) y se puede encontrar en trigo, centeno o cebada [23]. Actualmente, el único tratamiento eficaz es la eliminación total del gluten de la dieta. Como consecuencia, se dejan de consumir otros nutrientes como la fibra, lo que provoca efectos negativos en el organismo [23-25]. Tanto en las alergias alimentarias como en la enfermedad celíaca se ha visto un desequilibrio en la composición de la microbiota intestinal, lo cual tiene un efecto negativo sobre la salud. A este desequilibrio se le llama disbiosis [26,27] y será descrito con mayor detalle en el punto 6.2.

1.1 Nutrición personalizada

A raíz del masivo incremento en la incidencia de obesidad y sobrepeso surge la nutrición personalizada. Aunque no existe una definición consensuada de nutrición personalizada, se puede describir como un enfoque que utiliza información individual para desarrollar pautas, consejos, servicios o productos nutricionales específicos para una persona [28].

Con la creciente complejidad del sistema alimentario actual, no solo los consumidores, sino también la industria alimentaria, experimentan cada vez más problemas a la hora de tomar decisiones relacionadas con los alimentos. Además, las disparidades sociales en términos económicos y culturales son cada vez más pronunciadas. De esta forma, el conocimiento social debe integrarse en todo su ámbito, incluyendo el biológico, conductual y ambiental [29]. Todos estos factores jugarán un papel importante en una población a la hora de tomar decisiones de salud. Por lo tanto, es fundamental permitir o facilitar que los consumidores puedan tomar estas decisiones. Sin embargo, estas decisiones no solo deben centrarse en la propia salud o la alimentación saludable, sino que también deben permitir el uso de los aspectos sociales y ambientales de los alimentos de manera sostenible.

La nutrición personalizada aprovecha las tecnologías disponibles actualmente, como "Internet de las cosas", tecnologías emergentes, etc. La idea es crear un nuevo mundo de posibilidades innovadoras para mejorar la dieta y la salud de las personas al mismo tiempo que aumenta la sostenibilidad de los sistemas de producción de alimentos [30]. Hasta la fecha, muchos modelos industriales han desarrollado cierto grado de personalización en función de las preferencias alimentarias, médicas o éticas de los consumidores. Sin embargo, todos estos modelos se enfocan en un segmento de mercado más o menos grande, no individualmente. El objetivo de la nutrición personalizada es poder ofrecer una dieta específica para cada consumidor, adecuando la ingesta de nutrientes a cada persona. Para poder satisfacer las necesidades del individuo, tendría que tener en cuenta no sólo las preferencias alimentarias, sino que también tiene que considerar alergias alimentarias, intolerancias, aspectos culturales, religiosos, sociales, etc. Adicionalmente, tiene que estar basada en datos biológicos de la persona como género, peso, edad, nivel de actividad física, posibles deficiencias o

desordenes nutricionales, etc. Así, la nutrición personalizada tendría el potencial de prevenir deficiencias nutricionales, pero también, de servir como medida terapéutica frente a enfermedades no transmisibles, alergias alimentarias, etc. [28]. Para ello, tendría que hacer uso de la información genética (aunque no es imprescindible) y de la microbiota intestinal del consumidor, ya que juega un importante papel en el desarrollo de enfermedades crónicas o en el estado de salud de las personas [29]. La fisiología humana depende no solo de los genes humanos, sino también de los genes de los microorganismos que conforman la microbiota. Hay una comunidad microbiana en el intestino con muchas más células que las células eucariotas que componen nuestro cuerpo. El colon es el sitio principal para que la flora intestinal ejerza sus actividades metabólicas, lo que puede mejorar la extracción de energía de los nutrientes y afectar la síntesis, biodisponibilidad y función de los nutrientes [31]. La capacidad de adaptación del ser humano a cambios dietéticos estará determinada por la actividad metabólica de su microbiota intestinal y la del individuo propia, siendo la microbiota intestinal aquella parte del cuerpo humano más dúctil y adaptable. Por lo tanto, los cambios hacia una microbiota intestinal más sana son un elemento clave en la nutrición personalizada.

2. Tratamientos culinarios

La cocción de los alimentos se lleva a cabo exponiendo el alimento a una fuente de calor o radiación que altera física y/o químicamente la apariencia, textura, composición y valor nutritivo del alimento mediante la acción del calor para satisfacer demandas sensoriales como vista, gusto y olfato. Dicho de otra forma, la cocción es la aplicación de calor para hacer a un alimento más apetecible, digerible y más seguro sanitariamente. La tecnología culinaria es la parte de la tecnología de los alimentos que se centra en el conocimiento científico de todo aquello que pueda ocurrir durante un tratamiento culinario [32]. Tiene como objetivos el estudio científico de los cambios físicos y químicos producidos en el alimento, como establecer unas condiciones, tanto de manipulación de alimentos, como de trabajo, garantizando así una correcta calidad nutritiva, sanitaria y sensorial.

Los tipos de cocciones se dividen en tres grupos diferentes dependiendo del medio de transferencia de calor: cocciones en medio húmedo o acuoso, en medio no acuoso y cocciones mixtas [32,33].

2.1 Cocciones en medio acuoso

En estas cocciones se emplea un fluido acuoso como medio de transferencia de calor. El alimento entra en contacto con el agua, caldo corto, jarabe o vapor de agua [32]. Los diferentes tipos de cocciones en medio acuoso se describen a continuación [32,33]:

a) Escaldado

Se puede considerar una operación previa en la que se inactivan enzimas, se quitan pieles o se atenúa el sabor. Se trata de una cocción

incompleta llevada a cabo durante un corto periodo de tiempo (de 10 a 30 segundos). Si la esta se alarga en el tiempo, recibe el nombre de sancochado.

b) Hervido

Es la cocción de un alimento por inmersión en agua o caldo, que puede estar a diferentes grados de temperatura al inicio: fría, caliente o en ebullición.

c) Fuego lento

La principal diferencia con el hervido es que en este método no hay ebullición, encontrándose la temperatura entre los 90 ºC y los 98 ºC.

d) Vapor a presión normal

El calentamiento del alimento se realiza a unos 760 mm de Hg de presión y la temperatura es de 100 ºC. La cantidad de agua que se pone en contacto con el alimento es mínima, por lo que la disolución es baja.

e) Vapor a presión elevada

La diferencia con la anterior es que el punto de ebullición del agua se puede elevar por encima de los 100 ºC. Se lleva a cabo en una olla a presión o en un autoclave, ocupando así el vapor que antes ocupaba el aire, elevándose la presión rápidamente. Es necesario el aumento de temperatura al aumentar la presión para que así se pueda producir el vapor de agua.

2.2 Cocciones en medio no acuoso

a) Cocciones en medio no líquido

Son aquellas en las que el alimento se calienta a través de su superficie gracias al contacto con aire caliente. A su vez se pueden clasificar dependiendo de cómo el calor llega al alimento, pudiendo ser de forma directa como en el caso de la plancha o parrilla, o de forma indirecta como en el caso del horneado.

i. Plancha

Se lleva a cabo a una temperatura elevada mientras que el alimento está en contacto con una placa caliente de fundición. El calor se transfiere por conducción y la fuente calorífica es de gas o electricidad por lo general.

ii. Parrilla

La fuente calórica, que normalmente son ascuas, se encuentra a unos 30 cm del alimento, y todas las partes del mismo se encuentran a la misma distancia de la parrilla. La cocción es realizada mediante calentamiento continuo.

iii. Horneado

La forma de energía calórica producida en un horno se debe más a la convección que a la radiación y conducción, es por esto que se considera transferencia de calor de forma indirecta. El calor del horno produce la evaporación del agua del alimento, este vapor queda en el recinto y hace al aire caliento más húmedo.

b) Cocciones en medio lipídico

Son aquellas que usan un medio graso para la transferencia de calor. La temperatura usada siempre es muy elevada, dando un sabor y textura muy característicos a los alimentos, aportándoles además una importante palatabilidad.

i. Salteado

Es un método de cocción rápida, y puede ser total o parcial en el alimento. Normalmente se usa para alimentos crudos como un paso previo a otro método de cocción, o a alimentos que ya han sufrido una cocción previa para darles un sabor más agradable. La cantidad de aceite usada en este método culinario es pequeña.

ii. Fritura

Método de cocción total del alimento. Este se sumerge en un lípido que ha sido calentado previamente a temperaturas elevadas, entorno a los 180 ºC, provocando así en el alimento un calentamiento rápido y uniforme. Como resultado se obtienen preparaciones doradas y crujientes. Si la fritura se lleva a cabo sumergiendo solo una parte del alimento en el medio graso se le llama fritura superficial, ya que el resto del alimento se cuece por el vapor de agua que se genera en su interior. A veces se llevan a cabo una serie de preparaciones previas a la fritura para proteger al alimento de una deshidratación y de una penetración excesiva de calor. Entre estas preparaciones se pueden distinguir:

- **Rebozado**. El alimento se cubre con una capa de algunos ingredientes como la harina de trigo, gluten de trigo, proteína de soja, albúmina de huevo, leche, sal, etc.

- **Empanado**. El alimento se cubre con pan rallado.
- **Enharinado.** Se usa más en pescados. El alimento se cubre de harina.

2.3 Cocciones mixtas

Resultan de la combinación de una cocción en medio no acuoso y otra en medio húmedo y es muy utilizada en carnes y hortalizas. El medio no acuoso da lugar a modificaciones como la desecación de la superficie y la formación de productos del tostado que posteriormente dan palatabilidad, sabor y color de la preparación. A continuación, se agrega el medio acuoso y se completa la cocción a fuego lento. Este método es muy empleado en estofados y guisos [32,33].

3. Influencia del cocinado en alimentos

Los procesos de cocción llevan consigo otros procesos de transferencia de energía y masas, los cuales están muy influenciados por dos factores: naturaleza, forma y tamaño del alimento que se somete al cocinado, así como la intensidad del calor aplicado [33]. Es muy importante controlar tanto la temperatura como el tiempo de exposición a la misma del alimento para poder llegar al resultado deseado. Este aspecto será diferente en cada alimento.

El cocinado puede tener efectos tanto positivos como negativos, de ahí la importancia de que se lleve a cabo de una forma adecuada. Los aspectos beneficiosos del cocinado se pueden resumir de la siguiente manera:

i. **Inocuidad de los alimentos**: la inactivación de los patógenos, compuestos y microorganismos no deseados presentes en los alimentos es el principal efecto beneficioso del cocinado.

ii. **Higiene alimentaria:** prolongación de la vida útil del alimento, inactivación de toxinas y de enzimas naturales.

iii. **Valor nutricional:** alimentos más fácilmente digeribles y con mayor biodisponibilidad de nutrientes. Un buen ejemplo son las proteínas, que al desnaturalizarse por el calor son mucho más digeribles haciendo mucho más fácil la digestión por parte de enzimas digestivas. También ocurre con la gelatinización del almidón, que hace posible su hidrólisis por enzimas amilasa. Además, la destrucción de las pareces celulares en alimentos vegetales aumentará la disponibilidad de compuestos beneficiosos como son los carotenoides o los polifenoles. En cocciones grasas, si se emplea aceite de oliva virgen extra, el alimento se puede ver enriquecido en compuestos fenólicos por la penetración de aceite en el mismo, mejorando así su perfil

fenólico [34]. Así mismo, durante el calentamiento de los alimentos se da la **reacción de Maillard,** de la que se hablará con más profundidad en el punto 4. Es una reacción química entre los azúcares reductores y los grupos amino y que tiene como resultado la formación de compuestos con capacidad antioxidante [34].

iv. **Calidad sensorial:** aquí también intervine la reacción de Maillard, en la que se forman compuestos que aportan sabor, textura y olor y agentes colorantes [32,33].

El procesamiento de los alimentos también puede dañar la calidad de los mismos, llegando a tener consecuencias no deseadas como son las enumeradas a continuación (aspectos perjudiciales):

i. **Pérdidas de nutrientes debido a reacciones químicas:** cuando se utiliza un medio acuoso de cocción se dará una transfusión de nutrientes al mismo, viéndose el valor nutricional del alimento disminuido si el caldo de cocción no se consume [32]. De esta manera, se puede producir una pérdida de compuestos hidrosolubles como algunas vitaminas o compuestos fenólicos, provocando una disminución de la capacidad antioxidante del alimento [32,34]. Las cocciones en medio graso, al llegar a temperaturas muy elevadas, también pueden provocar una pérdida de compuestos termolábiles [34]. En la reacción de Maillard, no sólo se generan compuestos beneficiosos, sino que también se pueden provocar pérdidas en aminoácidos esenciales.

ii. **Formación de compuestos no deseados:** acrilamida, cloropropanodioles, aminas heterocíclicas, etc., muchos de ellos resultado del desarrollo de la reacción de Maillard.

iii. Formación de compuestos con efecto negativo en la percepción del sabor, pérdida de textura, decoloración: si el cocinado no se lleva a cabo de una forma adecuada.

Por estas razones se hace necesario que los procesos culinarios se optimicen de forma que los efectos positivos sean mayores que los negativos en la medida de lo posible. En la cocción de los sistemas alimentarios los procesos que se aplican son determinantes del producto cocido y se puede considerar dos procesos diferentes [32,33]:

El **proceso primario** es de naturaleza física fundamentalmente y afecta a dos mecanismos de transporte. El primero es la transferencia de energía que se produce al llegar el calor a la superficie del alimento yendo hacia el interior del mismo. En segundo lugar, la transferencia de masas que se da como consecuencia del movimiento de las moléculas en el interior del alimento. El **proceso secundario** tiene naturaleza física y química, ya que la cantidad de calor que recibe el alimento durante su cocción puede llevar cambios tanto físicos como químicos y van a afectar a la calidad del alimento. Las modificaciones que se producen se clasifican en:

a) Modificaciones de naturaleza física: son aquellas modificaciones visibles que afectan al aspecto externo del alimento, influyendo en la textura, apariencia y flavor del mismo. Se pueden dar pérdidas en el volumen por la pérdida de agua de constitución, así como ganancias por hidratación o la expansión de gases. A su vez, también se puede dar una pérdida de grasa por fusión. Además, se producen cambios en la coloración, dependiendo esto mucho de la composición y naturaleza del alimento y del método de cocción empleado. En este punto destacan cambios en el pH, pardeamiento de estructuras proteicas o caramelización de azúcares. También hay cambios en la consistencia por la coagulación y

desnaturalización de proteínas, dextrinización y coagulación del almidón y reblandecimiento de la celulosa. Otra modificación es la del sabor, ya que tras la cocción se dan transformaciones químicas que influyen en el aroma y sabor del alimento, aquí es muy importante hacer una buena elección del método culinario.

b) Modificaciones de las estructuras químicas: no tiene modificaciones externas, pero sí se dan modificaciones que afectan a la composición molecular del alimento.

1. Carbohidratos. Los azúcares sólidos, como la sacarosa, se funden con el calor, transformándose en caramelo a 170 ºC y cambiando su color. Por su parte, los polisacáridos como el almidón, tiene un comportamiento propio cuando se le aplica calor en medio acuoso. Una vez alcanzados los 50 ºC comienza a espesarse, provocando la gelatinización, la cual depende de otros factores como son agregado de otras sustancias, tipo de agua, reposo o agitación de las moléculas, o la propia combinación con otros alimentos en el mismo recipiente. Por su parte, si el medio de cocción es seco, se produce la dextrinización, provocando cambios en el color y el sabor.

2. Proteínas. Se produce desnaturalización proteica con coagulación y con pérdida de solubilidad hasta los 100 ºC. Además de reducción de tóxicos, mejora de la digestibilidad e inactivación enzimática. Entre los 100 y los 140 ºC se da la reacción de Maillard y reducción de digestibilidad ya que se forman compuestos covalentes, inter e intramoleculares. A una temperatura mayor de 140 ºC, se agrega la pérdida de valor nutritivo ya que los aminoácidos comienzan a destruirse.

3. Lípidos. Pasan de un estado sólido o semisólido a fundirse, cuando alcanzan su punto de fusión, el cual no es fácil de establecer ya que

su composición en diferentes ácidos grasos lo hace complicado. Por encima del punto de fusión se puede deshidratar el glicerol, llegar al punto de humo y descomponerse.

A modo de resumen, el medio de cocción, el tiempo y la temperatura serán factores que influyan en la calidad nutricional del alimento. Cada uno de ellos puede provocar efectos positivos o negativos. Del medio de cocción dependerá la pérdida de nutrientes, como ocurre cuando se utiliza un medio acuoso, o la ganancia, ya que cuando se utiliza aceite de oliva virgen extra en la fritura, los alimentos se ven enriquecidos en compuestos fenólicos y antioxidantes. Por su parte, el tiempo y la temperatura son determinantes para que se produzca la reacción de Maillard, en la que se pueden degradar compuestos, pero a la vez se generan otros que contribuyen capacidad antioxidante del alimento.

4. Reacción de Maillard

La reacción de Maillard es una reacción no enzimática que se da al formarse un enlace covalente entre el grupo carbonilo y grupos amino libres en un alimento, por lo tanto, ocurre generalmente en aquellos alimentos que contienen carbohidratos y proteínas o péptidos [35]. El grupo carbonilo procede principalmente de cetonas o aldehídos de azúcares reductores como son la fructosa o la glucosa, aunque también son generados tras la degradación por oxidación lipídica. En cuanto al grupo amino procede de proteínas o ácidos nucleicos [36]. La reacción de Maillard es considerada una de las reacciones secundarias que ocurren con más frecuencia por el calentamiento de productos alimentarios, sin necesidad de agregar compuestos químicos. Ocurre cuando los alimentos son procesados a elevadas temperaturas o son almacenados durante largos periodos de tiempo en condiciones inadecuadas. Su efecto en los alimentos se basa en cambios en el color, sabor, olor y estabilidad [35,37,38].

4.1 Etapas de la reacción de Maillard

La reacción de Maillard engloba una serie de reacciones complejas que se relacionan entre sí, y se dividen en tres etapas: etapa inicial, avanzada y final.

1. **Etapa inicial:** *glicosilación no enzimática.* Durante esta etapa las proteínas no sufren alteraciones importantes y tampoco hay producción de color. Se produce tras la unión de tipo covalente entre un grupo carbonilo y un grupo amino libre. El grupo carbonilo procede habitualmente de un azúcar reductor, aunque también puede proceder de productos generados durante la oxidación lipídica o de la degradación del ácido ascórbico. Por su parte, el grupo amino libre, no protonado, viene de un aminoácido, péptido o proteína.

El producto formado a partir de este enlace, pierde una molécula de agua y da lugar a una *base de Schiff,* la cual se transforma, por ciclación, en una glicosamina *N*-sustituida. Esta es muy inestable y sufre un reordenamiento irreversible llamado reordenamiento de Amadori o de Heyns. Si el azúcar de partida es una aldosa (D-galactosa) se forma el compuesto de Amadori (1-amino-1-desoxi-2-cetosa). Por el contrario, si se trata de una cetosa, da lugar al compuesto de Heyns (2-amino-2-desoxi-aldosa). Estos son los primeros productos estables de la reacción de Maillard [39-41].

2. Etapa avanzada: *formación de compuestos dicarbonilos y productos finales de la glicación.* En este punto se produce la degradación de azúcares y aminoácidos (degradación de Strecker). El compuesto de Amadori o Heyns puede sufrir varias reacciones irreversibles de degradación, a pesar de su estabilidad. Esto da lugar a la formación de compuestos intermedios de reacción, muchos de ellos con sabor y aroma característicos [42,43]. En esta etapa los azúcares se deshidratan por isomerización enólica produciendo furfural y derivados, responsables del aroma. También se produce la fragmentación de los azúcares enólicos, dando lugar a compuestos de bajo peso molecular como aldheídos, cetonas, aldoles, etc. muchos de ellos responsables del flavor [44].

3. Etapa final: *formación de melanoidinas.* En esta etapa se generan oligómeros y polímeros nitrogenados de alto peso molecular gracias a reacciones de deshidratación, ciclación, retroaldolización, isomerización, reordenamiento y condensación de los productos iniciales. Estos compuestos se denominan melanoidinas y son altamente coloreados e insolubles en agua [43]. Varían en peso molecular y tienen rasgos distintivos en la región visible del espectro [45]. Las melanoidinas poseen capacidad antioxidante de radical hidroxilo, superóxido y peróxido de hidrógeno, además de actividad quelante de metales [46,47].

En la **Figura 2** se puede ver de forma esquemática la reacción de Maillard a lo largo de las tres fases.

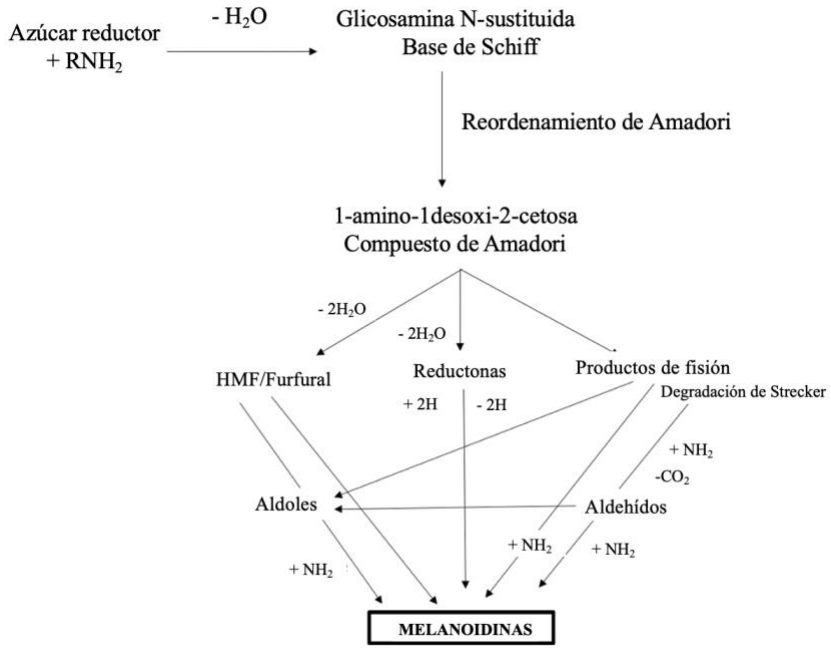


Figura 2. Reacción de Maillard.

4.2 Factores que afectan al progreso de la reacción de Maillard

El desarrollo de la reacción de Maillard se ve influenciado por una serie de factores que han de tenerse en cuenta tanto a la hora de almacenar el alimento, como cuando se llevan a cabo los cocinados. Estos factores se describen a continuación:

1. Actividad del agua

Es uno de los factores con más influencia sobre el desarrollo de la reacción de Maillard. Es expresado como actividad de agua (a_w) y la velocidad a la que ocurre la reacción aumenta exponencialmente cuando

también lo hace el contenido de agua del sistema [48,49]. Las actividades de agua comprendidas entre 0.2 y 0.3 son las idóneas para el almacenamiento de alimentos desecados. Esto se debe a que en estos casos el agua se encuentra fuertemente unida al alimento y por lo tanto no está disponible, haciendo la reacción de Maillard mínima [50,51]. Por el contrario, cuando la actividad de agua se encuentra comprendida entre 0.3 y 0.7 la reacción se ve favorecida, y cuando llega a 0.8 la reacción cesa debido a que los solutos se encuentran más diluidos en la fase acuosa [50,51].

Esta cinética tiene su explicación. Cuando la actividad del agua es muy baja, la difusión entre los reactantes se ve frenada. Al aumentar el agua presente se facilita la difusión de los mismos. Sin embargo, con cantidades mayores de agua la concentración de las sustancias reactivas disminuye, haciéndolo también la velocidad de reacción [52-55].

El estado fisicoquímico de los alimentos es también un factor influyente en la reacción de Maillard. Influirá el contenido en grasa del sistema, así como si es amorfo o cristalino. Un sistema de alimentos amorfo es capaz de interaccionar más con el agua que queda en el espacio entre moléculas, mientras que en un sistema cristalino esto sólo puede ocurrir de manera superficial [56]. La fase amorfa absorbe agua hasta que las moléculas consiguen el suficiente espacio y movilidad para formar la fase cristalina. Al iniciar la cristalización, el agua se expulsa y puede quedar atrapada en áreas localizadas del alimento, quedando por lo tanto disponible para interaccionar con otros componentes. De esta manera afecta a la reacción de Maillard, a no ser que se produzca su evaporación [57].

Los riesgos del pardeamiento en la reacción de Maillard aumentan generalmente con la deshidratación, en especial cuando el contenido de agua es inferior al 20% y la temperatura es alta [58].

2. Temperatura y tiempo de calentamiento

La temperatura es considerada como el factor más influyente en la reacción de Maillard [59]. El aumento de temperatura (como en la esterilización) y tiempo empleado favorecen de forma exponencial el pardeamiento [60]. A pesar de esto, es importante destacar que la reacción de Maillard también se puede llevar a cabo a temperatura ambiente durante el almacenamiento. Por lo tanto, se puede conseguir el mismo grado de pardeamiento si el alimento se calienta a una temperatura elevada y el tiempo de exposición es corto, como si la temperatura sobre el producto es más baja y el tiempo de exposición es más largo. En este punto, es importante destacar que, la carga de calor (cantidad de calorías aplicadas) es la mejor variable con la que se puede predecir el pardeamiento [59,60].

3. pH del medio

Tanto el pH de los reactantes como la capacidad tamponadora del sistema son condicionadores importantes del desarrollo de la reacción de Maillard. La velocidad de la reacción se ve aumentada cuando las condiciones son ligeramente básicas. La región entre pH 6 y 8 es la más favorable para el desarrollo de la reacción. Este rango se puede ampliar hasta pH 10, pero, a pH mayor de 10, la reacción se vuelve más lenta debido a una deficiencia de protones que son necesarios para catalizar los reordenamientos de Amadori y Heyns [61,62]. Por su parte, un pH ácido, cercano a 3, no influye positivamente en la velocidad de reacción, esta es mínima con estos valores de pH. A medida que la reacción avanza se produce una disminución del pH por la formación de ácidos grasos de cadena corta y la desaparición de aminoácidos alcalinos [63].

La reactividad del grupo amino se incrementa cuando el aminoácido se encuentra en forma aniónica y el valor depende del carácter ácido-base del aminoácido. Cuando los aminoácidos son ácidos, como es el caso del ácido glutámico y aspártico, la forma amónica se produce a pH 3, pero cuando los aminoácidos son básicos como la lisina o la arginina, es necesario un pH de 10 [64,65].

4. Naturaleza y concentración de los sustratos

Los sustratos principales de la reacción de Maillard son los grupos amino de proteínas, péptidos o aminoácidos libres y los grupos carbonilo de azúcares reductores o lípidos oxidados. Se trata de una reacción isomolecular en la que se produce una pérdida de azúcares al ocurrir simultáneamente la caramelización.

En cuanto a la reactividad del grupo amino es importante destacar que todas las proteínas tienen la capacidad de reaccionar con los azúcares, excepto aquellas insolubles. Además, algunos de ellos se encuentran ocultos en la cadena proteica, por lo que tampoco serían susceptibles a reaccionar. Los aminoácidos más reactivos son los del grupo amino situados en el extremo terminal, seguidos de los aminoácidos básicos [66].

Por su parte, la reacción será más o menos intensa en función del azúcar implicado. Los carbohidratos de bajo peso molecular son más reactivos que los de alto peso molecular, por su menor impedimento estérico [67-69]. La reactividad de los monosacáridos depende a su vez de otros factores como proporción de azúcar presente en disolución acuosa de forma acíclica o abierta (forma reactiva), electrofilicidad del grupo carbonilo y presencia de grupos cargados en la molécula de azúcar como, por ejemplo, grupos fosfato [70]. La capacidad de los azúcares para reaccionar disminuye

en el siguiente orden: aldopentosas, aldohexosas, cetohexosas, disacárisos y polisacáridos [71].

También es importante resaltar que, aquellos compuestos que poseen grupo carbonilo, como son los formados durante la degradación de los compuestos de Amadori (5-hidroximetilfurfural y furfural), y aquellos formados por la degradación de Strecker (aldehídos), tienen la capacidad de reaccionar con los grupos amino y así iniciar de nuevo la reacción sin necesidad de la presencia de azúcares [72].

Por último, en cuanto a la concentración de los sustratos, se ha visto que la reacción se ve favorecida cuando hay un exceso de azúcares en el medio, así como con el aumento de la reacción molar azúcar reductor/aminoácido [73].

5. Metales

La reacción de Maillard se ve afectada en presencia de cationes metálicos. Estos, a través de reacciones de oxidación hacen posible la formación de compuestos dicarbonílicos que participan en la reacción, así como otros complejos que pueden catalizarla [41,56]. Gracias a estos el proceso de pardeamiento se ve aumentado. Los primeros estudios hacen responsables a la disminución de pH producida en presencia de cationes metálicos [65].

4.3 Indicadores de la reacción de Maillard

Los indicadores de la reacción de Maillard son útiles para definir la calidad nutricional de un alimento, posible toxicidad y características organolépticas tras un procesado térmico o un almacenamiento prolongado. De esta forma se pueden optimizar estos procesos de cocinado y

almacenamiento y obtener productos con buen valor nutritivo y de calidad. En la **Figura 3** se puede ver de forma esquemática la formación de los indicadores de la reacción de Maillard.

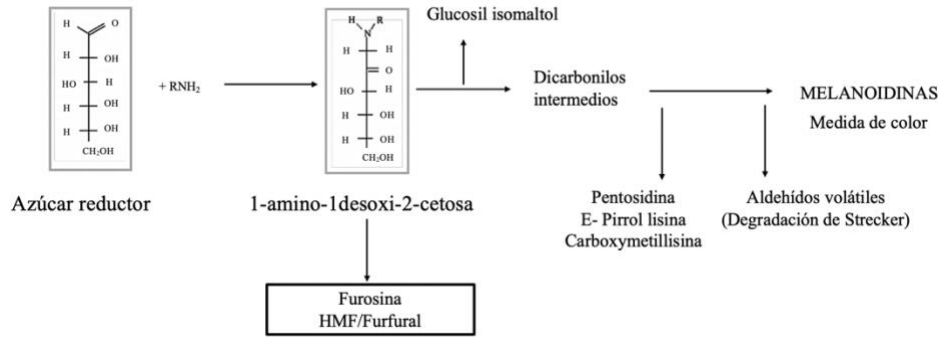


Figura 3. Indicadores de la reacción de Maillard.

Durante las etapas iniciales e intermedias de la reacción de Maillard se generan compuestos como la furosina, el 5-Hidroximetilfurfural (HMF) y el furfural. Estos se consideran marcadores de daño térmico en alimentos, por lo que son buenos indicadores de calidad del mismo.

- **Furosina**

Es uno de los indicadores de las primeras etapas de la reacción de Maillard [74]. También es utilizada para medir el daño en proteínas tras un procesamiento excesivo [75]. trata de un aminoácido que se obtiene por hidrólisis ácida del compuesto de Amadori, por lo tanto, su concentración dependerá de las condiciones de hidrólisis [74]. En realidad, la furosina se usa para cuantificar la c-N-deoxi-lactulosil-lisina (lactulosil-lisina), siendo esta el primer intermediario de la reacción de Maillard en la leche, y que se forma por el reordenamiento de Amadori de la lactulosil-amina. El problema para medir este compuesto es que la metodología es larga y tediosa, por lo que no es útil como método de control [76]. Tras la hidrólisis ácida de la

lactulosil-lisina se generan tres aminoácidos furosina, piridosina y lisina. Por lo tanto, si se considera que la formación de furosina es constante, su contenido en el alimento podría ser indicador de cómo ha transcurrido la reacción de Maillard, en cuanto a la intensidad de tratamiento térmico aplicado [74].

La técnica más usada para la determinación de furosina es la cromatografía líquida de alta resolución (HPLC). Para ello se propuso la separación de furosina en fase reversa con par iónico (columna C8) y posterior elución con gradiente [77]. Más adelante, Delgado et al., [78] pusieron a punto un método para separar furosina usando fase reversa con par iónico (columna C18) y posterior elución isocrática. Los dos métodos son rápidos, sensibles y reproducibles.

- **5-Hidroximetilfurfural (HMF)**

El HMF se puede generar por la fragmentación de azúcares (caramelización) y por la degradación de los productos de Amadori (reacción de Maillard) [75]. Sus reactivos precursores son hexosas reductoras en presencia de aminoácidos o proteínas [79]. Es un intermediario de la formación de pigmentos en las etapas avanzadas de la reacción de Maillard [74] y se genera también en la caramelización por deshidratación térmica catalizada por ácido a partir de fructosa, sacarosa y, en menor grado, de glucosa [79]. En ocasiones se utiliza como sustancia aromatizante en los alimentos. Desde 1950 se conoce la presencia de este compuesto en alimentos y se ha identificado en una gran variedad de alimentos modificados térmicamente. En algunos alimentos se usa como indicador de una buena conservación durante su almacenamiento [79], pero también es utilizado como indicador de daño térmico [74].

Para la determinación de HMF el método más utilizado es HPLC puesto que lo separa de diferentes interferencias y permite su medición de forma más exacta. Esto es porque las muestras líquidas son sometidas a una clarificación previamente [80]. Sin embargo, para alimentos con proteínas lácteas se ha visto que hay ciertos compuestos que coeluyen al HMF [81], por lo que se hace necesario controlar los componentes de las fases móviles o purificar la muestra con solventes orgánicos [82].

- **Furfural**

El furfural es un compuesto furánico producido en los alimentos procesados térmicamente, deshidratados o almacenados a temperaturas inapropiadas. Se relaciona con la aparición de aromas no deseables en alimentos, por lo tanto, también es un buen indicador para evaluar el alcance de la reacción de Maillard y de calidad [83]. Se produce en las etapas intermedias de la reacción de Maillard, en reacciones de degradación del ácido ascórbico y en la caramelización. Su determinación se lleva a cabo por cromatografía de gases, aunque es especialmente útil su determinación por HPLC, ya que es la única técnica que permite su cuantificación exacta de forma individual [84].

5. Actividad antioxidante

5.1 Radicales libres

Los radicales libres son especies químicas que tienen un electrón libre desapareado muy reactivo y situado en el orbital más extremo de su estructura atómica. Tienden a captar otro electrón a partir de otras moléculas cercanas, para así llegar a alcanzar su estabilidad electroquímica [85]. El electrón captado por el radical libre proviene de otra molécula, pasando ésta a ser un radical libre al quedarse con un electrón libre, iniciando así una reacción en cadena que va destruyendo nuestras células. La vida media de estas especies químicas es muy corta, pero tienen una gran capacidad de reacción con todas las moléculas que los rodean [86].

A los radicales libres derivados del oxígeno y a las moléculas no radicales que se forman como consecuencia de la reducción molecular del oxígeno se les llama especies reactivas del oxígeno (ROS) [87]. También existen especies oxidantes derivadas del nitrógeno. Algunas de las ROS con mayor significado biológico son: radical hidroxilo (•OH), anión superóxido ($\bullet O^{-}_{2}$), peroxinitrito ($ONOO^{-}$), óxido nítrico (NO•), radicales peroxilo (ROO•) y oxígeno singlete ($^{1}O_{2}$). Cada una de ellas tienen carácter endógeno, (mitocondria, peroxisomas, lipooxigenasas, NADPH oxidasa y citocromo P450). Dentro de las de origen exógeno se pueden encontrar radiaciones ionizantes y ultravioletas, medicamentos, tabaco, toxinas, etc. [88].

Las ROS a bajas concentraciones se relacionan con efectos beneficiosos para la salud ya que intervienen en la defensa ante agentes infecciosos, en la inducción de la respuesta mitogénica e intervienen en diferentes rutas de señalización celular [89]. Por el contrario, a altas concentraciones pueden provocar efectos perjudiciales derivados del estrés

oxidativo, provocando daños celulares en biomoléculas como lípidos, proteínas y ADN [90].

5.2 Estrés oxidativo

Durante el metabolismo celular aeróbico se van generando ROS. Este proceso, por lo general, está controlado por sistemas de defensa antioxidante endógenos, encargados de mantener el equilibrio redox [91]. Esto se puede ver influenciado por varias razones, como son estilo de vida, factores ambientales o incluso patologías, dando lugar a un desequilibrio llamado estrés oxidativo.

El estrés oxidativo se produce, o bien por un aumento de ROS, o por un déficit en los mecanismos antioxidantes. Se asocia a numerosas enfermedades (cáncer, enfermedad cardiovascular, diabetes, hipertensión, enfermedades neurodegenerativas, etc.) y con el proceso normal de envejecimiento [91]. En este punto, la dieta juega un importante papel, ya que gracias a ella hay un aporte al organismo de compuestos bioactivos que tienen efectos sinérgicos o aditivos [92] que mejoran la defensa antioxidante de forma directa o indirecta, debido a que potencian los sistemas antioxidantes y de detoxificación endógenos [93].

Un estado de estrés oxidativo puede provocar alteraciones en lípidos, carbohidratos, proteínas y ácidos nucleicos, dando lugar a alteraciones en el metabolismo y regulación celular. Esto a su vez puede producir disfunción mitocondrial, citotoxicidad, acumulación de agregados intracelulares y apoptosis [94]. La oxidación también afecta a los alimentos originando enranciamientos, deteriorándolos químicamente y reduciendo la calidad nutricional, color, textura, sabor e inocuidad [95].

5.3 Sistemas de defensa antioxidantes

Un compuesto antioxidante es aquel que neutraliza la acción oxidante de las ROS mediante la liberación de electrones en la sangre para ser catalizados posteriormente por dichos radicales. La continua exposición a ROS de diferentes fuentes ha hecho que los organismos desarrollen los siguientes mecanismos de defensa [96]:

1. Eliminación catalítica de radicales libres por enzimas.
2. Unión de proteínas a metales prooxidantes.
3. Protección contra daño (proteínas de choque térmico).
4. Estabilización de radicales libres con donadores de electrones o protones (glutatión, vitamina C o vitamina E).

Los sistemas de defensa antioxidante se pueden clasificar en sistemas antioxidantes endógenos y sistemas antioxidantes exógenos dependiendo de su origen. Estos últimos se adquieren a través de la dieta [97]. Los compuestos antioxidantes deben ser sustituidos a medida que se agotan. Cuando son de origen endógeno se irán reemplazando mediante su síntesis, pero si son de origen exógeno esto se tendrá que hacer a través de la alimentación [95].

Los **sistemas de defensa antioxidante endógenos** [97] se clasifican en sistemas enzimáticos y no enzimáticos. Los sistemas enzimáticos son la primera línea de defensa antioxidante, interaccionan directamente con las ROS para prevenir el daño oxidativo. Las enzimas que más destacan son la superóxido dismutasa, catalasa, y glutatión peroxidasa. Los sistemas no enzimáticos son la segunda línea de defensa antioxidante y son antioxidantes de bajo peso molecular. Estos forman un numeroso grupo de compuestos que previenen el daño oxidativo, ya que interactúan de directa o indirectamente

con las ROS. Aquí destacan la bilirrubina, el ácido úrico, el tripéptido glutatión y la histidina.

Los **sistemas de defensa antioxidante exógenos** son los antioxidantes procedentes de la dieta [97]. Los sistemas de defensa antioxidante endógenos son insuficientes, por lo que nuestro organismo necesita una ayuda externa. Aquellos nutrientes que consumimos a diario son una ayuda primordial para los sistemas endógenos para que no se den oxidaciones no deseadas. Pueden actuar como antioxidantes por sí solos o como cofactores de sistemas antioxidantes endógenos.

Hay varias estrategias de defensa celular frente a los procesos mediados por las ROS (**Figura 4**). Los primeros antioxidantes que actúan son los llamados **preventivos** o primarios y se encargan de evitar la formación de radicales libres. Tienen la habilidad de metabolizar las especies reactivas a estructuras más estables (mecanismo enzimático) y de secuestrar metales que participan en la formación de radicales libres (reacción no enzimática). A continuación, actúan los **eliminadores de los radicales libres** o secundarios, que se encargan de estabilizar dichos radicales y actúan cuando hay una superproducción de radicales libres y los sistemas primarios están desbordados. Dentro de este grupo hay tanto antioxidantes endógenos como exógenos procedentes de la dieta. Por último, actúan los enzimas sintetitazos de *novo* o **reparadores**, su función es reparar el daño en biomoléculas, reconstruyendo la membrana y subsanando el daño producido [98,99].

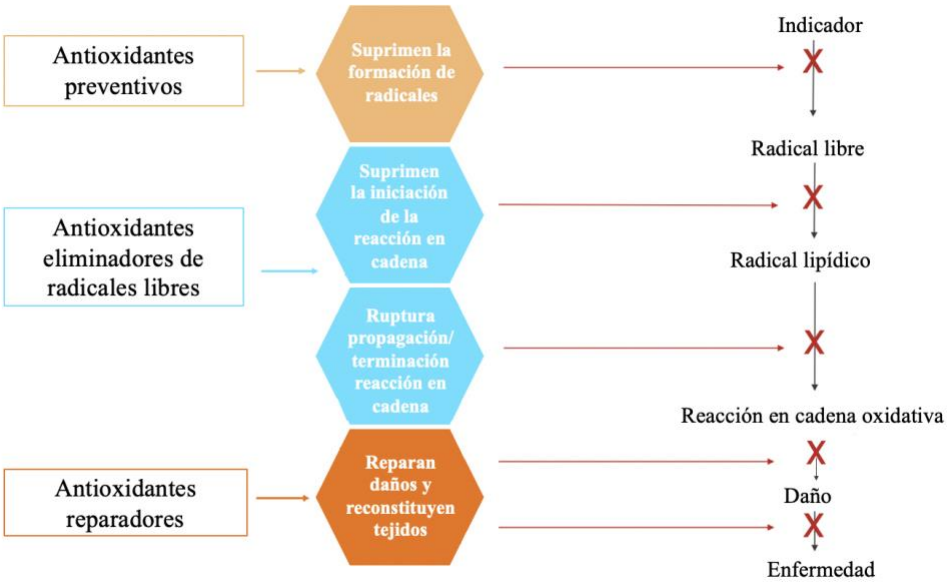


Figura 4. Clasificación de antioxidantes y su mecanismo de acción.

5.4 Antioxidantes de la dieta

En los alimentos que consumimos se encuentran nutrientes y compuestos no nutrientes con capacidad antioxidante, como por ejemplo polifenoles, terpenoides, compuestos de Maillard, minerales traza y vitaminas C, E o β-carotenos [100]. Estos antioxidantes se encuentran en el alimento de diversas formas y han sido clasificados de la siguiente manera [101]:

- Compuestos de bajo peso molecular.
- Compuestos físicamente en diferentes estructuras celulares.
- Compuestos de bajo peso molecular química o físicamente unidos a otras macromoléculas.
- Material antioxidante insoluble.

Es por esto que los alimentos de origen vegetal, como son frutas y hortalizas, se consideran promotores de la salud, no sólo por su valor nutricional general (bajo contenido en grasa y sal, alto contenido en fibra vitaminas y minerales y baja densidad energética) sino también por su contenido en fitoquímicos y componentes antioxidantes [102]. Además, la adición de algunas plantas a los productos alimentarios previene su estrés oxidativo, por inhibición del proceso de peroxidación lipídica y mejora de la calidad y valor nutricional de estos alimentos [103].

En los últimos años se ha despertado un interés por los compuestos antioxidantes procedentes de la dieta que se debe a tres razones principales:

1. El poder antioxidante de una amplia gama de fitoquímicos.

2. Los beneficios del consumo de antioxidantes naturales frente a enfermedades crónicas y degenerativas y el proceso de envejecimiento.

3. Inseguridad producida en las personas por el consumo de antioxidantes sintéticos.

En la población mundial hay una preferencia generalizada sobre consumir antioxidantes naturales frente a los sintéticos, siendo así, los naturales mucho más demandados comercialmente y aceptados [104]. El consumo de antioxidantes naturales de la dieta es un refuerzo considerable a las defensas antioxidantes endógenas.

- **Carotenoides**

Los carotenoides son los pigmentos que dan lugar a la coloración de aquellos alimentos amarillos, rojos, anaranjados de aquellos alimentos de origen vegetal. Químicamente se consideran terpenoides, los cuales están

formados por ocho unidades de isopreno, así la unión de cada unidad se convierte en el centro de cada molécula. En los carotenoides de origen natural sólo se encuentran tres elementos: carbono, hidrógeno y oxígeno. Este último puede estar presente como grupo hidroxilo carboxilo, hidroxilo, etc. El grupo de los carotenoides se divide en dos grupos: los carotenos (hidrocarburos), y las xantofilas (poseen oxígeno en su molécula). Algunos de los carotenoides son precursores de la vitamina A, como el β-caroteno, siendo el terpenoide con potentes propiedades antioxidantes [105].

La estructura de los carotenoides es la principal responsable se sus propiedades antioxidantes (**Figura 5**). Se forman por largas cadenas de dobles enlaces conjugados [106]. Esto le permite actuar como captadores de radicales libres. La inestabilidad de los carotenoides en los procesos de oxidación, se corresponde como una alta protección para otros compuestos frente a agentes oxidantes. El β-caroteno destaca en este punto ya que tiene la capacidad, que lo diferencia del resto de antioxidantes solubles en grasa, de ser más efectivo a presiones bajas de oxígeno [105].

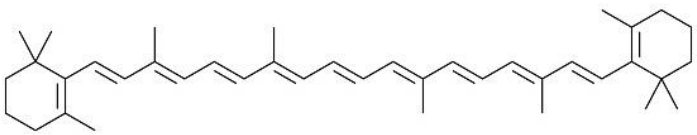


Figura 5. Estructura química del β-caroteno.

- **Vitamina C**

Se conoce como ácido L-ascórbico, compuesto de seis carbonos con carácter hidrofílico y que contiene un grupo enediol imprescindible para su actividad antioxidante. Gracias a él, el ácido ascórbico es un potente agente reductor que reacciona con radicales, neutralizándolos y transformándose de forma reversible en el radical dehidroascórbico.

La vitamina C, como ácido dehidroascórbico (**Figura 6**), su forma oxidada, es transportada al interior de la mitocondria y allí es reducida. La cadena respiratoria dona electrones para la reducción de la vitamina C, dando lugar a su estado reducido (acido ascórbico) gracias a la dihidroascorbato reductasa y glutatión reducido. El glutatión oxidado es regenerado por la glutatión reductasa a expensas de NADPH. El ácido ascórbico puede abandonar las mitocondrias por transporte activo; de esta manera, la molécula de ácido ascórbico actúa como un secuestrador de especies reactivas de oxígeno, protegiendo el genoma mitocondrial y evitando la despolarización de la membrana mitocondrial [107]. También, tiene la capacidad de prevenir o reducir la peroxidación lipídica inducida por el peróxido de hidrógeno y la formación de OH-deoxiguanosina, que se genera como consecuencia de la oxidación del ADN [108].

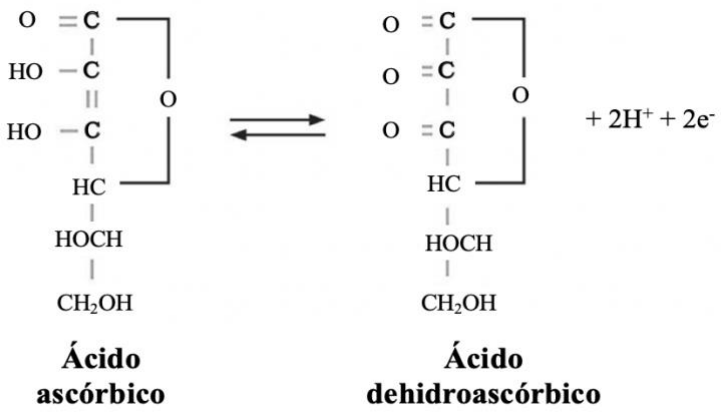


Figura 6. Estructura química de la vitamina C.

- **Vitamina E (tocoferol)**

La vitamina E actúa coordinándose con otras moléculas y enzimas para la defensa de las células de los efectos nocivos producidos por los radicales libres. Posee una gran capacidad de protección de las membranas

celulares, impidiendo su oxidación. Esta oxidación llevaría a una degradación del organismo. Su mecanismo de acción consiste en interrumpir las etapas de propagación y descomposición del proceso de autooxidación y en la reacción con el oxígeno singulete en el proceso de fotooxidación [109].

Esta vitamina ejerce una gran cantidad de efectos beneficiosos para la salud, como antioxidante, estimulante del sistema inmunitario, frena el desarrollo de la enfermedad de Alzheimer, además de ayudar a reducir los niveles de colesterol. La vitamina E como α-tocoferol (**Figura 7**), forma más activa de la vitamina E, puede llegar a inducir apoptosis en las células tumorales. Este compuesto actúa como antioxidante al transformarse en radical tocoferilo, que es más estable.

La vitamina E actúa de forma sinérgica con otros antioxidantes, como el ácido ascórbico, entre otros. Regeneran a la vitamina E y reducen el carácter prooxidante del tocoferilo [110].

El nivel de vitamina E en un alimento depende de sus condiciones de elaboración y almacenamiento. Durante este último se reduce el contenido de tocoferoles en los alimentos por la oxidación, humedad, presencia de luz, altas temperaturas, y acción de microorganismos [111].

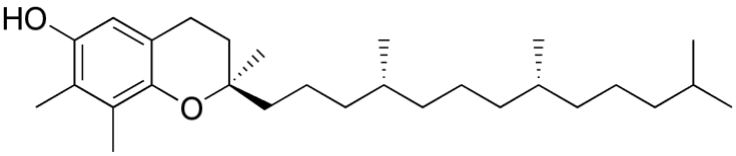


Figura 7. Estructura química del α-tocoferol.

- **Productos de la reacción de Maillard**

La capacidad antioxidante que se les atribuye a los productos de la reacción de Maillard fue observada por primera vez en 1954. Se descubrió un

aumento de la capacidad antioxidante de forma paralela al aumento del tiempo de calentamiento. La capacidad antioxidante aumenta con la aparición de productos derivados de las etapas intermedias y tardías de la reacción, así como con el tipo de azúcar involucrado [112]. Se ha propuesto que algunos de estos compuestos podrían ser utilizados como aditivos alimentarios para contribuir a la estabilidad oxidativa de los mismos y mejorando sus propiedades [113].

Sus mecanismos de acción como antioxidantes se basan en la inactivación de radicales libres lipídicos, inhibición de la formación de peróxidos, la acción como trampas de oxígeno y habilidad de captar iones metálicos y catalizadores de las reacciones de primer orden [114]. En cuanto a su propiedad quelante de metales, los productos de la reacción de Maillard retardan reacciones de oxidación que son catalizadas por los minerales [115].

- **Minerales**

Algunos minerales pueden tener propiedades antioxidantes, entre ellos se encuentran el hierro, cobre, zinc, selenio y manganeso. Estos actúan como cofactores de enzimas endógenas antioxidantes que están implicadas en reacciones que atrapan radicales superóxido, impiden la formación de radicales hidroxilo y favorecen la eliminación de H_2O_2 [116].

El **hierro** y el **cobre** poseen propiedades antioxidantes importantes y además pueden actuar como fuentes pro-oxidantes de radicales libres [117]. El **manganeso** protege de la peroxidación lipídica, promueve la síntesis de metalotioneínas y atrapa radicales hidroxilo y superóxido. Es importante destacar que tiene estas habilidades por formar parte de la superóxido dismutasa mitocondrial [118]. Por su parte, el **zinc** forma parte del centro activo de la superóxido dismutasa citosólica, junto con el **cobre** [119]. El

selenio ayuda a la absorción de vitamina E, aumenta la actividad de algunas enzimas, como la glutatión peroxidasa, y protege a las células frente al radical superóxido [120].

- **Polifenoles**

Se encuentran distribuidos en todas las plantas, pero sobre todo en frutas, hortalizas y sus alimentos derivados como cerveza, vino, aceite de oliva o zumos vegetales. El efecto beneficioso asociado al consumo de hortalizas y otros alimentos de origen vegetal se relacionan en su mayoría con estos compuestos y su capacidad antioxidante [102]. También se encuentran en cereales y legumbres. Actúan como antioxidantes mediante dos mecanismos:

- Debido a su facilidad para ceder a un radical libre un radical hidrógeno de un grupo hidroxilo aromático [121].

- Debido a la facilidad que poseen de quelar iones metálicos, como cobre o hierro, y así evitar la formación de radicales libres [122].

Los polifenoles influyen en gran medida en las características sensoriales de los alimentos. Además, tienen la capacidad de destruir o inhibir el crecimiento de bacterias, hongos y protozoos [123]. Estructuralmente poseen anillos aromáticos con dobles enlaces conjugados y sustituyentes hidroxilo, que normalmente aparecen en su forma glicosilada al combinarse con azúcares (**Figura 8**) [124]. En estado puro son difíciles de disolver en agua, pero las interacciones en las que participan en estado natural hacen posible cierta solubilidad en medio acuoso. Los polifenoles pueden ser clasificados en función de su número de anillos y a los elementos estructurales que se unen. Principalmente se clasifican en flavonoides (lo más abundantes), ácidos fenólicos, alcoholes fenólicos, estilbenos y lignanos [125] (**Tabla 1**).

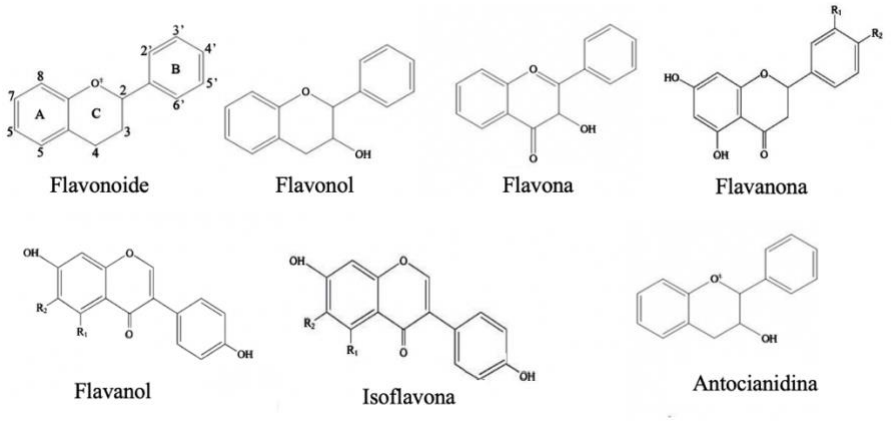


Figura 8. Estructura de los compuestos flavonoideos.

Tabla 1. Clasificación de los compuestos fenólicos.

GRUPO ESTRUCTURAL	COMPUESTO FENÓLICO
FLAVONOIDES	Flavonoles
	Flavonas
	Flavanonas
	Flavanoles
	Isoflavonas
	Antocianidinas
NO FLAVONOIDES	Ácidos fenólicos
	Acoholes fenólicos
	Estilbenos
	Lignanos

Algunos polifenoles, además de su propia actividad antioxidante, también pueden actuar potenciando la actividad de enzimas antioxidantes, tales como catalasa, glutatión peroxidasa, glutatión reductasa y superóxido

dismutasa [126]. A su vez, también inhiben la actividad de enzimas involucradas en procesos oxidativos como son la lipooxigenasa, ciclooxigenasa, xantina oxidasa y fosfolipasa A2. De esta forma evitan la generación de ROS [127].

5.5 Determinación de la capacidad antioxidante

Debido al aumento del interés por los efectos beneficiosos de los polifenoles en salud humana y en calidad alimentaria, se han desarrollado muchos métodos para determinar la capacidad antioxidante en los alimentos. Esta depende de las propiedades redox del propio alimento, de la capacidad del mismo como quelante de metales prooxidantes, así como de su capacidad de donar hidrógeno y de captar radicales libres. Sería ideal que cada una de estas propiedades se pudiera medir en cada componente para así evaluar la capacidad antioxidante total, pero en la práctica, resulta muy difícil.

Un buen método de determinación de capacidad antioxidante debe tener las siguientes características [128].

- Ser un método sencillo.
- Tener un mecanismo químico y un punto final fijo.
- Con un alto rendimiento de análisis.
- Tener buena reproducibilidad intra- e interlaboratorio.
- Ser adaptable a ensayos con antioxidantes hidrofílicos y lipofílicos.
- Poseer diferentes fuentes generadoras de radicales libres.

Se han desarrollado diferentes ensayos que conjugan estas propiedades en un método para determinar la capacidad antioxidante [129]. Así, existen dos tipos de métodos para la determinación de capacidad antioxidante:

- **Métodos directos**

Son aquellos que se llevan a cabo con un sustrato capaz de oxidarse [95,128]. Evalúan el efecto inhibitorio de una sustancia que es potencialmente antioxidante sobre la degradación oxidativa del sustrato usado. Esta aproximación se conjuga con un tipo de estrategia de medición basada en la detección de los productos de oxidación.

- **Métodos indirectos**

Son los más usados para determinar la capacidad antioxidante en alimentos de origen vegetal [128]. Se basan en la estabilización de un radical libre artificial, ya sea por transferencia de electrones o hidrógeno, o por la reducción de un metal de transición de carácter prooxidante, por transferencia de electrones. Para evaluar la capacidad antioxidante te una muestra de forma adecuada, deben combinarse al menos dos métodos, basados en distintos fundamentos. Algunos ejemplos de métodos indirectos son el ABTS, DPPH y ensayos de poder antioxidante por reducción del hierro férrico como es el FRAP. Se recomienda la combinación de ensayos basados en la capacidad de reducir el hierro, como el FRAP, junto con algún otro método que determine la capacidad de reducción de radicales libres [100]. Por su parte, algunos investigadores argumentan que existen métodos, como es el ensayo de fenoles totales por el método Folin-Ciocalteu, que, a pesar de no estar destinado directamente a medir capacidad antioxidante, podría ser útil para estos estudios, especialmente si se combinan con otros métodos [128]. Algunos

autores incluso lo han propuesto como un método de evaluación de capacidad antioxidante más, y estaría basado en la transferencia de electrones [131].

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

La oxidación del ABTS da lugar al radical $ABTS^{\cdot *}$ (**Figura 9**). Esta se puede generar de forma enzimática, química (persulfato potásico, dióxido de manganeso, radicales peróxido), o electroquímica [132]. El radical catiónico que se obtiene tiene una coloración verde-azulada estable con una absorbancia máxima a 734 nm. El método consiste en monitorizar la reducción del $ABTS^{\cdot *}$ por la adición de una muestra con antioxidantes. Esto se lleva a cabo midiendo la absorbancia del ABTS a 734 nm. Esta se compara con la del Trolox, que es un análogo sintético y soluble de la vitamina E. Es expresado como TEAC (capacidad antioxidante equivalente de Trolox) [132].

La principal ventaja de este método es que puede ser utilizado para muestras liposolubles e hidrosolubles, eligiendo el disolvente que corresponda en cada caso y que aporta rápidamente los resultados más reproducibles empleando un espectrofotómetro. Un aspecto a destacares que la longitud de onda a la que se realizan las medidas de absorbancia no es nada común en alimentos, haciendo de este método, un ensayo interesante para el estudio de extractos vegetales, eliminando la posibilidad de interferencias de color.

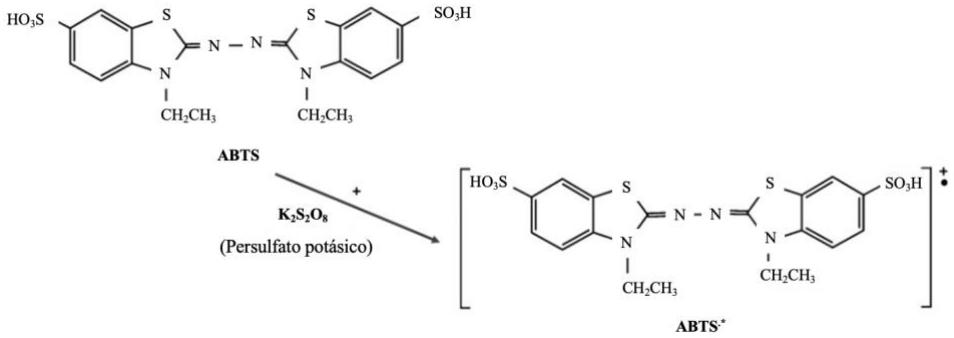


Figura 9. Formación del radical ABTS$^{\cdot *}$ desde ABTS y persulfato potásico.

1. No es representativo de la capacidad antioxidante, al igual que todos los métodos indirectos.
2. La reacción de algunos fenoles y productos naturales con el ABTS$^{\cdot *}$ es lenta. El resultado de la determinación del TEAC se espera que sea dependiente del tiempo de incubación, así como de la relación entre la cantidad de muestra y la concentración de ABTS$^{\cdot *}$.
3. Hay poca selectividad del ABTS$^{\cdot *}$ en la reacción con donadores de átomos de hidrógeno. Esto es debido a que se produce una reacción con el grupo hidroxilo de un compuesto aromático de forma independiente a su potencial antioxidante real.

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

Por la simplicidad y alta sensibilidad de este ensayo, se ha hecho muy popular para el estudio de antioxidantes naturales [133]. Está basado en la teoría de que cualquier donador de hidrógeno es antioxidante. El DPPH· capta un hidrógeno del antioxidante para formar DPPH **(figura 10)**, por lo tanto, el poder antioxidante será proporcional a la pérdida o desaparición de DPPH·.

Hay varios métodos que permiten su monitorización. El más común es el que se lleva a cabo con espectrofotometría ultravioleta, por su precisión y facilidad. El radical tiene un máximo de absorción a 517 nm, y torna a color amarillo cuando se forma DPPH, por lo que sus efectos antioxidantes se pueden ver fácilmente por la pérdida de absorción de la molécula. Sin embargo, algunos antioxidantes pueden causar interferencias al presentar un espectro de absorción similar al del DPPH·, como por ejemplo los carotenoides. Los resultados, al igual que en ABTS, se pueden expresar como TEAC, aunque también se pueden interpretar como % de desaparición de DPPH.

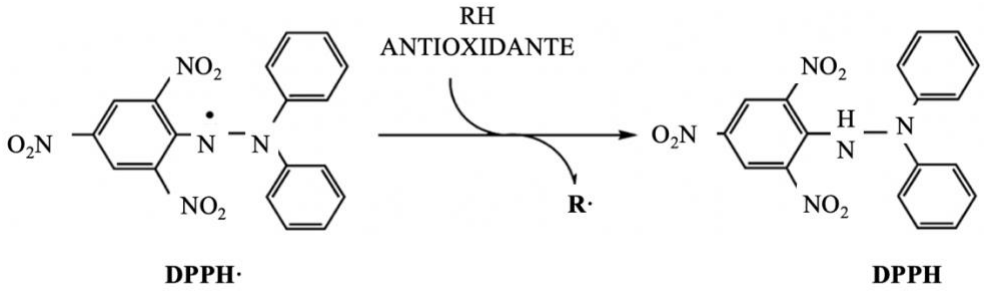


Figura 10. Captación del radical libre del DPPH· y formación del DPPH.

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

Este método está basado en la reducción del hierro férrico (Fe^{3+}, prooxidante) a hierro ferroso (Fe^{2+}) por un antioxidante y en condiciones de acidez (**Figura 11**). La reducción puede ser cuantificada por la acción del cloruro de 2,3,5-trifeniltetrazolio (TPTZ), que es un compuesto químico capaz de quelar al hierro. El complejo Fe^{3+}-TPTZ posee una coloración azul intensa con un máximo de absorción a 595 nm. Por su parte, el complejo Fe^{2+}-TPTZ posee una coloración amarilla [134]. Por lo tanto, la evaluación de la

capacidad antioxidante se llevará a cabo monitorizando esto con un espectrofotómetro.

Con este ensayo se obtienen resultados reproducibles y de forma rápida. La única desventaja que tiene es que se debe realizar en matriz acuosa, por lo que se debe usar un antioxidante de referencia hidrosoluble, como el Trolox o el ácido ascórbico. En cuanto a la expresión de los resultados, se pueden expresar por lo tanto como TEAC (si se usa Trolox) o CEAC (si se usa ácido ascórbico). También pueden ser interpretados como cantidad de hierro ferroso producido.

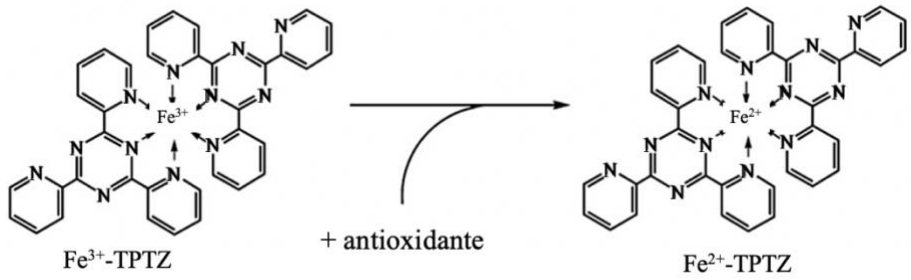


Figura 11. Captación del complejo (Fe^{2+}-TPTZ) desde el complejo (Fe^{3+}-TPTZ).

- **Métodos de determinación de polifenoles**

  - **Método de Folin-Ciocalteu. Polifenoles totales.**

Se usa para determinar el contenido de fenoles y polifenoles totales que están presentes en un alimento. Como estándar se emplea el ácido gálico. Está basado en la reducción de los fenoles totales por una mezcla de ácido fosfomolíbdico y fosfotúngstico, generando una mezcla de óxidos de molibdeno y tungsteno de color azul, con un máximo de absorción a 765 nm, proporcional al contenido en compuestos fenólicos [135]. El mecanismo se basa en una transferencia de electrones del medio alcalino, por parte de los

polifenoles (y otras especies reductoras presentes en la muestra) al reactivo Folin-Ciocalteu [136]. Se trata de un método simple y reproducible, pero tiene el inconveniente de que puede sufrir interferencias con sustancias no fenólicas como fructosa, sacarosa, proteínas, algunos ácidos orgánicos, algunos aminoácidos y algunas sales orgánicas [137]. Los resultados son expresados en mg/L de ácido gálico.

- **UHPLC/TOF-MS. Especies fenólicas.**

Además del contenido fenólico total, también es importante medir su composición en compuestos fenólicos de forma individual [138]. Para ello se usa la cromatografía líquida de ultra alta resolución (UHPLC). La cromatografía UHPLC es una técnica física de separación en la cual, los componentes de una muestra se dividen en dos fases: una fase móvil y una fase estacionaria. Deben ser de polaridad opuesta, y todos los solutos serán de igual polaridad que la fase móvil. Todo soluto que entra en la columna, ha de salir de ella (propagación) y, como objetivo, los solutos se separan entre sí, a lo que se le llama migración diferencial [139].

La espectrometría de masas se basa en extraer iones de moléculas orgánicas en fase gaseosa, y una vez extraídos los iones, se separan según su masa y carga y finalmente se detectan. El espectro de masas será información bidimensional que representa los parámetros asociados con la abundancia de diferentes tipos de iones en función de la relación masa-carga de cada ion. Los procesos que tienen lugar en un espectrómetro de masas son de naturaleza química, por lo que la presencia y aparición de iones en el espectro dependerá de la estructura química de cada compuesto [140].

6. Microbiota intestinal

La microbiota es definida como una comunidad de microorganismos vivos que viven en una relación de simbiosis tanto de tipo comensal como de mutualismo, y que residen en un nicho ecológico determinado. Por lo tanto, la microbiota intestinal, es el conjunto de microorganismos vivos que habitan y conviven en el intestino [141-143]. En el caso de los humanos, está formada por trillones de células microbianas y cientos de especies diferentes, siendo una de las comunidades más densamente pobladas. Debido a esto, el ser humano puede ser considerado un "super organismo". En ella se pueden encontrar microorganismos que pertenecen principalmente a cuatro phyla: *Firmicutes* (Gram-positivos), *Bacteroides* (Gram-negativos), *Actinobacteria* (Gram-positivos) y *Proteobacteria* (Gram-negativos). A parte de estos cuatro, se presentan grupos minoritarios constituidos por otros phyla como *Verrucomicrobia*, y otros reinos como Fungi y Archaea [144,145].

6.1 Microbiota intestinal y metabolismo

La microbiota intestinal se puede considerar un "órgano metabólico" que lleva a cabo funciones bioquímicas y fisiológicas muy variadas con influencia sobre el metabolismo del huésped. Los microorganismos que la conforman poseen diversas enzimas que permiten transformar hidratos de carbono (polisacáridos, oligosacáridos y almidón resistente) y otros nutrientes y componentes alimentarios que no pueden ser digeridos ni absorbidos en el intestino. Estos, en su mayoría hidratos de carbono, llegan al colon donde son fermentados, con lo que se permite que estos nutrientes y componentes alimentarios se vuelvan disponibles para el organismo, y no se pierdan con las deposiciones [144,145].

Como resultado de dicha fermentación, aparecen los productos finales de la misma, como son monosacáridos, ácidos grasos de cadena corta, compuestos fenólicos, así como ciertos gases, entre los que podemos encontrar CO_2, hidrógeno y metano [146]. Los ácidos grasos de cadena corta, son cadenas lineales o ramificadas de uno a seis átomos de carbono. La producción de metabolitos por parte de la microbiota intestinal depende por tanto de los sustratos disponibles [147,148].

Los principales metabolitos derivados de hidratos de carbono son los ácidos acético, propiónico y butírico. Otros ácidos orgánicos, principalmente láctico y succínico, participan el proceso conocido como "cross-feeding" [149]. Este proceso consiste, de manera sencilla, en que los metabolitos producidos por unas bacterias pueden ser usados como sustrato por otras. Así, los ácidos láctico y succínico pueden ser producidos por algunos géneros y usados por otros para la producción de acético, propiónico o butírico. Por su parte, como consecuencia del metabolismo de proteínas, destaca la producción de amoniaco, aminas, fenoles (ácidos hidroxifenilacéticos, hidroxifenilpropiónicos), tioles e indoles. Además, destaca la producción de ácidos grasos de cadena ramificada como los ácidos isobutírico o isovalérico. Estos compuestos derivados del metabolismo de proteínas son potencialmente perjudiciales [150].

Los ácidos **acético**, **propiónico** y **butírico (Figura 12)** juegan un papel positivo para la salud humana [145,151,152], entre sus efectos se puede destacar:

- Refuerzan la barrera intestinal y nutren la mucosa protectora del intestino.
- Mejoran el tránsito en el intestino grueso.
- Contribuyen a equilibrar el nivel de glucemia.

- Previenen enfermedades no transmisibles como la obesidad y el cáncer de colon, reducen el colesterol y ayudan al sistema inmune.
- Sirven como sustrato energético a los colonocitos.
- Cerca del 10% de los requerimientos energéticos de la dieta son cubiertos por ellos.

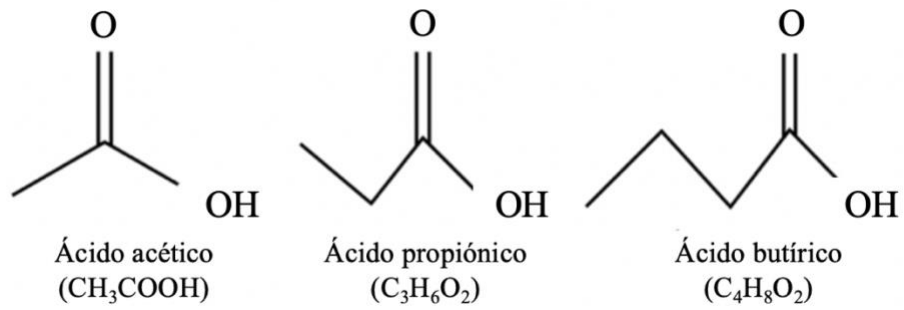


Figura 12. Ácidos acético, propiónico y butírico.

Concretamente, el ácido **butírico** ha captado la mayor atención. Es el principal sustrato y fuente energética para los colonocitos, proporcionándoles entre el 60 y el 70% de la energía que necesitan para su proliferación y diferenciación. Este ácido graso es importante también para mantener la formación de epitelio colónico a través de su función como agente antiinflamatorio para prevenir la producción de especies reactivas de oxígeno y especies reactivas de nitrógeno generadas en caso de estrés oxidativo. Además, puede desempeñar un papel en el metabolismo lipídico, así como ejercer efectos antitumorígenos en algunas líneas celulares de cáncer [150,152]. Las principales bacterias productoras de butirato en el intestino humano pertenecen a la fila *Firmicutes* (géneros *Faecalibacterium y Eubacterium,* entro otros), los cuales son anaerobios, por lo que las bajas concentraciones de oxígeno en el colon hacen que sea un nicho favorable para ellos [149].

A su vez, el **propionato** se absorbe y llega al hígado siendo usado para la gluconeogénesis [149]. El **acetato**, por su parte, entra en la circulación periférica para llegar a los tejidos periféricos, donde se utiliza como sustrato principal para la síntesis de colesterol y puede interferir directamente en el metabolismo de los lípidos. Además, puede cruzar la barrera hematoencefálica para activar la acetil-CoA carboxilasa y la expresión de neuropéptidos, lo que induce la activación neuronal hipotalámica y suprime el apetito [150,152]. Es importante destacar que el uso de propionato como precursor gluconeogénico, inhibe el uso de acetato para la posterior formación de lípidos y síntesis de colesterol, reduciendo la lipogénesis hepática y las concentraciones de triglicéridos y colesterol en suero tras el consumo de hidratos de carbono fermentables [150].

En el tracto gastrointestinal humano la concentración más alta de ácidos grasos de cadena corta se encuentra en el colon, en una relación molar de aproximadamente 60:25:15 para acetato:propionato:butirato. Sin embargo, la cuantificación de los ácidos grasos de cadena corta intestinales humanos solo proporciona niveles constantes y puede no reflejar con precisión el nivel de producción bacteriana. Esto es porque la mayoría de los ellos son producidos en la luz del colon (90-95%) y se absorben por la mucosa intestinal [152,153]. Aun así, el análisis de ácidos grasos de cadena corta en muestras fecales se utiliza como una aproximación de los niveles intestinales.

La microbiota intestinal también es capaz de **metabolizar compuestos fenólicos**. El metabolismo de los compuestos fenólicos por parte de los microbios intestinales se ha estudiado ampliamente, ya que muchos de sus beneficios para la salud se atribuyen, de hecho, a los metabolitos microbianos [154]. Actualmente se estima que sólo entre el 10 y el 20% de los polifenoles de la dieta se absorben en el intestino delgado [155]. Por lo

tanto, la mayoría de ellos llegan al colon y son metabolizados por las bacterias intestinales en compuestos más pequeños [149].

Sin embargo, se ha demostrado que el tratamiento con antibióticos puede alterar la metabolización de las catequinas por parte de los microbios intestinales [156]. Según estos autores, los ratones tratados con antibióticos mostraron mayores niveles de epigalocatequina en sangre, hígado y orina, probablemente debido a la eliminación de los microbios encargados de la metabolización de las catequinas. Por lo tanto, la biodisponibilidad de las catequinas estará determinada, al menos en parte, por la microbiota intestinal.

6.2 Disbiosis

La disbiosis disbiosis es un desequilibrio en la abundancia de distintos géneros bacterianos intestinales llevando a un desequilibrio en la comunidad microbiana. Esto podría provocar una respuesta adversa en el hospedador. Actualmente, se reconoce que la disbiosis es un importante mecanismo de etiopatogenia, por lo que es necesario encontrar herramientas terapéuticas que restauren la composición microbiana intestinal y su actividad metabólica, como pueden ser intervenciones dietéticas [145,150].

Entre las posibles causas de cambios en la microbiota intestinal o disbiosis, se encuentran los antibióticos, comúnmente usados como remedio para infecciones. Es común que, cuando se toman para tratar una infección extraintestinal puedan provocar cambios a lo largo del tracto gastrointestinal, incluyendo modificaciones en la microbiota intestinal por la reducción drástica de *Lactobacillus*, así como por una promoción de *Proteobacteria spp* [157]. Además, la dieta también puede ser una importante causa de disbiosis, ya que los microorganismos que residen en el intestino humano se alimentan de aquellos nutrientes que les llegan a través de la alimentación del individuo.

Esto hace que, haya más predominio de unas especies u otras dependiendo de la alimentación [158].

La variabilidad interindividual y la plasticidad de la microbiota intestinal ha dificultado los esfuerzos para identificar una microbiota "sana", aunque los marcadores de la estabilidad de la microbiota, como la riqueza y diversidad, son a menudo usados como indicadores de la salud intestinal debido a su inversa asociación con ciertas patologías [144]. De esta manera, se hace interesante su estudio en aquellas personas que padezcan alguna enfermedad, como, por ejemplo, celiaquía, alergias alimentarias, así como obesidad. En la actualidad, cada vez hay más literatura científica que defiende que, en estas personas, se produce disbiosis, lo que puede estar relacionado en gran medida con la patología que padezcan.

Recientemente, se han descrito alteraciones en la composición de la microbiota de pacientes celiacos [159]. La enfermedad celíaca es un trastorno inflamatorio del intestino delgado caracterizado por una intolerancia permanente al gluten que se ingiere con la alimentación y que tiene manifiesto en individuos con predisposición genética. Se han encontrado diferencias entre la composición y la diversidad de la microbiota intestinal de adultos con síntomas intestinales clásicos de enfermedad celíaca y las de adultos con síntomas extraintestinales, indicando que la composición de la microbiota residente en la mucosa duodenal de los pacientes difería basándose en las manifestaciones de la enfermedad celíaca [160]. Es posible que ciertos cambios en la composición de la microbiota a nivel duodenal se deban a las consecuencias del patrón inflamatorio destructivo, lo que se hace más evidente en la atrofia vellositaria duodenal más avanzada [161].

La obesidad, por su parte, no sólo se relaciona actualmente con el factor genético como determinante de la obesidad, sino que la investigación

está señalando a la microbiota intestinal como uno de los factores que afectan al desarrollo de esta enfermedad [150]. Apoyando estas investigaciones, existen estudios en roedores y en humanos donde la obesidad se relaciona con alteraciones en la composición de la microbiota: una disminución de 50% de la población de *Bacteroides* y un aumento proporcional en los *Firmicutes*, comparada con la microbiota de personas sanas no obesas [162]. Es considerado que, aquellas dietas que se caracterizan por un consumo elevado de grasa y bajo en fibra, pueden ser causantes de disbiosis en la microbiota intestinal, lo que lleva a la reducción de la integridad de la barrera intestinal; alteración que a su vez puede predisponer a la obesidad [158].

En estudios realizados como intervenciones con personas voluntarias obesas sometidas a una dieta baja en calorías durante un año, se observaron cambios de composición en su microbiota con una menor proporción de microorganismos Gram positivos y mayor proporción de Gram negativos. Esto tiene como resultado un cambio en la relación de microorganismos *Firmicutes/Bacteroides*, provocando así una disbiosis intestinal, que consiste en la alteración de los distintos grupos de microorganismos que conforman la microbiota intestina [163,164]. Esto a su vez está relacionado directamente con los cambios de peso corporal del individuo. Este cambio de composición de la microbiota genera un aumento plasmático de lipopolisacáridos que favorecen los procesos inflamatorios y como consecuencia una mayor resistencia a la insulina que promueve el desarrollo de diabetes, además de favorecer el aumento de adipocitos, dislipidemia y un desequilibrio en el peso corporal en comparación con individuos de una mayor diversidad microbiológica [165].

También se ha observado que la microbiota del individuo obeso presenta una menor biodiversidad microbiana en comparación con individuos

sin patologías (no obesos). En este sentido, se ha visto que la influencia que tiene la microbiota intestinal en el hospedero está relacionada con las funciones de tipo metabólico, tróficas, secreción de hormonas intestinales y reguladoras del sistema inmunitario, además de tener implicación en la regulación de los depósitos de grasa corporal en tejido adiposo [163,164]. Los mecanismos exactos por lo cual la microbiota contribuye a la obesidad, no son del todo esclarecidos [166].

Asimismo, los desórdenes alérgicos también han sido asociados con disbiosis. En las últimas décadas incluso se ha estudiado el uso de probióticos como una posible intervención dietética para interrumpir la progresión de los síntomas clásicos, como son eccema, rinitis y asma [162,167].

6.3 Metodologías de evaluación de la microbiota intestinal

Para el estudio de la microbiota intestinal humana se han utilizado diferentes metodologías, desde técnicas *in vitro* [168] hasta intervenciones en humanos. A continuación, se van a explicar los experimentos más comunes para el estudio de la microbiota intestinal:

1. **Estudio de la microbiota intestinal *in vitro***: los modelos *in vitro* proporcionan información en diferentes etapas del proceso de fermentación al permitir un muestreo dinámico a lo largo del tiempo en diferentes regiones consecutivas del colon humano. Además, como están estandarizados, proporcionan resultados con una alta reproducibilidad [169]. Es importante destacar que no hay restricciones éticas para su uso, de modo que se puedan usar patógenos, compuestos tóxicos o radioactivos sin aprobación ética. Por lo tanto, los enfoques *in vitro* ofrecen la posibilidad de estudios mecánicos y el desarrollo de hipótesis que deben ser probadas (o

refutadas) en ensayos clínicos en humanos. Sin embargo, es importante crear condiciones que se parezcan mucho a la situación *in vivo* [169]. Cuanto mejor pueda simular un sistema la situación real, mayor será la importancia fisiológica de la información obtenida.

Los modelos *in vitro* más simples y utilizados con mayor frecuencia para estudiar el microbioma intestinal son las fermentaciones estáticas [168]. Por lo general, éstos se realizan en pequeños vasos o tubos de ensayo, en los que se analiza una variedad de cultivos, como cepas específicas, comunidades microbianas intestinales o fecales de origen animal o humano, para determinar su capacidad para metabolizar diferentes sustratos.

- Ventajas: barato, fácil de realizar, pocos problemas éticos, permite evaluar muchas muestras diferentes.
- Inconvenientes: poca similitud con la realidad, acumulación de metabolitos microbianos que inhiben la actividad microbiana adicional.

2. **Estudio de la microbiota intestinal con sistemas continuos**: un ejemplo es aquél que se lleva a cabo mediante un simulador del ecosistema microbiano intestinal (SHIME) [169,170]. Es un método que intenta simular el tracto gastrointestinal humano y con ello se puede llevar a cabo la estabilización de la microbiota humana. Con un donante de heces se hace posible la extracción de microbiota intestinal, la cual será alimentada durante un periodo de tiempo para permitir su supervivencia y reproducción. Durante esta estabilización y al final de la misma se puede llevar a cabo el estudio de la microbiota y así ver cómo va cambiando, e incluso, se puede cambiar la alimentación de la misma para ver las variaciones. Esto lo podemos ver, por ejemplo, con los ácidos grasos de cadena corta producidos.

- Ventajas: se asemeja más a la realidad.
- Inconvenientes: sólo permite el estudio de una muestra a la vez.

3. **Intervenciones en animales o personas**: los ensayos *in vivo* en animales o humanos están limitados para detectar los efectos y el posible mecanismo de acción de ciertos ingredientes o medicamentos dietéticos, ya que se enfrentan a la alta complejidad del entorno tracto gastrointestinal y al difícil acceso al intestino [169].

- Ventajas: es ideal porque los resultados son reales.
- Inconvenientes: es un método muy caro, puede acarrear problemas éticos y hace difícil el estudio de alimentos concretos.

Referencias bilbiográficas

1. Katz, S.H.; Weaver, W.W. Encyclopedia of Food and Culture; Gale virtual reference library; Scribner: New York, 2003; ISBN 978-0-684-31416-7.

2. Konner, M.; Eaton, S.B. Paleolithic Nutrition: Twenty-Five Years Later. Nutr. Clin. Pract. Off. Publ. Am. Soc. Parenter. Enter. Nutr. 2010, 25, 594-602, doi:10.1177/0884533610385702.

3. Sonnenburg, E.D.; Smits, S.A.; Tikhonov, M.; Higginbottom, S.K.; Wingreen, N.S.; Sonnenburg, J.L. Diet-Induced Extinctions in the Gut Microbiota Compound over Generations. Nature 2016, 529, 212-215, doi:10.1038/nature16504.

4. Rabassa, M.; Zamora-Ros, R.; Urpi-Sarda, M.; Bandinelli, S.; Ferrucci, L.; Andres-Lacueva, C.; Cherubini, A. Association of Habitual Dietary Resveratrol Exposure with the Development of Frailty in Older Age: The Invecchiare in Chianti Study12. Am. J. Clin. Nutr. 2015, 102, 1534-1542, doi:10.3945/ajcn.115.118976.

5. de Bont, J.; Bennett, M.; León-Muñoz, L.M.; Duarte-Salles, T. Prevalencia e incidencia de sobrepeso y obesidad en 2,5 millones de niños y adolescentes en España. Rev. Esp. Cardiol. 2022, 75, 300-307, doi:10.1016/j.recesp.2021.06.030.

6. Basterra-Gortari, F.J.; Bes-Rastrollo, M.; Ruiz-Canela, M.; Gea, A.; Martinez-Gonzalez, M.Á. Prevalencia de obesidad y diabetes en adultos españoles, 1987-2012. Med. Clínica 2017, 148, 250-256, doi:10.1016/j.medcli.2016.11.022.

7. León-Muñoz, L.M.; Guallar-Castillón, P.; Graciani, A.; López-García, E.; Mesas, A.E.; Aguilera, M.T.; Banegas, J.R.; Rodríguez-Artalejo, F. Adherence to the Mediterranean Diet Pattern Has Declined in Spanish Adults. J. Nutr. 2012, 142, 1843-1850, doi:10.3945/jn.112.164616.

8. Aesan - Agencia Española de Seguridad Alimentaria y Nutrición. Disponible en https://www.aesan.gob.es/AECOSAN/web/nutricion/subseccion/vigilancia_obesidad_infantil.htm (conultado el 6 de septiembre de 2022)

9. Warschburger, P. The Unhappy Obese Child. Int. J. Obes. 2005, 29, S127-S129, doi:10.1038/sj.ijo.0803097.

10. Oguoma, V.M.; Coffee, N.T.; Alsharrah, S.; Abu-Farha, M.; Al-Refaei, F.H.; Al-Mulla, F.; Daniel, M. Prevalence of Overweight and Obesity, and Associations with Socio-Demographic Factors in Kuwait. BMC Public Health 2021, 21, 667, doi:10.1186/s12889-021-10692-1.

11. Varela-Moreiras, G.; Ávila, J.M.; Cuadrado, C.; del Pozo, S.; Ruiz, E.; Moreiras, O. Evaluation of Food Consumption and Dietary Patterns in Spain by the Food Consumption Survey: Updated Information. Eur. J. Clin. Nutr. 2010, 64, S37-S43, doi:10.1038/ejcn.2010.208.

12. Hawkes, C.; Smith, T.G.; Jewell, J.; Wardle, J.; Hammond, R.A.; Friel, S.; Thow, A.M.; Kain, J. Smart Food Policies for Obesity Prevention. The Lancet 2015, 385, 2410-2421, doi:10.1016/S0140-6736(14)61745-1.

13. Rao, M.; Afshin, A.; Singh, G.; Mozaffarian, D. Do Healthier Foods and Diet Patterns Cost More than Less Healthy Options? A Systematic Review and Meta-Analysis. BMJ Open 2013, 3, e004277, doi:10.1136/bmjopen-2013-004277.

14. Barosh, L.; Friel, S.; Engelhardt, K.; Chan, L. The Cost of a Healthy and Sustainable Diet--Who Can Afford It? Aust. N. Z. J. Public Health 2014, 38, 7-12, doi:10.1111/1753-6405.12158.

15. Imamura, F.; O'Connor, L.; Ye, Z.; Mursu, J.; Hayashino, Y.; Bhupathiraju, S.N.; Forouhi, N.G. Consumption of Sugar Sweetened Beverages, Artificially Sweetened Beverages, and Fruit Juice and Incidence of Type 2 Diabetes: Systematic Review, Meta-Analysis, and Estimation of Population Attributable Fraction. BMJ 2015, 351, h3576, doi:10.1136/bmj.h3576.

16. Popkin, B.M. The Nutrition Transition in Low-Income Countries: An Emerging Crisis. Nutr. Rev. 1994, 52, 285-298, doi:10.1111/j.1753-4887.1994.tb01460.x.

17. Paeratakul, S.; Popkin, B.M.; Keyou, G.; Adair, L.S.; Stevens, J. Changes in Diet and Physical Activity Affect the Body Mass Index of Chinese Adults. Int. J. Obes. Relat. Metab. Disord. J. Int. Assoc. Study Obes. 1998, 22, 424-431, doi:10.1038/sj.ijo.0800603.

18. Popkin, B.M. Urbanization, Lifestyle Changes and the Nutrition Transition. World Dev. 1999, 27, 1905-1916, doi:10.1016/S0305-750X(99)00094-7.

19. Bermudez, O.I.; Gao, X.; Valdes, V. Nutrition Transition in Panama: Adult Overweight and Obesity Are Linked to Urbanization, Ethnicity and Poverty Status. FASEB J. 2008, 22, 866.5-866.5, doi:10.1096/fasebj.22.1_supplement.866.5.

20. Royo-Bordonada, M.Á.; Rodríguez-Artalejo, F.; Bes-Rastrollo, M.; Fernández-Escobar, C.; González, C.A.; Rivas, F.; Martínez-González, M.Á.; Quiles, J.; Bueno-Cavanillas, A.; Navarrete-Muñoz, E.M.; et al., Políticas Alimentarias Para Prevenir La Obesidad y Las Principales Enfermedades No Transmisibles En España: Querer Es Poder. Gac. Sanit. 2019, 33, 584-592, doi:10.1016/j.gaceta.2019.05.009.

21. Du Toit, G.; Foong, R.-X.; Lack, G. The Role of Dietary Interventions in the Prevention of IgE-Mediated Food Allergy in Children. Pediatr. Allergy Immunol. 2017, 28, 222-229, doi:10.1111/pai.12711.

22. NIAID-Sponsored Expert Panel; Boyce, J.A.; Assa'ad, A.; Burks, A.W.; Jones, S.M.; Sampson, H.A.; Wood, R.A.; Plaut, M.; Cooper, S.F.; Fenton, M.J.; et al., Guidelines for the Diagnosis and Management of Food Allergy in the United States: Report of the NIAID-Sponsored Expert Panel. J. Allergy Clin. Immunol. 2010, 126, S1-58, doi:10.1016/j.jaci.2010.10.007.

23. Sierra, M.; Hernanz, N.; Alonso, I.G. y L. Enfermedad celiaca. Medicine (Baltimore) 2020, 13, 9-15, doi:10.1016/j.med.2020.01.002.

24. Hoffmanová, I.; Sánchez, D.; Tučková, L.; Tlaskalová-Hogenová, H. Celiac Disease and Liver Disorders: From Putative Pathogenesis to Clinical Implications. Nutrients 2018, 10, 892, doi:10.3390/nu10070892.

25. Alhassan, E.; Yadav, A.; Kelly, C.P.; Mukherjee, R. Novel Nondietary Therapies for Celiac Disease. Cell. Mol. Gastroenterol. Hepatol. 2019, 8, 335-345, doi:10.1016/j.jcmgh.2019.04.017.

26. Lee, G.J.; Kao, J.Y. Recent Advances in Pediatric Celiac Disease. Expert Rev. Gastroenterol. Hepatol. 2017, 11, 583-592, doi:10.1080/17474124.2017.1309288.

27. Zhao, W.; Ho, H.; Bunyavanich, S. The Gut Microbiome in Food Allergy. Ann. Allergy. Asthma. Immunol. 2019, 122, 276-282, doi:10.1016/j.anai.2018.12.012.

28. Ordovas, J.M.; Ferguson, L.R.; Tai, E.S.; Mathers, J.C. Personalised Nutrition and Health. BMJ 2018, 361, bmj.k2173, doi:10.1136/bmj.k2173.

29. Hinojosa-Nogueira, D.; Pérez-Burillo, S.; Navajas-Porras, B.; Ortiz-Viso, B.; de la Cueva, S.P.; Lauria, F.; Fatouros, A.; Priftis, K.N.; González-Vigil, V.; Rufián-Henares, J.Á. Development of an Unified Food Composition Database for the European Project "Stance4Health." Nutrients 2021, 13, 4206, doi:10.3390/nu13124206.

30. Ordovas, J.M.; Ferguson, L.R.; Tai, E.S.; Mathers, J.C. Personalised Nutrition and Health. The BMJ 2018, 361, bmj.k2173, doi:10.1136/bmj.k2173.

31. Tremaroli, V.; Bäckhed, F. Functional Interactions between the Gut Microbiota and Host Metabolism. Nature 2012, 489, 242-249, doi:10.1038/nature11552.

32. Bello Gutiérrez, J. Ciencia y Tecnología Culinaria; Díaz de Santos, S.A.; Madrid, Spain, 1998.

33. Rita Garda, María. Técnicas Del Manejo de Los Alimentos; 3a edición.; Eudeba.; Ciudad Autónoma de Buenos Aires, Argentina, 2020.

34. Ramírez-Anaya, J.D.P.; Samaniego-Sánchez, C.; Castañeda-Saucedo, M.C.; Villalón-Mir, M.; de la Serrana, H.L.-G. Phenols and the Antioxidant Capacity of Mediterranean Vegetables Prepared with Extra Virgin Olive Oil Using Different Domestic Cooking Techniques. Food Chem. 2015, 188, 430-438, doi:10.1016/j.foodchem.2015.04.124.

35. Nooshkam, M.; Varidi, M.; Verma, D.K. Functional and Biological Properties of Maillard Conjugates and Their Potential Application in Medical and Food: A Review. Food Res. Int. Ott. Ont 2020, 131, 109003, doi:10.1016/j.foodres.2020.109003.

36. Voyer, L.E.; Alvarado, C. Reacción de Maillard: Efectos Patogénicos. Med. B. Aires 2019, 79, 137-143.

37. Hwang, I.G.; Kim, H.Y.; Woo, K.S.; Lee, J.; Jeong, H.S. Biological Activities of Maillard Reaction Products (MRPs) in a Sugar-Amino Acid Model System. Food Chem. 2011, 126, 221-227, doi:10.1016/j.foodchem.2010.10.103.

38. Koschinsky, T.; He, C.J.; Mitsuhashi, T.; Bucala, R.; Liu, C.; Buenting, C.; Heitmann, K.; Vlassara, H. Orally Absorbed Reactive Glycation Products (Glycotoxins): An Environmental Risk Factor in Diabetic Nephropathy. Proc. Natl. Acad. Sci. U. S. A. 1997, 94, 6474-6479, doi:10.1073/pnas.94.12.6474.

39. Chuyen, N.V. Maillard Reaction and Food Processing. Application Aspects. Adv. Exp. Med. Biol. 1998, 434, 213-235, doi:10.1007/978-1-4899-1925-0_18.

40. Hodge, J.E. The Amadori Rearrangement. In Advances in Carbohydrate Chemistry; Wolfrom, M.L., Ed.; Academic Press, 1955; Vol. 10, pp. 169-205.

41. Zhang, Q.; Ames, J.M.; Smith, R.D.; Baynes, J.W.; Metz, T.O. A Perspective on the Maillard Reaction and the Analysis of Protein Glycation by Mass Spectrometry: Probing the Pathogenesis of Chronic Disease. J. Proteome Res. 2009, 8, 754-769, doi:10.1021/pr800858h.

42. Ledl, F.; Schleicher, E. New Aspects of the Maillard Reaction in Foods and in the Human Body. Angew. Chem. Int. Ed. Engl. 1990, 29, 565-594, doi:10.1002/anie.199005653.

43. Friedman, M. Food Browning and Its Prevention: An Overview. J. Agric. Food Chem. 1996, 44, 631-653, doi:10.1021/jf950394r.

44. Henle, T. Protein-Bound Advanced Glycation Endproducts (AGEs) as Bioactive Amino Acid Derivatives in Foods. Amino Acids 2005, 29, 313-322, doi:10.1007/s00726-005-0200-2.

45. Clark, A.V.; Tannenbaum, S.R. Isolation and Characterization of Pigments from Protein-Carbonyl Browning Systems. Isolation, Purification and Properties. J. Agric. Food Chem. 1970, 18, 891-894.

46. Lertittikul, W.; Benjakul, S.; Tanaka, M. Characteristics and Antioxidative Activity of Maillard Reaction Products from a Porcine Plasma Protein-Glucose Model System as Influenced by PH. Food Chem. 2007, 2, 669-677, doi:10.1016/j.foodchem.2005.09.085.

47. Maillard, M.N.; Billaud, C.; Chow, Y.N.; Ordonaud, C.; Nicolas, J. Free Radical Scavenging, Inhibition of Polyphenoloxidase Activity and Copper Chelating Properties of Model Maillard Systems. Food Sci. Technol. 2007.

48. Labuza, T.P.; Saltmarch, M. Kinetics of Browning and Protein Quality Loss in Whey Powders During Steady State and Nonsteady State Storage Conditions. J. Food Sci. 1982, 47, 92-96, doi:10.1111/j.1365-2621.1982.tb11035.x.

49. Ames, J.M. Control of the Maillard Reaction in Food Systems. Trends Food Sci. Technol. 1990, 1, 150-154, doi:10.1016/0924-2244(90)90113-D.

50. Loncin, M.; Bimbenet, J.J.; Lenges, J. Influence of the Activity of Water on the Spoilage of Foodstuffs. Int. J. Food Sci. Technol. 1968, 3, 131-142, doi:10.1111/j.1365-2621.1968.tb01448.x.

51. Kato, H.; Hayase, F.; Shin, D.B.; Oimomi, M.; Baba, S. 3-Deoxyglucosone, an Intermediate Product of the Maillard Reaction. Prog. Clin. Biol. Res. 1989, 304, 69-84.

52. Schwartz, H.M.; Lea, C.H. The Reaction between Proteins and Reducing Sugars in the `dry' State. Relative Reactivity of the α- and ∈-Amino Groups of Insulin. Biochem. J. 1952, 50, 713-716.

53. Wolfrom, M.; Rooney, C. Chemical Interactions of Amino Compounds and Sugars. VIII. Influence of Water - Correction. J. Am. Chem. Soc. 1953, 75, 6363-6363, doi:10.1021/ja01120a664.

54. Labuza, T.P.; Tannenbaum, S.R.; Karel, M. Water Content and Stability of Low-Moisture & Intermediate-Moisture Foods. Food Technol. 1970.

55. Eichner, K.; Karel, M. Influence of Water Content and Water Activity on the Sugar-Amino Browning Reaction in Model Systems under Various Conditions. J. Agric. Food Chem. 1972, 20, 218-223, doi:10.1021/jf60180a025.

56. O'Brien, J.; Morrissey, P.A. Metal Ion Complexation by Products of the Maillard Reaction. Food Chem. 1997, 58, 17-27, doi:10.1016/S0308-8146(96)00162-8.

57. Erbersdobler, H.F.; Dehn, B.; Nangpal, A.; Reuter, H. Determination of furosine in heated milk as a measure of heat intensity during processing. J. Dairy Res. 1987, 54, 147-151, doi:10.1017/S0022029900025255.

58. Purlis, E. Browning Development in Bakery Products - A Review. J. Food Eng. 2010, 99, 239-249, doi:10.1016/j.jfoodeng.2010.03.008.

59. Carabasa-Giribet, M.; Ibarz-Ribas, A. Kinetics of Colour Development in Aqueous Glucose Systems at High Temperatures. J. Food Eng. 2000, 44, 181-189.

60. Kwon, S.Y.; Baek, H.H. Effects of Temperature, PH, Organic Acids, and Sulfites on Tagatose Browning in Solutions during Processing and Storage. Food Sci. Biotechnol. 2014, 23, 677-684, doi:10.1007/s10068-014-0092-6.

61. Namiki, Mitsuo.; Oka, Michiko.; Otsuka, Miki.; Miyazawa, Teruo.; Fujimoto, Kenshiro.; Namiki, Kazuko. Weak Chemiluminescence at an Early Stage of the Maillard Reaction. J. Agric. Food Chem. 1993, 41, 1704-1709, doi:10.1021/jf00034a035.

62. Ajandouz, E.H.; Puigserver, A. Nonenzymatic Browning Reaction of Essential Amino Acids: Effect of PH on Caramelization and Maillard Reaction Kinetics. J. Agric. Food Chem. 1999, 47, 1786-1793, doi:10.1021/jf980928z.

63. Lea, C.H.; Hannan, R.S. Studies of the Reaction between Proteins and Reducing Sugars in the "Dry" State: I. The Effect of Activity of Water, of PH and of Temperature on the Primary Reaction between Casein and Glucose. Biochim. Biophys. Acta 1949, 3, 313-325, doi:10.1016/0006-3002(49)90100-6.

64. Song, P.-S.; Chichester, C.O. Kinetic Behavior and Mechanism of Inhibition in the Maillard Reaction. III. Kinetic Behavior of the Inhibition in the Reaction Between D-Glucose and Glycine. J. Food Sci. 1967, 32, 98-106, doi:10.1111/j.1365-2621.1967.tb01967.x.

65. Powell, R.C.T.; Spark, A.A. Effects of Zirconium and Aluminium Compounds and PH on the Maillard Reaction. J. Sci. Food Agric. 1971, 22, 596-599, doi:10.1002/jsfa.2740221110.

66. Oliver, C.M.; Melton, L.D.; Stanley, R.A. Creating Proteins with Novel Functionality via the Maillard Reaction: A Review. Crit. Rev. Food Sci. Nutr. 2006, 46, 337-350, doi:10.1080/10408690590957250.

67. Lee, C.M.; Lee, T.-C.; Chichester, C.O. Kinetics of the Production of Biologically Active Maillard Browned Products in Apricot and Glucose-L-Tryptophan. J. Agric. Food Chem. 1979, 27, 478-482, doi:10.1021/jf60223a034.

68. Nacka, F.; Chobert, J.-M.; Burova, T.; Léonil, J.; Haertlé, T. Induction of New Physicochemical and Functional Properties by the Glycosylation of Whey Proteins. J. Protein Chem. 1998, 17, 495-503, doi:10.1023/A:1022530904218.

69. Prabhakaram, M.; Ortwerth, B.J. Determination of Glycation Crosslinking by the Sugar-Dependent Incorporation of [14C] Lysine into Protein. Anal. Biochem. 1994, 216, 305-312, doi:10.1006/abio.1994.1046.

70. Bunn, H.F.; Higgins, P.J. Reaction of Monosaccharides with Proteins: Possible Evolutionary Significance. Science 1981, 213, 222-224, doi:10.1126/science.12192669.

71. Yeboah, F.K.; Alli, I.; Yaylayan, V.A. Reactivities of D-Glucose and D-Fructose during Glycation of Bovine Serum Albumin. J. Agric. Food Chem. 1999, 47, 3164-3172, doi:10.1021/jf981289v.

72. Montgomery, M.W.; Day, E.A. Aldehyde-Amine Condensation Reaction: A Possible Fate of Carbonyls in Foods. J. Food Sci. 1965, 30, 828-832, doi:10.1111/j.1365-2621.1965.tb01849.x.

73. Warmbier, H.C.; Schnickels, R.A.; Labuza, T.P. Nonenzymatic Browning Kinetics in an Intermediate Moisture Model System: Effect of Glucose to Lysine Ratio. J. Food Sci. 1976, 41, 981-983, doi:10.1111/j.1365-2621.1976.tb14372.x.

74. Ferrer, E.; Alegría, A.; Farré, R.; Abellán, P.; Romero, F. Revision: Indicadores Del Deterioro de La Calidad Proteica y Del Valor Nutritivo de La Leche / Review: Indicators of Damage of Protein Quality and Nutritional Value of Milk. Food Sci. Technol. Int. 1999.

75. Li, Y.; Wu, Y.; Quan, W.; Jia, X.; He, Z.; Wang, Z.; Adhikari, B.; Chen, J.; Zeng, M. Quantitation of Furosine, Furfurals, and Advanced Glycation End Products in Milk Treated with Pasteurization and Sterilization Methods Applicable in China. Food Res. Int. Ott. Ont 2021, 140, 110088, doi:10.1016/j.foodres.2020.110088.

76. Olano, A.; Santa-Maria, G.; Corzo, N.; Calvo, M.M.; Martinez-Castro, I.; Jimeno, M.L. Determination of Free Carbohydrates and Amadori Compounds Formed at the Early Stages of Non-Enzymic Browning. Food Chem. U. K. 1992.

77. Resmini, P.; Pellegrino, L.; Battelli, G. Accurate Quantification of Furosine in Milk and Dairy Products by a Direct HPLC Method. Ital. J. Food Sci. 1990, 2, 173-183.

78. Delgado, T.; Corzo, N.; Santa Maria, G.; Jimeno, M.L.; Olano, A. Determination of furosine in milk samples by ion-pair reversed phase liquid chromatography. Chromatographia 1992, 33, 374-376, doi:10.1007/bf02275921.

79. Abraham, K.; Gürtler, R.; Berg, K.; Heinemeyer, G.; Lampen, A.; Appel, K.E. Toxicology and Risk Assessment of 5-Hydroxymethylfurfural in Food. Mol. Nutr. Food Res. 2011, 55, 667-678, doi:10.1002/mnfr.201000564.

80. Garcia-Villanova, Belen.; Guerra-Hernandez, Eduardo.; Martinez-Gomez, Elena.; Montilla, Javier. Liquid Chromatography for the Determination of 5-(Hydroxymethyl)-2-Furaldehyde in Breakfast Cereals. J. Agric. Food Chem. 1993, 41, 1254-1255, doi:10.1021/jf00032a017.

81. van Boekel, M. a. J.S. Formation of Flavour Compounds in the Maillard Reaction. Biotechnol. Adv. 2006, 24, 230-233, doi:10.1016/j.biotechadv.2005.11.004.

82. Rufian-Henares, J.A.; Garcia-Villanova, B.; Guerra-Hernandez, E. Determination of Furfural Compounds in Enteral Formula. J. Liq. Chromatogr. Relat. Technol. 2001, 24, 3049-3061, doi:10.1081/JLC-100107356.

83. Lee, H. s.; Nagy, S. Quality Changes and Nonenzymic Browning Intermediates in Grapefruit Juice During Storage. J. Food Sci. 1988, 53, 168-172, doi:10.1111/j.1365-2621.1988.tb10201.x.

84. Albalá-Hurtado, S.; Veciana-Nogués, M.T.; Izquierdo-Pulido, M.; Vidal-Carou, M.C. Determination of Free and Total Furfural Compounds in Infant Milk Formulas by High-Performance Liquid Chromatography. J. Agric. Food Chem. 1997, 45, 2128-2133, doi:10.1021/jf960770n.

85. Gutiérrez, V.; R, J. Daño Oxidativo, Radicales Libres y Antioxidantes. Rev. Cuba. Med. Mil. 2002, 31, 126-133.

86. Cheeseman, K.H.; Slater, T.F. An Introduction to Free Radical Biochemistry. Br. Med. Bull. 1993, 49, 481-493, doi:10.1093/oxfordjournals.bmb.a072625.

87. Halliwell, B. Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. Plant Physiol. 2006, 141, 312-322, doi:10.1104/pp.106.077073.

88. Finkel, T.; Holbrook, N.J. Oxidants, Oxidative Stress and the Biology of Ageing. Nature 2000, 408, 239-247, doi:10.1038/35041687.

89. Seifried, H.E.; Anderson, D.E.; Fisher, E.I.; Milner, J.A. A Review of the Interaction among Dietary Antioxidants and Reactive Oxygen Species. J. Nutr. Biochem. 2007, 18, 567-579, doi:10.1016/j.jnutbio.2006.10.007.

90. Aruoma, O.I. Methodological Considerations for Characterizing Potential Antioxidant Actions of Bioactive Components in Plant Foods. Mutat. Res. 2003, 523-524, 9-20, doi:10.1016/s0027-5107(02)00317-2.

91. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M.T.D.; Mazur, M.; Telser, J. Free Radicals and Antioxidants in Normal Physiological Functions and Human Disease. Int. J. Biochem. Cell Biol. 2007, 39, 44-84, doi:10.1016/j.biocel.2006.07.001.

92. Liu, R.H. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. J. Nutr. 2004, 134, 3479S-3485S, doi:10.1093/jn/134.12.3479S.

93. Masella, R.; Di Benedetto, R.; Varì, R.; Filesi, C.; Giovannini, C. Novel Mechanisms of Natural Antioxidant Compounds in Biological Systems: Involvement of Glutathione and Glutathione-Related Enzymes. J. Nutr. Biochem. 2005, 16, 577-586, doi:10.1016/j.jnutbio.2005.05.013.

94. Hicks, J.J.; Torres-Ramos, Y.D.; Sierra-Vargas, M.P. Estrés oxidante. Concepto y clasificación. Rev. Endocrinol. Nutr. 2006, 14, 223-226.

95. Laguerre, M.; Lecomte, J.; Villeneuve, P. Evaluation of the Ability of Antioxidants to Counteract Lipid Oxidation: Existing Methods, New Trends and Challenges. Prog. Lipid Res. 2007, 46, 244-282, doi:10.1016/j.plipres.2007.05.002.

96. Londoño Londoño, J.A. Antioxidantes: importancia biológica y métodos para medir su actividad. In Desarrollo y transversalidad serie Lasallista Investigación y Ciencia; Corporación Universitaria Lasallista, 2012; pp. 129-162.

97. Ugartondo Casadevall, V. Caracterización de derivados polifenólicos obtenidos de fuentes naturales. Citotoxicidad y capacidad antioxidante frente a estrés oxidativo en modelos celulares. Ph.D. Thesis, Universitat de Barcelona, 2009.

98. Willcox, J.K.; Ash, S.L.; Catignani, G.L. Antioxidants and Prevention of Chronic Disease. Crit. Rev. Food Sci. Nutr. 2004, 44, 275-295, doi:10.1080/10408690490468489.

99. Pacifici, R.E.; Davies, K.J. Protein, Lipid and DNA Repair Systems in Oxidative Stress: The Free-Radical Theory of Aging Revisited. Gerontology 1991, 37, 166-180, doi:10.1159/000213257.

100. Pérez-Jiménez, J.; Saura-Calixto, F. Effect of Solvent and Certain Food Constituents on Different Antioxidant Capacity Assays. Food Res. Int. 2006, 39, 791-800, doi:10.1016/j.foodres.2006.02.003.

101. Naczk, M.; Shahidi, F. Phenolics in Cereals, Fruits and Vegetables: Occurrence, Extraction and Analysis. J. Pharm. Biomed. Anal. 2006, 41, 1523-1542, doi:10.1016/j.jpba.2006.04.002.

102. Balasundram, N.; Sundram, K.; Samman, S. Phenolic Compounds in Plants and Agri-Industrial by-Products: Antioxidant Activity, Occurrence, and Potential Use. Food Chem. 2006, 99, 191-203, doi:10.1016/j.foodchem.2005.07.042.

103. Mariutti, L.R.B.; Barreto, G.P. de M.; Bragagnolo, N.; Mercadante, A.Z. Free radical scavenging activity of ethanolic extracts from herbs and spices commercialized in Brazil. Braz. Arch. Biol. Technol. 2008, 51, doi:10.1590/s1516-89132008000600018.

104. Dorman, H.J.D.; Hiltunen, R. Fe(III) Reductive and Free Radical-Scavenging Properties of Summer Savory (Satureja Hortensis L.) Extract and Subfractions. Food Chem. 2004, 88, 193-199, doi:10.1016/j.foodchem.2003.12.039.

105. Meléndez-Martínez, A.J.; Vicario, I.M.; Heredia, F.J. Estabilidad de los pigmentos carotenoides en los alimentos. Arch. Latinoam. Nutr. 2004, 54, 209-215.

106. Namitha, K.K.; Negi, P.S. Chemistry and Biotechnology of Carotenoids. Crit. Rev. Food Sci. Nutr. 2010, 50, 728-760, doi:10.1080/10408398.2010.499811.

107. Mandl, J.; Szarka, A.; Bánhegyi, G. Vitamin C: Update on Physiology and Pharmacology. Br. J. Pharmacol. 2009, 157, 1097-1110, doi:10.1111/j.1476-5381.2009.00282.x.

108. Yen, G.-C.; Duh, P.-D.; Tsai, H.-L. Antioxidant and Pro-Oxidant Properties of Ascorbic Acid and Gallic Acid. Food Chem. 2002, 79, 307-313, doi:10.1016/S0308-8146(02)00145-0.

109. Gómez, A.S.; Marín, M.I.; López, R.A.; Millán, M.T.M. Vitamina E y aceites vegetales. Grasas Aceites 2007, 58, 74-86.

110. Burton, G.W.; Ingold, K.U.; Cheeseman, K.H.; Slater, T.F. Application of Deuterated Alpha-Tocopherols to the Biokinetics and Bioavailability of Vitamin E. Free Radic. Res. Commun. 1990, 11, 99-107, doi:10.3109/10715769009109672.

111. Tiwari, U.; Cummins, E. Nutritional Importance and Effect of Processing on Tocols in Cereals. Trends Food Sci. Technol. 2009, 20, 511-520, doi:10.1016/j.tifs.2009.06.001.

112. Chen, X.-M.; Kitts, D.D. Correlating Changes That Occur in Chemical Properties with the Generation of Antioxidant Capacity in Different Sugar-Amino Acid Maillard Reaction Models. J. Food Sci. 2011, 76, C831-837, doi:10.1111/j.1750-3841.2011.02215.x.

113. Bressa, F.; Tesson, N.; Dalla Rosa, M.; Sensidoni, A.; Tubaro, F. Antioxidant Effect of Maillard Reaction Products: Application to a Butter Cookie of a Competition Kinetics Analysis. J. Agric. Food Chem. USA 1996.

114. Yen, W.-J.; Wang, B.-S.; Chang, L.-W.; Duh, P.-D. Antioxidant Properties of Roasted Coffee Residues. J. Agric. Food Chem. 2005, 53, 2658-2663, doi:10.1021/jf0402429.

115. Wijewickreme, A. n.; Kitts, D. d. Oxidative Reactions of Model Maillard Reaction Products and α-Tocopherol in a Flour-Lipid Mixture. J. Food Sci. 1998, 63, 466-471, doi:10.1111/j.1365-2621.1998.tb15765.x.

116. Bravo, A.; Araujo, S.; Vargas, M.E.; Mesa, J.; Souki, A.; Bermúdez, V.; Cano, C. Actividad de la enzima antioxidante superóxido dismutasa y niveles de cobre y zinc en pacientes con diabetes mellitus tipo 2. Arch. Venez. Farmacol. Ter. 2007, 26, 37-41.

117. Turi, J.L.; Yang, F.; Garrick, M.D.; Piantadosi, C.A.; Ghio, A.J. The Iron Cycle and Oxidative Stress in the Lung. Free Radic. Biol. Med. 2004, 36, 850-857, doi:10.1016/j.freeradbiomed.2003.12.008.

118. Molina, E. El papel de los antioxidantes como desaceleradores del envejecimiento. ReNut 2012, 1109-1119.

119. Prohaska, J.R. Biochemical Changes in Copper Deficiency. J. Nutr. Biochem. 1990, 1, 452-461, doi:10.1016/0955-2863(90)90080-5.

120. Tinggi, U. Selenium: Its Role as Antioxidant in Human Health. Environ. Health Prev. Med. 2008, 13, 102-108, doi:10.1007/s12199-007-0019-4.

121. Duthie, G.G.; Gardner, P.T.; Kyle, J.A.M. Plant Polyphenols: Are They the New Magic Bullet? Proc. Nutr. Soc. 2003, 62, 599-603, doi:10.1079/PNS2003275.

122. Khokhar, S.; Owusu Apenten, Richard.K. Iron Binding Characteristics of Phenolic Compounds: Some Tentative Structure-Activity Relations. Food Chem. 2003, 81, 133-140, doi:10.1016/S0308-8146(02)00394-1.

123. Lee, H.C.; Jenner, A.M.; Low, C.S.; Lee, Y.K. Effect of Tea Phenolics and Their Aromatic Fecal Bacterial Metabolites on Intestinal Microbiota. Res. Microbiol. 2006, 157, 876-884, doi:10.1016/j.resmic.2006.07.004.

124. Harborne, J.B. Phenolic Compounds. In Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis; Harborne, J.B., Ed.; Springer Netherlands: Dordrecht, 1973; pp. 33-88.

125. D'Archivio, M.; Filesi, C.; Di Benedetto, R.; Gargiulo, R.; Giovannini, C.; Masella, R. Polyphenols, Dietary Sources and Bioavailability. Ann. Ist. Super. Sanita 2007, 43, 348-361.

126. Fernández-Pachón, M.S.; Berná, G.; Otaolaurruchi, E.; Troncoso, A.M.; Martín, F.; García-Parrilla, M.C. Changes in Antioxidant Endogenous Enzymes (Activity and Gene Expression Levels) after Repeated Red Wine Intake. J. Agric. Food Chem. 2009, 57, 6578-6583, doi:10.1021/jf901863w.

127. Sun, A.Y.; Wang, Q.; Simonyi, A.; Sun, G.Y. Botanical Phenolics and Brain Health. Neuromolecular Med. 2008, 10, 259-274, doi:10.1007/s12017-008-8052-z.

128. Roginsky, V.; Lissi, E.A. Review of Methods to Determine Chain-Breaking Antioxidant Activity in Food. Food Chem. 2005, 92, 235-254, doi:10.1016/j.foodchem.2004.08.004.

129. Gorinstein, S.; Haruenkit, R.; Poovarodom, S.; Vearasilp, S.; Ruamsuke, P.; Namiesnik, J.; Leontowicz, M.; Leontowicz, H.; Suhaj, M.; Sheng, G.P. Some Analytical Assays for the Determination of Bioactivity of Exotic Fruits. Phytochem. Anal. PCA 2010, 21, 355-362, doi:10.1002/pca.1207.

130. Díaz-García, M.C.; Obón, J.M.; Castellar, M.R.; Collado, J.; Alacid, M. Quantification by UHPLC of Total Individual Polyphenols in Fruit Juices. Food Chem. 2013, 138, 938-949, doi:10.1016/j.foodchem.2012.11.061.

131. Huang, D.; Ou, B.; Prior, R.L. The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem. 2005, 53, 1841-1856, doi:10.1021/jf030723c.

132. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radic. Biol. Med. 1999, 26, 1231-1237, doi:10.1016/s0891-5849(98)00315-3.

133. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. LWT - Food Sci. Technol. 1995, 28, 25-30, doi:10.1016/S0023-6438(95)80008-5.

134. Benzie, I.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal. Biochem. 1996, 239, 70-76, doi:10.1006/abio.1996.0292.

135. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 1965, 16, 144-158.

136. Magalhães, L.M.; Segundo, M.A.; Reis, S.; Lima, J.L.F.C. Methodological Aspects about in Vitro Evaluation of Antioxidant Properties. Anal. Chim. Acta 2008, 613, 1-19, doi:10.1016/j.aca.2008.02.047.

137. Prior, R.L.; Wu, X.; Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. J. Agric. Food Chem. 2005, 53, 4290-4302, doi:10.1021/jf0502698.

138. Tomás-Barberán, F.A.; Espín, J.C. Phenolic Compounds and Related Enzymes as Determinants of Quality in Fruits and Vegetables. J. Sci. Food Agric. 2001, 81, 853-876, doi:10.1002/jsfa.885.

139. Garcia-Sartal, C.; Taebunpakul, S.; Stokes, E.; Barciela-Alonso, M. del C.; Bermejo-Barrera, P.; Goenaga-Infante, H. Two-Dimensional HPLC Coupled to ICP-MS and Electrospray Ionisation (ESI)-MS/MS for Investigating the Bioavailability in Vitro of Arsenic Species from Edible Seaweed. Anal. Bioanal. Chem. 2012, 402, 3359-3369, doi:10.1007/s00216-011-5483-4.

140. Jordana-Lluch, E.; Martró Català, E.; Ausina Ruiz, V. La espectrometría de masas en el laboratorio de microbiología clínica. Enfermedades Infecc. Microbiol. Clínica 2012, 30, 635-644, doi:10.1016/j.eimc.2012.01.012.

141. Lagier, J.-C.; Khelaifia, S.; Alou, M.T.; Ndongo, S.; Dione, N.; Hugon, P.; Caputo, A.; Cadoret, F.; Traore, S.I.; Seck, E.H.; et al., Culture of Previously Uncultured Members of the Human Gut Microbiota by Culturomics. Nat. Microbiol. 2016, 1, 16203, doi:10.1038/nmicrobiol.2016.203.

142. Keeney, K.M.; Yurist-Doutsch, S.; Arrieta, M.-C.; Finlay, B.B. Effects of Antibiotics on Human Microbiota and Subsequent Disease. Annu. Rev. Microbiol. 2014, 68, 217-235, doi:10.1146/annurev-micro-091313-103456.

143. Selber-Hnatiw, S.; Rukundo, B.; Ahmadi, M.; Akoubi, H.; Al-Bizri, H.; Aliu, A.F.; Ambeaghen, T.U.; Avetisyan, L.; Bahar, I.; Baird, A.; et al., Human Gut Microbiota: Toward an Ecology of Disease. Front. Microbiol. 2017, 8, 1265, doi:10.3389/fmicb.2017.01265.

144. gentil, C.L.; Weir, T.L. The Gut Microbiota at the Intersection of Diet and Human Health. Science 2018, 362, 776-780, doi:10.1126/science.aau5812.

145. Icaza-Chávez, M.E. Microbiota intestinal en la salud y la enfermedad. Rev. Gastroenterol. México 2013, 78, 240-248, doi:10.1016/j.rgmx.2013.04.004.

146. Jandhyala, S.M.; Talukdar, R.; Subramanyam, C.; Vuyyuru, H.; Sasikala, M.; Reddy, D.N. Role of the Normal Gut Microbiota. World J. Gastroenterol. WJG 2015, 21, 8787-8803, doi:10.3748/wjg.v21.i29.8787.

147. Macfarlane, S.; Macfarlane, G.T. Regulation of Short-Chain Fatty Acid Production. Proc. Nutr. Soc. 2003, 62, 67-72, doi:10.1079/PNS2002207.

148. Sartor, R.B. Microbial Influences in Inflammatory Bowel Diseases. Gastroenterology 2008, 134, 577-594, doi:10.1053/j.gastro.2007.11.059.

149. Rowland, I.; Gibson, G.; Heinken, A.; Scott, K.; Swann, J.; Thiele, I.; Tuohy, K. Gut Microbiota Functions: Metabolism of Nutrients and Other Food Components. Eur. J. Nutr. 2018, 57, 1-24, doi:10.1007/s00394-017-1445-8.

150. Etxeberria, U.; Milagro, F.I.; González-Navarro, C.J.; Martínez, J.A. Role of gut microbiota in obesity. An Real Acad Farm. 2016, 26 234-259.

151. Sánchez Almaraz, R.; Martín Fuentes, M.; Palma Milla, S.; López Plaza, B.; Bermejo López, L.M.; Gómez Candela, C. Indicaciones de Diferentes Tipos de Fibra En Distintas Patologías. Nutr. Hosp. 2015, 31, 2372-2383, doi:10.3305/nh.2015.31.6.9023.

152. Parada Venegas, D.; De la Fuente, M.K.; Landskron, G.; González, M.J.; Quera, R.; Dijkstra, G.; Harmsen, H.J.M.; Faber, K.N.; Hermoso, M.A. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. Front. Immunol. 2019, 10, 277, doi:10.3389/fimmu.2019.00277.

153. Ochoa, G.M.A.; Giraldo, N.A.G.; Causil, C.J.B.; Duque, B.E.V. Microbiota intestinal y ácidos grasos de cadena corta en pacientes críticos. Perspect. Nutr. Humana 2016, 18, 205-222, doi:10.17533/udea.penh.v18n2a06.

154. Chen, H.; Sang, S. Biotransformation of Tea Polyphenols by Gut Microbiota. J. Funct. Foods 2014, 7, 26-42, doi:10.1016/j.jff.2014.01.013.

155. Kawabata, K.; Yoshioka, Y.; Terao, J. Role of Intestinal Microbiota in the Bioavailability and Physiological Functions of Dietary Polyphenols. Molecules 2019, 24, 370, doi:10.3390/molecules24020370.

156. Liu, A.B.; Tao, S.; Lee, M.-J.; Hu, Q.; Meng, X.; Lin, Y.; Yang, C.S. Effects of Gut Microbiota and Time of Treatment on Tissue Levels of Green Tea Polyphenols in Mice. BioFactors 2018, 44, 348-360, doi:10.1002/biof.1430.

157. Paula, H.; Grover, M.; Halder, S.L.; Locke, G.R.; Schleck, C.D.; Zinsmeister, A.R.; Talley, N.J. Non-Enteric Infections, Antibiotic Use, and Risk of Development of Functional Gastrointestinal Disorders. Neurogastroenterol. Motil. Off. J. Eur. Gastrointest. Motil. Soc. 2015, 27, 1580-1586, doi:10.1111/nmo.12655.

158. Basain Valdés, J.M.; Valdés Alonso, M. del C.; Miyar Pieiga, E.; Linares Valdés, H.; Martínez Izquierdo, A. Alteraciones en la microbiota intestinal por la dieta y su repercusión en la génesis de la obesidad. Medisan 2015, 1536-1546.

159. Ghosh, S. Advances in Our Understanding of the Pathogenesis of Celiac Disease. Can. J. Gastroenterol. 2011, 25, 186.

160. Green, P.H.R.; Cellier, C. Celiac Disease. N. Engl. J. Med. 2007, 357, 1731-1743, doi:10.1056/NEJMra071600.

161. Girón Fernández-Crehuet, F.; Tapia-Paniagua, S.; Moriñigo Gutiérrez, M.A.; Navas-López, V.M.; Juliana Serrano, M.; Blasco-Alonso, J.; Sierra Salinas, C. La composición de la microbiota duodenal en niños con enfermedad celíaca activa está influenciada por el grado de enteropatía. An. Pediatría 2016, 84, 224-230, doi:10.1016/j.anpedi.2015.06.014.

162. Zamudio-Vázquez, V.P.; Ramírez-Mayans, J.A.; Toro-Monjaraz, E.M.; Cervantes-Bustamante, R.; Zárate-Mondragón, F.; Montijo-Barrios, E.; Cadena-León, J.F.; Cázares-Méndez, J.M.; Zamudio-Vázquez, V.P.; Ramírez-Mayans, J.A.; et al., Importancia de la microbiota gastrointestinal en pediatría. Acta Pediátrica México 2017, 38, 49-62, doi:10.18233/apm1no1pp49-621323.

163. Angelakis, E.; Armougom, F.; Million, M.; Raoult, D. The Relationship between Gut Microbiota and Weight Gain in Humans. Future Microbiol. 2012, 7, 91-109, doi:10.2217/fmb.11.142.

164. Brahe, L.K.; Astrup, A.; Larsen, L.H. Can We Prevent Obesity-Related Metabolic Diseases by Dietary Modulation of the Gut Microbiota? Adv. Nutr. Bethesda Md 2016, 7, 90-101, doi:10.3945/an.115.010587.

165. Davila, A.-M.; Blachier, F.; Gotteland, M.; Andriamihaja, M.; Benetti, P.-H.; Sanz, Y.; Tomé, D. Intestinal Luminal Nitrogen Metabolism: Role of the Gut Microbiota and Consequences for the Host. Pharmacol. Res. 2013, 68, 95-107, doi:10.1016/j.phrs.2012.11.005.

166. Suárez-Diéguez, T.; Galván, M.; López-Rodríguez, G.; Olivo, D.; Olvera Najera, M. EFECTO DE LA DIETA SOBRE LA MODULACIÓN DE LA MICROBIOTA EN EL DESARROLLO DE LA OBESIDAD. RESPYN Rev. Salud Pública Nutr. 2018, 17, doi:10.29105/respyn17.1-5.

167. Van Niel, C.W.; Feudtner, C.; Garrison, M.M.; Christakis, D.A. Lactobacillus Therapy for Acute Infectious Diarrhea in Children: A Meta-Analysis. Pediatrics 2002, 109, 678-684, doi:10.1542/peds.109.4.678.

168. Pérez-Burillo, S.; Molino, S.; Navajas-Porras, B.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; López-Maldonado, A.; Pastoriza, S.; Rufián-Henares, J.Á. An in Vitro Batch Fermentation Protocol for Studying the Contribution of Food to Gut Microbiota Composition and Functionality. Nat. Protoc. 2021, 16, 3186-3209, doi:10.1038/s41596-021-00537-x.

169. Venema, K.; van den Abbeele, P. Experimental Models of the Gut Microbiome. Best Pract. Res. Clin. Gastroenterol. 2013, 27, 115-126, doi:10.1016/j.bpg.2013.03.002.

170. Daguet, D.; Pinheiro, I.; Verhelst, A.; Possemiers, S.; Marzorati, M. Arabinogalactan and Fructooligosaccharides Improve the Gut Barrier Function in Distinct Areas of the Colon in the Simulator of the Human Intestinal Microbial Ecosystem. J. Funct. FOODS 2016, 20, 369-se 379, doi:10.1016/j.jff.2015.11.005.

OBJETIVOS

Objetivo general

El objetivo general de esta tesis doctoral consiste en evaluar la capacidad antioxidante de diferentes alimentos de origen vegetal y animal tras ser digeridos y fermentados *in vitro*, para así ver los cambios que se producen sobre la funcionalidad de la microbiota intestinal, y además, estudiar los cambios producidos por el procesado térmico aplicado a dichos alimentos, viendo cómo afectan, tanto a la capacidad antioxidante de los mismos como a la funcionalidad de la microbiota intestinal.

Objetivos específicos

1. Estudiar la capacidad antioxidante de los diferentes alimentos de origen vegetal y animal con diferentes procesados térmicos aplicados tras la digestión y fermentación *in vitro* con microbiota intestinal de adultos sin patologías, niños sin patologías, niños obesos, niños celíacos y niños con alergia a las proteínas de la leche de vaca.

2. Evaluar el consumo diario y por ración, a través del consumo de alimentos, de compuestos con capacidad antioxidante, tanto para adultos como para niños.

3. Comparar la ingesta diaria y por ración de compuestos con capacidad antioxidante entre los distintos grupos de niños.

4. Evaluar el efecto del procesado térmico sobre los alimentos, estudiando indicadores como furosina, HMF y furfural.

5. Correlacionar la capacidad antioxidante de los alimentos tras la digestión y fermentación *in vitro*, con los indicadores de daño térmico anteriormente nombrados.

General objective

The general objective of this doctoral thesis is to evaluate the antioxidant capacity of different foods of plant and animal origin after being digested and fermented *in vitro* in order to unravel the changes they produce on the functionality of the gut microbiota. In addition, to study the changes produced by thermal processing applied to these foods, to decipher how they affect both their antioxidant capacity and the functionality of the gut microbiota.

Specific objectives

1. To study the antioxidant capacity of different foods of plant and animal origin with different thermal processes applied after digestion and *in vitro* fermentation with the gut microbiota of healthy adults, healthy children, obese children, celiac disease children and children with allergy to cow's milk proteins.

2. To evaluate the daily and per serving consumption, through food consumption, of compounds with antioxidant capacity, both for adults and children.

3. To compare the daily and per serving intake of antioxidant capacity among different groups of children.

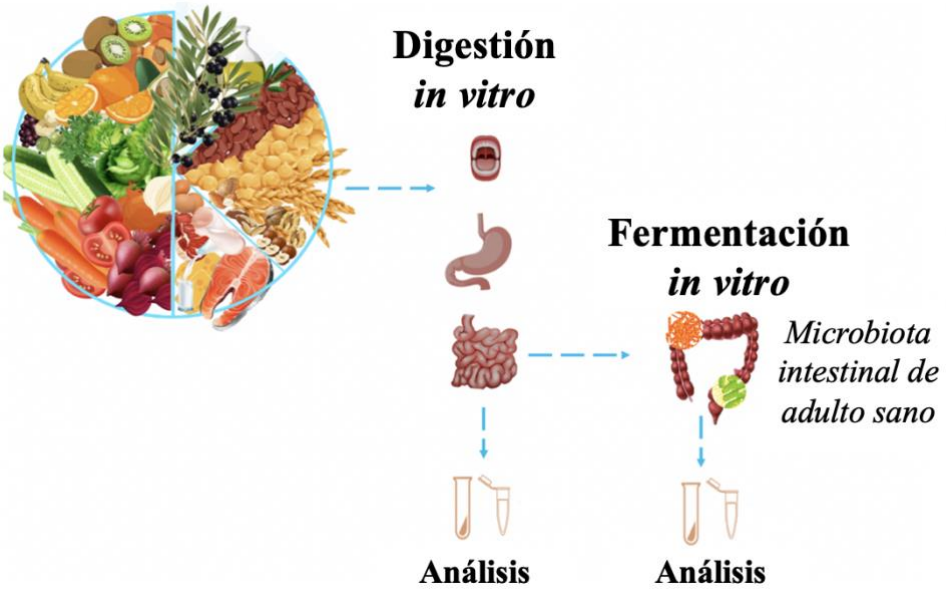
4. To evaluate the effect of thermal processing on food, studying chemical indicators such as furosine, HMF and furfural.

5. To correlate the antioxidant capacity of foods after digestion and *in vitro* fermentation with the indicators of thermal damage mentioned above.

PARTE EXPERIMENTAL

CAPÍTULO I:

Estudio de la funcionalidad de la microbiota intestinal de adultos sanos



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Relationship of quality parameters, antioxidant capacity and total phenolic content of EVOO with ripening state and olive variety

En este trabajo se estudió la evolución de los parámetros de calidad, la composición de ácidos grasos, la capacidad antioxidante y el contenido fenólico total del aceite de oliva virgen extra obtenido a partir de dos variedades de aceituna con diferente tiempo de maduración. Tanto la capacidad antioxidante como el contenido fenólico total se midieron tras someter a las muestras de aceite a digestión y fermentación *in vitro* con microbiota de adulto sano, para acercarnos lo máximo posible a las condiciones fisiológicas. Este fue el primer trabajo de esta tesis que se llevó a cabo con microbiota de adulto sano y en el que estudió la funcionalidad de la misma con dos variedades de aceite diferentes.

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Relationship of quality parameters, antioxidant capacity and total phenolic content of EVOO with ripening state and olive variety

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Abstract

The aim of this work was to study the evolution of quality parameters, fatty acids composition, antioxidant capacity, and total phenolic content during maturation of two different olive cultivars, Manzanilla and Picual. In order to mimic physiological conditions, antioxidant capacity and total phenolic content were measured after submitting olive oil to an *in vitro* digestion followed by an *in vitro* gut microbial fermentation. Quality parameters were always within the legal limits for olive oil to be called Extra Virgin Olive Oil. Antioxidant capacity, total phenolic content, SFA and MUFA decreased during maturation whereas PUFA increased in both cultivars. Manzanilla showed higher PUFA content, whereas Picual had higher MUFA concentration. Antioxidant capacity and total phenolic content was higher in Picual cultivar. On the other hand, the statistical approach demonstrated that, at least involving Manzanilla and Picual, the type of cultivar is more important than collection date regarding fatty acids composition and antioxidant capacity.

Keywords: olive oil, antioxidant capacity, polyphenols, harvest, *in vitro* digestion, *in vitro* fermentation

1. Introduction

Olive oil, especially extra virgin olive oil (EVOO), is the main fat source of the Mediterranean diet with Europe still holding most of the production and, more specifically, Spain [1]. The importance of olive oil reflects on the fact that its nutritional, sensorial, and commercial aspects are regulated by the International Olive Council (IOC), European legislation (EC), and the Codex Alimentarius [1].

Nowadays EVOO consumption is not limited to Europe or the Mediterranean countries but instead it is a highly demanded product around the world [2]. This growing attention is mainly attributed to its health benefits [3]. Those benefits were first linked to its high content in monounsaturated fatty acids (MUFA) but more recently, the unsaponifiable fraction have proven to play a major role in health [4]. This fraction, which accounts for ~1% of the oil, is composed of polyphenolic substances, tocopherols, chlorophylls, alcohols, etc. On the other hand, the increasing demand of EVOO cannot only be attributed to its healthy effects, but also to its organoleptic attributes [2]. These make EVOO clearly distinguishable from other vegetable oils due to its content in aromatic substances among other compounds [5].

All these characteristics that makes EVOO unique are heavily influenced by several agronomic factors, being key two of them: cultivar and, degree of ripening [6]. During ripening, different metabolic pathways are in motion prompting several changes in composition that affects fatty acids, phenolic content, etc. [4]. These changes in composition could also affect quality parameters used to classify olive oils: free acidity and those related to oxidative stability (peroxide value, K_{232} and K_{270}). There are several studies that focused on the evolution of such parameters along ripening of olive

drupes of certain varieties or cultivars. However, there are some contradictory results. For instance, Nikolaos Gougoulias' group studied the cultivar Amfissis, and found that antioxidant capacity and phenolic content increase during ripening until harvest [7]. On the other hand, Vincenzo Sicari found that olives which are not completely ripe give oil with higher content in phenolic compounds for cultivars Ottobratica, Sinopolese and Roggianella [6]. Regarding quality parameters, there is also opposite results depending on the cultivar. Whereas for Gemlik and Halhalı cultivars peroxide values were found to increase during ripening [5], for the Sayali cultivar, results were the other way around [8]. Similar tendencies were found for K_{232} and K_{270}. Fatty acids composition during ripening have also showed to behave differently depending on the cultivar. Sayali cultivar showed an increase on MUFA [8], whereas Azeradj cultivar suffered a decrease in MUFA content during ripening [4].

However, the olive variety could be just as important as the ripening stage, since different varieties can evolve diversely during ripening; therefore the results should not be extrapolated from one variety to another. In order to shed some light on this matter, the aim of this paper was to follow the ripening evolution of quality parameters such as fatty acid composition, antioxidant capacity and total phenolic content of EVOO from two of the most used varieties in Spain, Manzanilla and Picual. In order to get results closer to physiological conditions, the antioxidant capacity and total phenolic content was measured for the first time after submitting EVOO to an *in vitro* digestion followed by *in vitro* colonic fermentation. Finally, calculations were performed in order to check the contribution to daily antioxidant capacity and total phenols provided by the consumption of EVOO in the Spanish population.

2. Materials and methods

2.1 Chemicals

Pancreatin from porcine pancreas was from Alpha Aesar (UK). The rest of the reagents, including salts and enzymes for *in vitro* digestion-fermentation as well as reagents and solvents for the analysis of anti- oxidant capacity were of analytical grade and purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2. Samples

Olives were collected every 15 days for three months (from November 1st, 2018 till February 2nd, 2019) in the region of Montefrío (Granada, Spain), from two different olive (Olea europaea L.) varieties, Manzanilla and Picual. Olives were hand picked (from three olive trees of each variety) and oil extraction was performed under similar industrial conditions using an Abencor system, equipped with a hammer crusher, malaxer and a centrifuge. Prior to oil extraction, olives were manually cleaned and sorted keeping only the healthy ones. Right after, olives were crushed and mixed for 30 min at 25 °C. After that, the obtained paste was centrifuged at 3000 rpm for 10 min. The liquid phase, composed of oil and waste, was allowed to naturally decant to separate the oil. The oil was then stored in bottles and kept away from light until analysis.

2.3. Fatty acids composition. Methylated fatty acids analysis by GC-MS chromatography.

Fatty acids composition was determined according to the method proposed by the International Olive oil Council (IOC) [9], based on converting fatty acids into fatty acids methyl esters in order to make them

more volatile and being able to analyzed them by GC-MS. Derivatization process and GC-MS analysis was carried out as in a previous work [10]. The analyses were realized by using a high-resolution gas chromatograph (model: 7890ª of Agilent (USA)), coupled to a triple quadrupole mass spectrometer (model: Quatto microGC of Waters (USA)).

The GC conditions were as follows: ZB-FAME capillary column (30 mx 0.25 mm ID x 0.25 um film), injector in Split mode (10:1), injector temperature 250 ºC, transfer line temperature 240 ºC, helium carrier gas (1 mL / min), program temperature 100 ºC (2 min) up to 210 ºC (5 min) at 4 º / minutes. The MSD conditions were as follows: source temperature 240 ° C; Fullscan from 45 D to 450 Da; ionization by electronic impact (EI +) at 70 eV.

Fatty acids were identified by comparing the retention time with reference compounds. Analyses was carried out in triplicate.

2.4. Quality parameters

Free acidity, peroxide index, and spectrometric UV at 232 and 270 (K_{232} and K_{270}) were performed according to the methodology described in EEC Regulations 2568/91 and the following amendments [11].

Free acidity was expressed as % of oleic acid, and peroxide index was expressed as meq of active oxygen/ kg of oil. The UV measurements were performed with a spectrophotometer (LS 55, Perkin-Elmer, Waltham, USA) with one-centimeter quartz glass cuvettes (QS-1000 Suprasil, Hellma GmbH & Co, Germany). Analyses were carried out in triplicate.

2.5. In vitro gastrointestinal digestion

All samples used for the analysis of antioxidant capacity and total phenolic content were submitted to an *in vitro* digestion-fermentation in order to mimic physiological processes in the human gut according to the protocol previously described [12]. For the experiment, 5 g of olive oil were submitted, in triplicate, to *in vitro* gastrointestinal digestion followed by *in vitro* fermentation. The *in vitro* fermentation was carried out using fecal samples from five healthy donors (who had not taken antibiotics for three months prior to the assay, with a mean Body Mass Index = 21.3); the fecal samples were pooled together to reduced inter-individual variability. A separate fermentation was performed using only the fecal fermentation solution (composed of peptone, cysteine, and resazurin) as control. The fermentation was carried out at 37 °C for 24 h. After the *in vitro* gastrointestinal digestion and fermentation, two fractions were obtained: digestion supernatant (fraction available for absorption at the small intestine), and fermentation supernatant (fraction available for absorption at the large intestine).

2.6. Antioxidant assays

The antioxidant capacity was measured in the two fractions above described for each oil sample: (i) supernatant obtained after *in vitro* gastrointestinal digestion; (ii) soluble phase (supernatant) obtained after *in vitro* microbial fermentation. The sum of the two fractions represents the total antioxidant capacity of each sample that could exert its reducing-antiradical activity within the human being [13]. The antioxidant capacity was determined using three different methods [14]:

$TEAC_{ABTS}$ assay (Trolox equivalent antioxidant capacity against $ABTS^{\cdot+}$ radicals). It measures the scavenging capacity of samples against the

artificial radical ABTS through the method described in a previous work [15] and adapted to a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Briefly, 20 µL of either digestion or fermentation supernatant were added, in triplicate, to a 96 well plate and mixed with 280 µL ABTS reagent, which was prepared the day before according to the followed protocol [15]. The antioxidant reaction was monitored for 30 min. Calibration curve was prepared with Trolox at a concentration ranging from 0.01 to 0.4 mg/mL. Results were expressed as mmol Trolox Equivalent/kg of oil.

$TEAC_{FRAP}$ 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 [16] and adapted to a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Briefly, 20 µL of either digestion or fermentation supernatant were added, in triplicate, to a 96 well plate and mixed with 280 µL FRAP reagent, which was prepared the day of the experiment according to Benzie and Strain [16]. The antioxidant reaction was monitored for 30 min. Calibration curve was prepared with Trolox at a concentration ranging from 0.01 to 0.4 mg/mL. Results were expressed as mmol Trolox Equivalent/kg of oil.

$TEAC_{DPPH}$ assay (Trolox equivalent antioxidant capacity against DPPH radicals). This method was conducted according to the procedure described by Yen & Chen [17] and adapted to a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Briefly, 20 µL of either digestion or fermentation supernatant were added, in triplicate, to a 96 well plate and mixed with 280 µL DPPH reagent, which was prepared the day of the experiment according to Yen and Chen [17]. The antioxidant reaction was monitored for 60 min. Calibration curve was prepared with Trolox at a

concentration ranging from 0.01 to 0.4 mg/mL. Results were expressed as mmol Trolox Equivalent/kg of oil.

2.7. Determination of total phenolic content

Total phenolic content was also analyzed in the supernatant obtained after *in vitro* gastrointestinal digestion and microbial fermentation. The sum of both fractions would be the total amount of phenolic compounds released. Total phenolic content was estimated following the procedure described by Singleton & Rossi [18], adapted to a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Briefly, 30 µL of either digestion or fermentation supernatant were added, in triplicate, to a 96 well plate and mixed with 15 µL of Folin-Ciocalteu reagent, 60 µL of sodium carbonate 10% (w/v), and 195 µL of milli-Q water. The antioxidant reaction was monitored for 60 min. Calibration curve was prepared with gallic acid at a concentration ranging from 0.01 to 1.00 mg/mL. The results obtained are expressed as mg Gallic acid equivalents (GAE) per kg of oil.

2.8. *Calculations of daily antioxidant capacity and total phenol intake per serving*

Dietary antioxidant capacity and total phenol intake was calculated as the individual contribution of each olive oil [19] taking into account the amount of oil per serving and their daily consumption [20]. In this sense, the antioxidant capacity and total phenols of each olive oil referred to the usual serving size in Spain [21]. The contribution of each olive oil to the daily intake of antioxidant activity and polyphenols was referred to the results previously published by Saura-Calixto and Goñi [22].

2.9. *Statical 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 ($p < 0.05$). Multivariate analysis was performed in three steps: an exploratory method (Principal Component Analysis; PCA), an interpretive method (distance based redundancy analysis; db-RDA) and a discriminatory method (orthogonal projections to latent structures discriminant analysis; OPLS-DA). db-RDA is a type of constrained ordination that assesses how much of the variation in one set of variables can be explained by the variation in another set of variables. OPLS-DA is a discriminatory multivariate analysis, with the aim to identify the variables (in this case fatty acids or antioxidant capacity and total phenolic content) most affected by either type of variety or collection date. Specifically, OPLS-DA was performed to compare varieties (Manzanilla vs Picual) whereas OPLS regression model was performed to identify the most affected variables by collection date. Due to the different scales and units, a pareto scale was performed. Model fitting was evaluated by the parameters R^2Y and Q^2. The first one reflects the percentage of variation explained by the response variables and the latter explains the proportion of variance in the data predictable by the model [23]. One-way analysis of the variance was performed using Statgraphics Plus software, version 5.1, 2001 whereas multivariate analysis was performed by R, version 3.6.1.

3. Results and discussion

3.1. *Evolution of quality parameters during ripening*

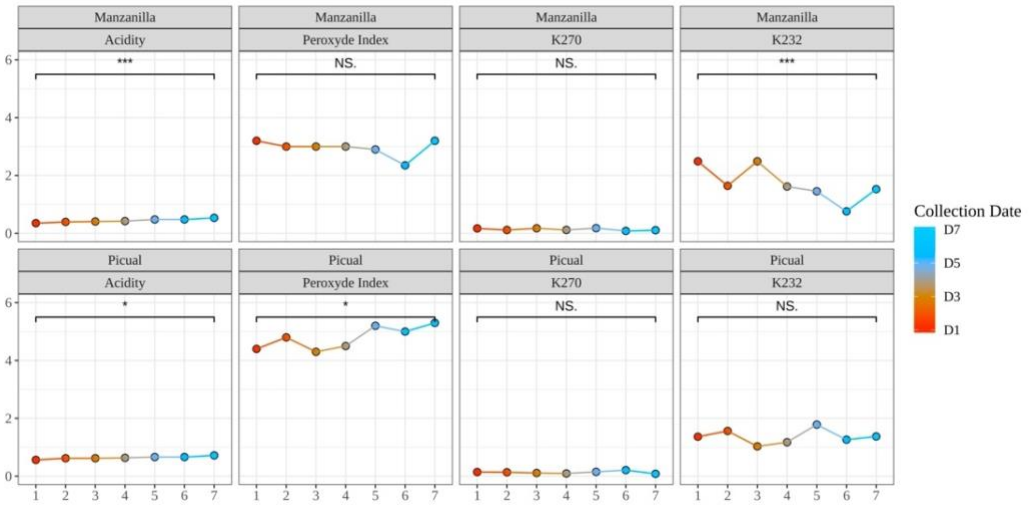
Free acidity, peroxide index and spectrophotometric UV absorption at 232 and 270 were measured throughout ripening for the Manzanilla and Picual varieties. All time points tested for both varieties showed quality values within the legal limits for olive oil to be branded as "Extra virgin olive oil" (**Figure 13A**). Acidity level, which is related with the presence of free fatty acids, increased with ripening for both the Manzanilla and Picual varieties, being significantly higher at the latter collection date ($p < 0.05$) than at the earlier one. These results are in line with those reported by other authors [1,4,8].

On the other hand, the peroxide index, which measures the primary products of autoxidation (hydroperoxides), showed different tendencies depending on the varieties: whereas for the Picual variety peroxide value it tended to increase significantly along ripening, for the Manzanilla variety such an index did not change significantly (**Figure 13A**). Finally, the analysis of absorbance in the UV region, K_{232} and K_{270}, is related to the presence of conjugated dienes and trienes as well as other oxidation products like carbonyl compounds [1], though their presence needs to be assessed by FTIR (Fourier-transform infrared spectroscopy) analysis. For the Manzanilla variety K_{270} did not change significantly along ripening, while K_{232} showed a significant decreasing tendency ($p < 0.05$). However, for Picual no clear tendencies were found during ripening (**Figure 13A**).

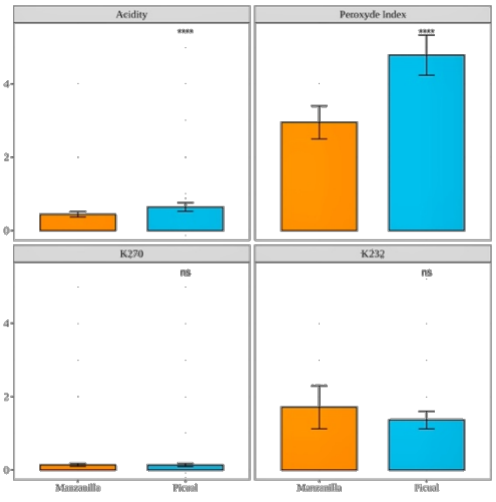
Comparing both the Picual and Manzanilla varieties, acidity and peroxide index were significantly higher in Picual olive oil (**Figure 13B**), whereas K_{232} and K_{270} did not show significant differences between both

varieties. In order to unravel if quality parameters are enough to differentiate both varieties, a PCA multivariate analysis was carried out (**Figure 13C**). As depicted in the figure, both varieties were fairly separated from one another, underlying the importance of the variety in quality parameters. Since both ripening and variety type have an influence on quality parameters, a multivariate interpretative statistical analysis (distance based redundancy analysis with Euclid distance, db-RDA) was carried out in order to unravel which variable (ripening or variety) has a higher influence. Only the variety variable was statistically significant ($p < 0.05$) whereas the ripening variable was not ($p = 0.297$). After applying a variance partitioning analysis, the variable variety explained a 40% of the variance whereas ripening only an 11% (**Figure 13D**).

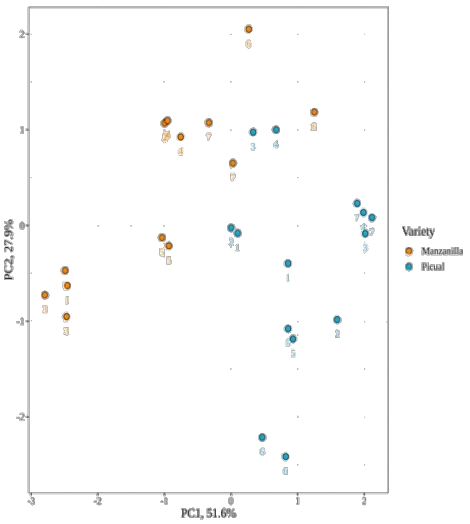
A



B



C



(Figure 13 continued)

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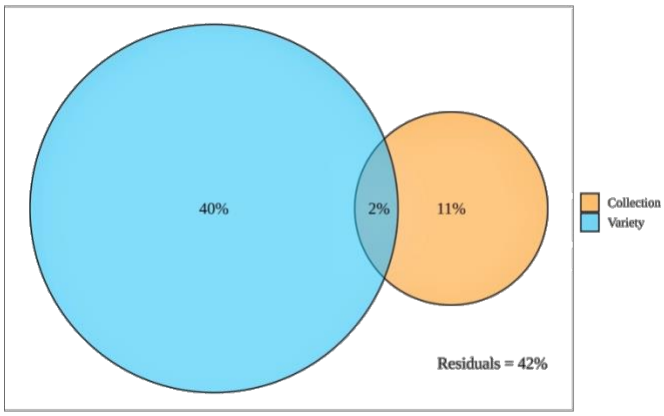


Figure 13. 13A: Evolution of quality parameters during ripening. Acidity results are expressed as % of Oleic acid, peroxyde index results are expressed as meq active O_2/kg of oil. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant. Statistical analysis was performed comparing D1 (earliest collection date) with D7 (latest collection date). Analyses were carried out in triplicate. Abbreviations: D1 to D7: collection date 1 (earliest) to collection date 7 (latest). **13B**: Average quality values for each cultivar. Acidity results are expressed as % of Oleic acid, peroxyde index results are expressed as meq active O_2/kg of oil. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant. Statistical significance is shown for tests that used Manzanilla cultivar as the reference group. **13C:** PCA of the quality parameters. The numbers beneath the points indicate the collection date (1-7). **13D**: Analysis of the variance of the db-RDA. It depicts the relative contribution of explanatory variables (quality parameters) to the overall variability in the dataset.

3.2. Evolution of fatty acids composition during ripening

Both varieties experienced a significant ($p < 0.05$) decrease in palmitic (main saturated fatty acid), stearic and oleic acids (main monounsaturated fatty acid) along ripening, whereas linoleic acid increased (**Table 2**). Oleic and linoleic acid behavior are related to the activity of the enzyme oleate desaturase, which transforms oleic acid into linoleic acid during triglyceride biosynthesis [4]. Regarding minor fatty acids, both varieties experienced a significant increase in linolenic, palmitoleic and arachidic acids.

Fatty acid composition has a great influence on EVOO oxidative stability [24], with the ratio of MUFA:PUFA as a key factor. For both varieties, this ratio decreased significantly along ripening (**Table 2**). Such a change in the MUFA:PUFA ratio is related to the above-mentioned decrease in oleic acid and increase in linoleic acid, but also due to the increase in linolenic acid. As a consequence, lower values could result in an increased oxidative susceptibility, and therefore in an oil with a shorter shelf life.

Comparing both, the Manzanilla variety showed significantly higher amounts of stearic, linoleic and arachidic acids ($p < 0.05$) whereas the Picual variety showed higher amounts ($p < 0.05$) of palmitoleic, oleic and linolenic acids (**Table 2**). These results are in accordance with those showed by Gómez-González et al., [10] who reported that saturated fatty acids were in higher amounts in the Manzanilla than the Picual variety. The PCA analysis (**Figure 14A**) showed a clear separation between varieties. In this case, the type of variety as well as ripening stage had a significant ($p < 0.05$) influence on the variance. However, the type of variety had a stronger influence (59%) on fatty acid composition than ripening stage (36%) as demonstrated by variance partitioning analysis (**Figure 14B**).

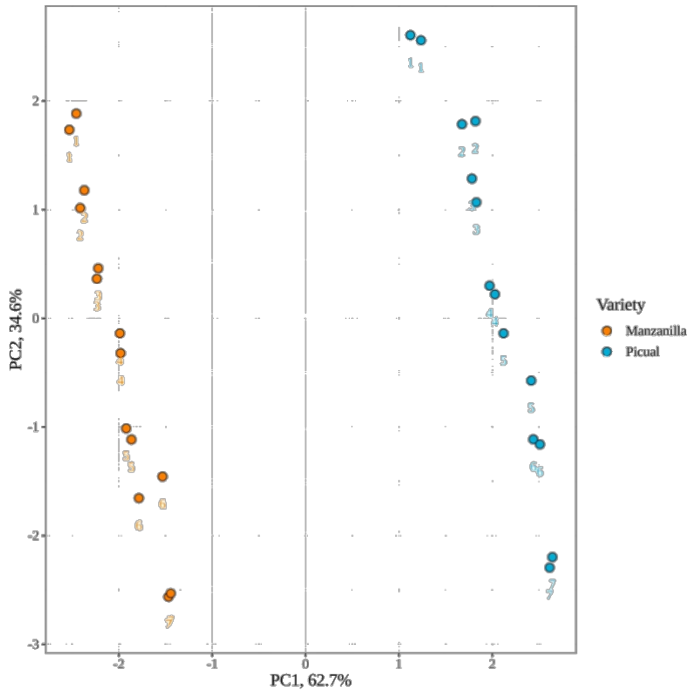
Table 2. Fatty acids composition of Manzanilla and Picual variety depending on the collection date

	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Arachidic acid	MUFA	PUFA	SFA	MUFA/PUFA
Sample	%	%	%	%	%	%	%	%	%	%	%
Manzanilla (01/11/2018)	9.82 ± 0.08	0.31 ± 0.01	3.26 ± 0.06	79.56 ± 0.32	8.26 ± 0.02	0.48 ± 0.01	0.36 ± 0.00	79.87 ± 0.31	8.74 ± 0.01	13.45 ± 0.14	9.14 ± 0.04
Manzanilla (14/11/2018)	9.49 ± 0.12	0.32 ± 0.00	3.14 ± 0.01	78.86 ± 0.02	8.61 ± 0.14	0.49 ± 0.00	0.37 ± 0.00	79.18 ± 0.02	9.10 ± 0.14	13.00 ± 0.11	8.70 ± 0.13
Manzanilla (01/12/2018)	9.06 ± 0.04*	0.34 ± 0.00	3.11 ± 0.02	78.51 ± 0.31	8.79 ± 0.01	0.51 ± 0.00	0.37 ± 0.00	78.85 ± 0.31	9.30 ± 0.00	12.54 ± 0.03	8.48 ± 0.03
Manzanilla (15/12/2018)	8.76 ± 0.16*	0.36 ± 0.00*	3.07 ± 0.04	78.31 ± 0.04	8.91 ± 0.06	0.53 ± 0.00	0.39 ± 0.00	78.67 ± 0.05	9.44 ± 0.06	12.22 ± 0.13	8.33 ± 0.05
Manzanilla (02/01/2019)	8.40 ± 0.02*	0.38 ± 0.00*	2.91 ± 0.01	77.38 ± 0.01	9.07 ± 0.04	0.54 ± 0.00	0.41 ± 0.00	77.76 ± 0.01	9.62 ± 0.05	11.72 ± 0.03	8.09 ± 0.04
Manzanilla (16/01/2019)	8.20 ± 0.05*	0.40 ± 0.00*	2.85 ± 0.05*	77.92 ± 1.14	9.44 ± 0.41*	0.56 ± 0.00*	0.44 ± 0.00	78.32 ± 1.14	10.00 ± 0.41*	11.49 ± 0.09*	7.83 ± 0.21
Manzanilla (02/02/2019)	8.02 ± 0.01*	0.41 ± 0.01*	2.79 ± 0.01*	76.77 ± 0.16*	9.91 ± 0.26*	0.62 ± 0.01*	0.46 ± 0.00*	77.19 ± 0.14	10.53 ± 0.27*	11.27 ± 0.03*	7.33 ± 0.17*
Total	8.82 ± 0.65*	0.36 ± 0.04a	3.01 ± 0.17*	78.20 ± 0.95*	9.00 ± 0.54*	0.53 ± 0.05*	0.40 ± 0.04*	78.54 ± 0.92*	9.53 ± 0.59*	12.24 ± 0.77*	8.27 ± 0.57*
Picual (01/11/2018)	9.75 ± 0.07	0.40 ± 0.01	2.82 ± 0.07	82.85 ± 0.08	3.78 ± 0.01	0.55 ± 0.00	0.14 ± 0.01	83.25 ± 0.09	4.33 ± 0.01	12.71 ± 0.15	19.21 ± 0.08
Picual (14/11/2018)	9.50 ± 0.12	0.42 ± 0.00	2.43 ± 0.02	82.67 ± 0.09	3.90 ± 0.03	0.57 ± 0.01	0.17 ± 0.01*	83.10 ± 0.10	4.47 ± 0.02	12.10 ± 0.11	18.59 ± 0.10
Picual (01/12/2018)	9.24 ± 0.04	0.44 ± 0.00	2.42 ± 0.07	82.13 ± 0.22	4.01 ± 0.01	0.59 ± 0.00	0.19 ± 0.01*	82.57 ± 0.22	4.60 ± 0.01	11.84 ± 0.10	17.95 ± 0.08*
Picual (15/12/2018)	8.83 ± 0.07*	0.49 ± 0.01	2.35 ± 0.00	81.51 ± 0.11*	4.19 ± 0.02*	0.60 ± 0.00	0.21 ± 0.00*	82.00 ± 0.12	4.79 ± 0.01	11.39 ± 0.06	17.12 ± 0.02*
Picual (02/01/2019)	8.56 ± 0.16*	0.55 ± 0.03*	2.29 ± 0.05*	81.32 ± 0.06*	4.22 ± 0.01*	0.61 ± 0.00*	0.23 ± 0.01*	81.87 ± 0.09*	4.83 ± 0.01*	11.08 ± 0.21*	16.95 ± 0.03*
Picual (16/01/2019)	8.30 ± 0.06*	0.59 ± 0.01*	2.21 ± 0.01*	81.02 ± 0.21*	4.52 ± 0.02*	0.63 ± 0.01*	0.27 ± 0.00*	81.61 ± 0.20*	5.14 ± 0.03*	10.78 ± 0.05*	15.87 ± 0.12*
Picual (02/02/2019)	7.81 ± 0.03*	0.62 ± 0.01*	2.12 ± 0.01*	80.36 ± 0.14*	5.12 ± 0.04*	0.66 ± 0.01*	0.30 ± 0.00*	80.98 ± 0.15*	5.77 ± 0.03*	10.23 ± 0.02*	14.03 ± 0.05*
Total	8.85 ± 0.66*	0.50 ± 0.08b	2.37 ± 0.22*	81.70 ± 0.87*	4.25 ± 0.43*	0.60 ± 0.3*	0.22 ± 0.05*	82.20 ± 0.79*	4.84 ± 0.46*	11.44 ± 0.81*	17.10 ± 1.68*

*Statistically significant differences ($p < 0.05$) with respect to the earliest collection date (D1).

*Within the "Total" row means statistically significant differences ($p < 0.05$) between Manzanilla Total and Picual Total.

A



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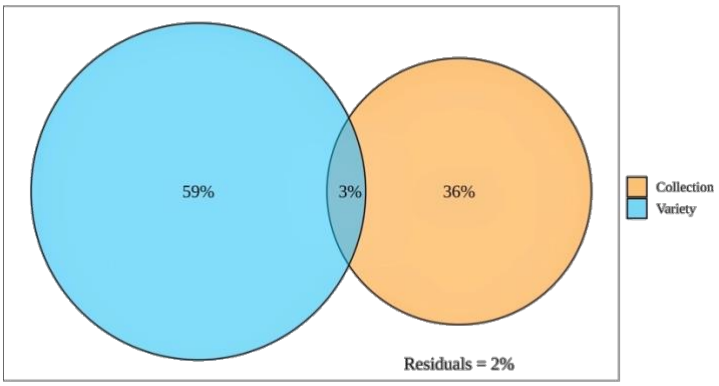


Figure 14. 14A: PCA of fatty acids composition. The numbers beneath the points indicate the collection date (17). **14B:** Analysis of the variance of the db-RDA. It depicts the relative contribution of explanatory variables (fatty acid concentrations) to the overall variability in the dataset.

3.3. Evolution of antioxidant capacity and phenolic content during ripening

In order to simulate the physiological extraction of antioxidant capacity and phenolic compounds, they were measured after submitting olive oil to an *in vitro* gastrointestinal digestion followed by an *in vitro* fermentation with human fecal samples. Antioxidant capacity was measured through the ABTS, FRAP, and DPPH methods, whereas total phenolics were measured through the Folin-Ciocalteu method.

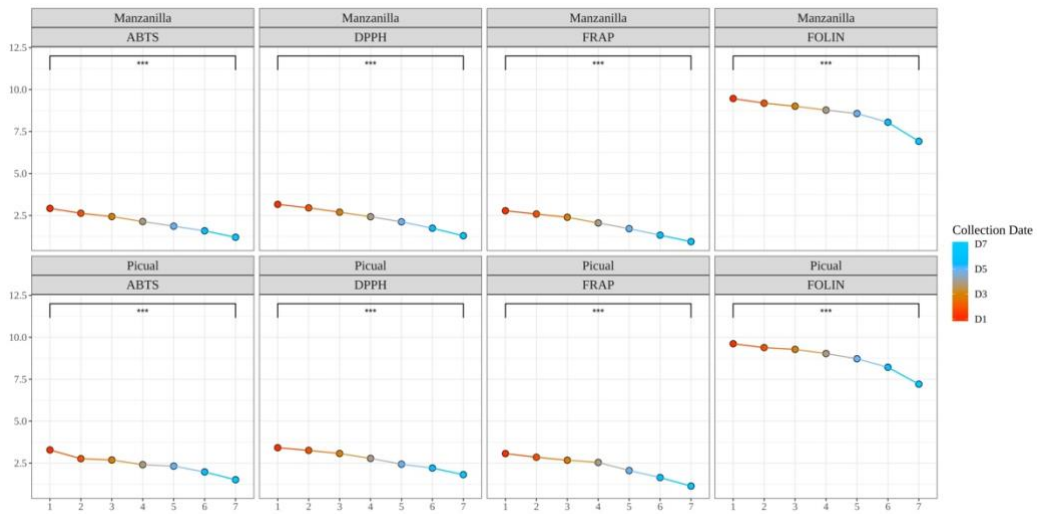
Antioxidant capacity as well as total phenolics, either released after gastrointestinal digestion or fermentation, decreased in a significant manner ($p < 0.05$) along ripening, showing the same behaviour with all the methods tested (**Figure 15A and 15B**). These results are in accordance with those reported by other authors [4-6,8]. However, Gougoulias et al., [7] reported the opposite, stating that phenolic compounds increased along ripening. These contradictions could be related, as previously stated, to the study of different varieties. Moreover, as stated by other authors, the decrease of antioxidant capacity along ripening could be due to a loss in phenolic compounds, but could also be related to an increase in the MUFA:PUFA ratio [24]. The increased MUFA:PUFA ratio would make oil more susceptible to oxidation processes catalyzed by free-radicals and thus, requiring higher antioxidant potential to keep their fatty acids from being oxidized. Yun and Surh [24] also stated that oxidative stability of olive oil could be affected by chlorophylls and carotenoids acting as prooxidants, releasing radicals due to their conjugated double bond. However, these conditions were studied under induced oxidative stress and, on the other, olive oil initial quality was not specified which could make this study not comparable with ours.

The antioxidant capacity released after *in vitro* gastrointestinal digestion (**Figure 15A**) was lower than that released after *in vitro* fermentation (**Figure 15B**). According to bibliography, the main compounds responsible for olive oil antioxidant properties are polyphenols [4], including phenolic acids, flavonols, flavones, lignans or secoiridoids such as oleuropein [25]. However, a large amount of polyphenols usually escapes absorption in the small intestine, reaching the large one. Most of these compounds can be used by gut microbes as substrates and yield others, usually smaller, phenolic compounds [26,27]. All these newly formed compounds could increase the antioxidant capacity after gut microbes' fermentation as it has been stated for other foodstuffs [12].

On the other hand, antioxidant capacity released after *in vitro* gastrointestinal digestion was very similar in both the Picual and Manzanilla varieties, with no significant differences (**Figure 16A**). However, antioxidant capacity released by Picual oil after *in vitro* fermentation was significantly higher than that of Manzanilla. The same behavior was found for all three antioxidant methods and Folin-Ciocalteu method. Therefore, no clear separation was observed in PCA of the digestion antioxidant values, whereas a clear separation was found for the fermentation antioxidant capacity (**Figure 16B**). Accordingly, a possible explanation could be that those compounds responsible for the main differences between varieties would not be solubilized during digestion and therefore, not absorbed, reaching the large intestine, where they could be metabolized by gut bacteria [28]. Further studies would be needed to identify the specific com- pounds released after gut microbial fermentation of different olive oil varieties to check whether consuming one or another could actually have a different impact on human health.

As stated above (in the previous section) the Picual MUFA:PUFA ratio was twice as high as that of the Manzanilla (**Table 2**). Thus, the Picual variety could be associated with a higher oxidative stability whereas the Manzanilla would be more susceptible to suffer fat oxidations. Therefore, antioxidant capacity could be invested in protecting fatty acids from oxidation. In this sense, variance quantity that could be explained by the type of variety and collection date was also tested. Regarding gastrointestinal digestion antioxidant values, collection date explained 87% of the variance whereas the type of variety explained only 8%. However, regarding antioxidant capacity released during fermentation, it was the variable type of variety that explained most of the variance (48%) while collection date explained a 39% (**Figure 16C**).

A



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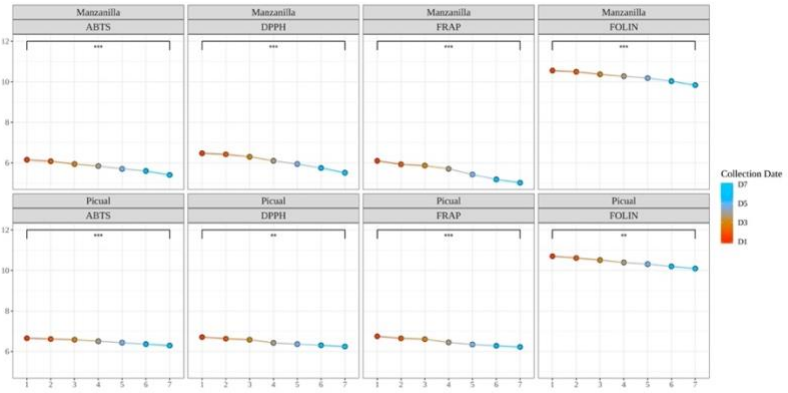

Figure 15. 15A: Evolution of antioxidant capacity and total phenolic content obtained after *in vitro* digestion during ripening. Results are log_2 transformed to improve visualization. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant. Statistical analysis was performed comparing D1 (earliest collection date) with D7 (latest collection date). Analyses were carried out in triplicate. ABTS, FRAP and DPPH mmol Trolox Equivalent/kg of oil. Folin-Ciocalteu values are expressed as mg Gallic acid equivalent/kg of oil. Abbreviations: D1 to D7: collection date 1 (earliest) to collection date 7 (latest). **15B:** Evolution of antioxidant capacity and total phenolic content obtained after *in vitro* gut microbial fermentation during ripening. Results are log_2 transformed to improve visualization. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant. Statistical analysis was performed comparing D1 (earliest collection date) with D7 (latest collection date). Analyses were carried out in triplicate. ABTS, FRAP and DPPH mmol Trolox Equivalent/kg of oil. Folin-Ciocalteu values are expressed as mg Gallic acid equivalent/kg of oil. Abbreviations: D1 to D7: collection date 1 (earliest) to collection date 7 (latest).

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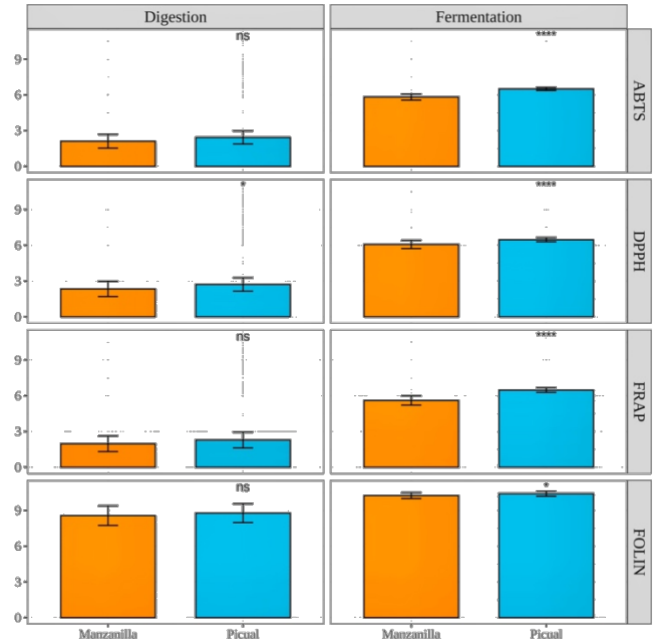

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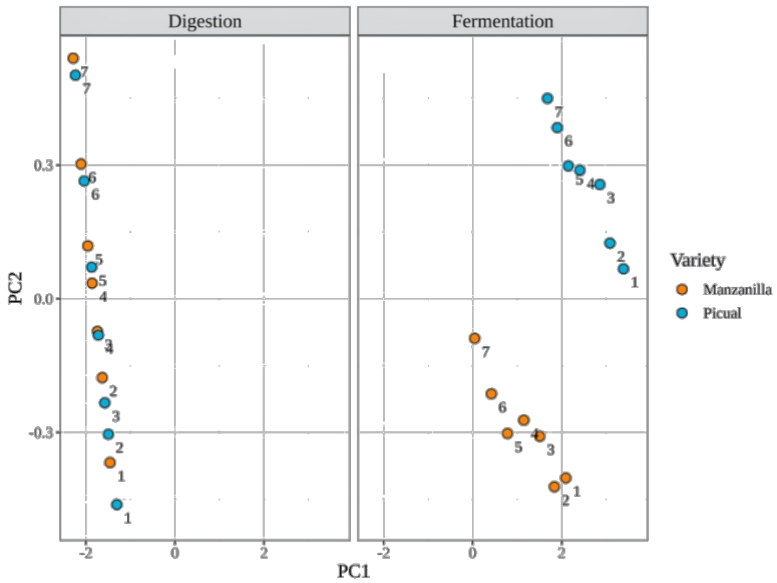

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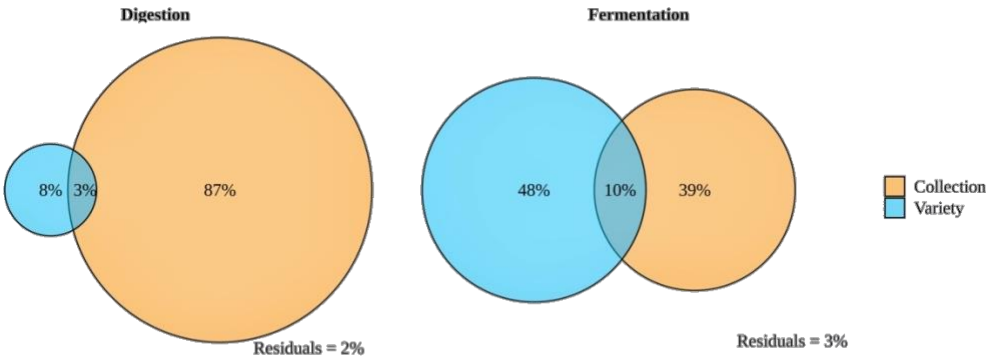


Figure 16. 16A: Average antioxidant and total phenolic values for each variety. Results are $\log_2$ transformed to improve visualization. Statistical significance: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant. Statistical significance is shown for tests that used Manzanilla variety as the reference group. ABTS, FRAP and DPPH mmol Trolox Equivalent/kg of oil. Folin-Ciocalteu values are expressed as mg Gallic acid equivalent/kg of oil. **16B:** PCA of the antioxidant capacity and total phenolic values. Left panel shows PCA involving *in vitro* digestion values. Right panel shows PCA involving *in vitro* fermentation values. The numbers beneath the points indicate the collection date (1-7). **16C**: Analysis of the variance of the db-RDA. It depicts the relative contribution of explanatory variables (antioxidant capacity and total phenolic content) to the overall variability in the dataset. Left panel refers to *in vitro* gastrointestinal values. Right panel refers to *in vitro* gut microbial fermentation values.

3.4 Contribution of olive oil consumption to the daily antioxidant capacity and total phenolic compound intake.

Extra virgin olive oil is the main fat addition in the Mediterranean diet, which favors a lower incidence of different chronic non-communicable diseases such as cardiovascular disease, diabetes, cancer, neurodegenerative diseases, etc. [29]. Therefore, once the antioxidant capacity and total phenols data were available, the contribution of olive oil consumption to the daily intake of antioxidant capacity and polyphenols in Spain was calculated. To do so, a mean olive oil intake in Spain of 2.5 L/inhabitant/year [20], olive oil portion size of 10 mL [21] and a recommended daily intake of 4.5 portions/day of olive oil [29] were used.

The consumption of the analyzed olive oils could mean a daily antioxidant capacity intake ranging from 306 to 758 μmol Trolox equivalents for Manzanilla olive oil and 236-794 μmol Trolox equivalents for Picual variety (**Table 3**). Green varieties released a higher antioxidant capacity than ripen ones. In Spain, the mean daily intake of antioxidant capacity ranges from 6014 to 3549 μmol Trolox equivalents for FRAP and ABTS methods, respectively [22]. Therefore, the consumption of the studied oils means a contribution of a 3.9-15.2% of the daily antioxidant activity intake for the ABTS method and an 8.8-21.3% for the FRAP method (**Table 3**). These calculations were carried out taking into account the mean olive oil consumption in Spain, but if the calculations are performed per serving, then the anti- oxidant capacity provided increases, reaching a daily contribution up to 31.2%. Even more, when the recommended olive oil intake is used for calculations, then the daily contribution to antioxidant capacity exceeds 100% for Manzanilla olive oil and rises to 86.7% for Picual (**Table 3**). This information stresses the relevance of olive oil consumption for human beings.

In the case of polyphenols, the daily intake ranged from 7.1 to 16.8 mg polyphenols/day (**Table 3**). Since the daily polyphenols intake in Spain is 1171 mg gallic acid [22], olive oil consumption contributes just a 0.6-1.3% of total daily polyphenols. However, the intake of polyphenols increased if a serving is used for calculations (up to 24.5 mg; 2.1% contribution) or the recommended olive oil intake (up to 110 mg polyphenols; 9.4% contribution).

Table 3. Contribution of olive oil consumption to the daily antioxidant capacity (AOX) and polyphenols intake in the Spanish diet.

Type of olive oil	**Analytical assay**	**AOX/daily intake[1]** (μmol Trolox/day)	**Mean contribution to daily antioxidant capacity intake** (%)	**AOX/serving intake[2]** (μmol Trolox/serving)	**Contribution to daily antioxidant capacity intake per serving** (%)	**AOX/recommended intake** (μmol Trolox/4.5 servings)	**Contribution to daily antioxidant capacity intake recommended** (%)
Manzanilla	*ABTS-Green*	538.4	15.2	786	22.1	3537	99.7
	ABTS-Ripen	305.5	8.6	446	12.6	2007	56.6
	FRAP-Green	757.6	21.3	1106	31.2	4977	140.2
	FRAP-Ripen	556.9	15.7	813	22.9	3659	103.1
Picual	*ABTS-Green*	517.2	8.6	755	12.6	3398	56.5
	ABTS-Ripen	235.6	3.9	344	5.7	1548	25.7
	FRAP-Green	793.9	13.2	1159	19.3	5216	86.7
	FRAP-Ripen	527.5	8.8	770	12.8	3465	57.6
Type of olive oil	**Analytical assay**	**Polyphenols/daily intake[1]** (mg/day)	**Mean contribution to daily polyphenols intake** (%)	**Polyphenols/serving intake[2]** (mg/serving)	**Contribution to daily polyphenols intake per serving** (%)	**Polyphenols/ recommended intake** (μmol Trolox/4.5 servings)	**Contribution to daily polyphenols intake recommended** (%)
Manzanilla	*FOLIN-Green*	15.1	1.3	22.1	1.9	99	8.5
	FOLIN-Ripen	7.1	0.6	10.4	0.9	47	4.0
Picual	*FOLIN-Green*	16.8	1.4	24.5	2.1	110	9.4
	FOLIN-Ripen	8.5	0.7	12.4	1.1	56	4.8

[1]Considering olive oil consumption for a whole year.

[2]Consideing the intake of 1 serving.

[2]Consideing the intake of 4.5 servings.

3.5 Selection of olive oil variety and collection date

Taking all the information provided in the previous section into account, it is clear that it is important to select olive oil depending on the variety type and collection date (green vs. ripen). In order to study the importance of both variables, a distance-based redundancy analysis with Euclid distance was carried out (**Figure 17A**). Even though there were some changes depending on the variety and collection date, these variables do not

play a relevant role in quality parameters, since all samples, from both varieties, were within the legal limits for olive oil to be branded as Extra Virgin Olive Oil.

The Manzanilla variety showed higher amounts of PUFA and SFA (saturated fatty acids) than the Picual class; however, MUFA concentration was higher in the Picual variety (**Figure 17A**). Moreover, whereas SFA and MUFA were higher (in both varieties) in early collection dates, PUFA concentration increased during maturation. Therefore, for higher PUFA content, the best choice would be Manzanilla olives at the end of their maturation (ripened), whereas for higher MUFA content the choice would be Picual olive oils at early maturation stages (green). On the other hand, due to the importance of the w3:w6 relation for human health, the choice of one oil type over the other could be made based on that. The w3:w6 relation will determine the formation of anti-inflammatory or pro-inflammatory substances, or coagulation and gene expression related substances.

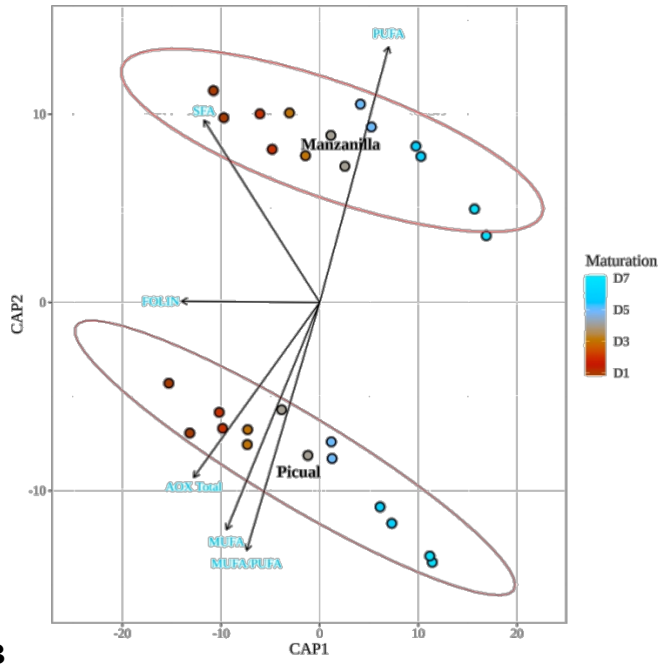
Regarding antioxidant capacity, the Picual variety showed higher values than the Manzanilla one. In both varieties, the antioxidant capacity as well as phenolic content was higher in early maturation stages. Therefore, for higher antioxidant power, the best choice would be Picual olives from early maturation stages. The phenolic content was similar in both varieties, though it decreased during maturation. Thus, early harvested olives also provide more phenolics (**Figure 17A**).

Both variables, olive variety and collection date, had an important influence explaining variance. However, variety explained a 53% of variance whereas collection dates just a 33% (**Figure 17B**). Therefore, regarding antioxidant capacity and fatty acid composition, there was a larger difference between varieties than between collection dates, at least for Manzanilla and

Picual varieties, and thus, the first question when choosing an olive oil should be "Which variety should we choose?".

Finally, an OPLS-DA (a discriminatory multivariate analysis) was performed with the aim to identify the variables (fatty acids, antioxidant capacity and phenolic content) most affected by either variety type or collection date. Model fitting was evaluated by the parameters R2Y and Q^2. The first parameter (R^2Y) reflects the percentage of variation explained by the response variables and the latter (Q^2)) explains what variance portion in the data is predictable by the model [23]. When the model was "Manzanilla vs. Picual", it showed an almost perfect reliability ($R^2Y = 0.998$) and predictive ability ($Q^2 = 0.998$). The most affected variables (top five) by the type of variety were fatty acids, and more specifically (in the following order): linoleic acid, total PUFA, relation MUFA/PUFA, total MUFA and arachidic acid. On the other hand, the model to compare the different collection dates also showed great fitting values ($R^2Y = 0.999$ and $Q^2 = 0.998$ for Manzanilla; $R^2Y = 0.991$ and $Q^2 = 0.989$ for Picual). For both varieties, the variable most affected by collection date was, by far, total phenol concentration measured by Folin-Ciocalteu method, followed by antioxidant capacity (total and individual values for each method tested).

A



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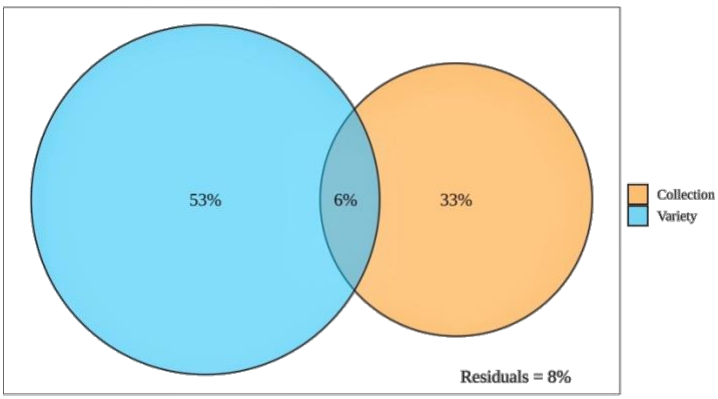

Figure 17. 17A: Distance-based Redundancy Analysis with Euclid distance. The arrows point to where the variable is higher. Abbreviations: D1 to D7: collection date 1 (earliest) to collection date 7 (latest). **17B:** Analysis of the variance of the db-RDA. It depicts the relative contribution of explanatory variables (fatty acids, antioxidant capacity and total phenolic content) to the overall variability in the dataset.

4. Conclusions

This paper describes the evolution of quality parameters, fatty acids composition, antioxidant capacity and total phenolic content of olive oils obtained from olives with different maturation times and two different varieties Manzanilla and Picual. Quality parameters were al- ways within the legal limits for olive oil to be called "Extra Virgin Olive Oil". SFA and MUFA decreased during maturation whereas PUFA increased in both varieties. The Manzanilla variety showed higher PUFA content, while Picual had higher MUFA concentration. On the other hand, antioxidant capacity along with total phenolic content decreased during maturation. While antioxidant capacity was higher in the Picual variety, total phenolic content was very similar in both of them. Under the physiological point of view, the antioxidant capacity of olive oil after gut microbiota fermentation was 3-4 times higher than that released after *in vitro* digestion. In this sense, it has been shown that olive oil consumption could contribute a 26-140% of the daily antioxidant capacity recommended for the Spanish population, depending on the maturity stage of the olives used for oil extraction. Finally, the statistical approach demonstrated that, at least involving Manzanilla and Picual, the type of variety is more important than collection date regarding fatty acid composition and antioxidant capacity. Thus, the type of variety as well as collection date have a higher influence on fatty acid composition than on antioxidant capacity or quality parameters.

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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.

References

1. Mn, F.; J, S.; C, D.M.; M, M.; D, M.-V. Influence of the fruit's ripeness on virgin olive oil quality. J. Oleo Sci. 2015, 64, doi:10.5650/jos.ess14148.

2. Ouni, Y.; Flamini, G.; Zarrouk, M. The Chemical Properties and Volatile Compounds of Virgin Olive Oil from Oueslati Variety: Influence of Maturity Stages in Olives. J. Am. Oil Chem. Soc. 2016, 93, 1265-1273, doi:10.1007/s11746-016-2863-0.

3. Köseoğlu, O.; Sevim, D.; Kadiroğlu, P. Quality Characteristics and Antioxidant Properties of Turkish Monovarietal Olive Oils Regarding Stages of Olive Ripening. Food Chem. 2016, 212, 628-634, doi:10.1016/j.foodchem.2016.06.027.

4. Bakhouche, A.; Lozano-Sánchez, J.; Bengana, M.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Time Course of Algerian Azeradj Extra-Virgin Olive Oil Quality during Olive Ripening. Eur. J. Lipid Sci. Technol. 2015, 117, 389-397, doi:10.1002/ejlt.201400197.

5. Bozdogan Konuskan, D.; Mungan, B. Effects of Variety, Maturation and Growing Region on Chemical Properties, Fatty Acid and Sterol Compositions of Virgin Olive Oils. J. Am. Oil Chem. Soc. 2016, 93, 1499-1508, doi:10.1007/s11746-016-2904-8.

6. Vincenzo, S. Antioxidant Potential of Extra Virgin Olive Oils Extracted from Three Different Varieties Cultivated in the Italian Province of Reggio Calabria. J. Appl. Bot. Food Qual. 2017, 90, 76-82, doi:10.5073/JABFQ.2017.090.011.

7. Gougoulias, N.; Liviu, G.; Vagelas, I.; Wogiatzi, E.; Ntalla, M.-N. Changes in Total Phenol Content and Antioxidant Activity of Greek Table Olive Cultivar Amfissis during Maturation. Stud. Univ. Babeș-Bolyai Chem. 2017, 62, 387-396, doi:10.24193/subbchem.2017.2.31.

8. Nsir, H.; Taamalli, A.; Valli, E.; Bendini, A.; Gallina Toschi, T.; Zarrouk, M. Chemical Composition and Sensory Quality of Tunisian 'Sayali' Virgin Olive Oils as Affected by Fruit Ripening: Toward an Appropriate Harvesting Time. J. Am. Oil Chem. Soc. 2017, 94, 913-922, doi:10.1007/s11746-017-3000-4.

9. International olive oil council. Method of analysis. COI/T.20/Doc. no24, 2001. Retrieved from http://www.internationaloliveoil.org/downloads/ orga6.pdf (accessed on 6 December 2019)

10. Gómez-González, S.; Ruiz-Jiménez, J.; Luque de Castro, M.D. Oil Content and Fatty Acid Profile of Spanish Cultivars During Olive Fruit Ripening. J. Am. Oil Chem. Soc. 2011, 88, 1737-1745, doi:10.1007/s11746-011-1840-x.

11. European Community. Council Regulation No. 1513/2001 of 23 July 2001 Amending Regulations No. 136/66/EEC and (EC) No. 1638/98 as Regards the Extension of the Period of Validity of the Aid Scheme and the Quality Strategy for Olive Oil. Off J Eur Comm., L201, 4-7.

12. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an Improved Global Antioxidant Response Method (GAR+): Physiological-Resembling in Vitro Antioxidant Capacity Methods. Food Chem. 2018, 239, 1263-1272, doi:10.1016/j.foodchem.2017.07.063.

13. Pastoriza, S.; Delgado-Andrade, C.; Haro, A.; Rufián-Henares, J.A. A Physiologic Approach to Test the Global Antioxidant Response of Foods. The GAR Method. Food Chem. 2011, 129, 1926-1932, doi:10.1016/j.foodchem.2011.06.009.

14. Marfil, R.; Giménez, R.; Martínez, O.; Bouzas, P.R.; Rufián-Henares, J.A.; Mesías, M.; Cabrera-Vique, C. Determination of Polyphenols, Tocopherols, and Antioxidant Capacity in Virgin Argan Oil (Argania Spinosa, Skeels). Eur. J. Lipid Sci. Technol. 2011, 113, 886-893, doi:10.1002/ejlt.201000503.

15. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radic. Biol. Med. 1999, 26, 1231-1237, doi:10.1016/s0891-5849(98)00315-3.

16. Benzie, I.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal. Biochem. 1996, 239, 70-76, doi:10.1006/abio.1996.0292.

17. Yen, G.-C.; Chen, H.-Y. Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity. J. Agric. Food Chem. 1995, 43, 27-32, doi:10.1021/jf00049a007.

18. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 1965, 16, 144-158.

19. Jiménez-Zamora, A.; Delgado-Andrade, C.; Rufián-Henares, J.A. Antioxidant Capacity, Total Phenols and Color Profile during the Storage of Selected Plants Used for Infusion. Food Chem. 2016, 199, 339-346, doi:10.1016/j.foodchem.2015.12.019.

20. Mercasa. La alimentación en España, 2019. Available in https:// alimentacionenespaña2019.es/ae18/. (accessed on 12 December 2019).

21. Salvadó, J.S.; Sanjaume, A.B. i; Casañas, R.T.; Solà, M.E.S. i; Peláez, R.B. Nutrición y dietética clínica; Elsevier, 2019; ISBN 978-84-9113-303-2.

22. Saura-Calixto, F.; Goñi, I. Antioxidant Capacity of the Spanish Mediterranean Diet. Food Chem. 2006, 94, 442-447, doi:10.1016/j.foodchem.2004.11.033.

23. Deiana, P.; Santona, M.; Dettori, S.; Culeddu, N.; Dore, A.; Molinu, M.G. Multivariate Approach to Assess the Chemical Composition of Italian Virgin Olive Oils as a Function of Variety and Harvest Period. Food Chem. 2019, 300, 125243, doi:10.1016/j.foodchem.2019.125243.

24. Yun, J.-M.; Surh, J. Fatty Acid Composition as a Predictor for the Oxidation Stability of Korean Vegetable Oils with or without Induced Oxidative Stress. Prev. Nutr. Food Sci. 2012, 17, 158-165, doi:10.3746/pnf.2012.17.2.158.

25. Klikarová, J.; Rotondo, A.; Cacciola, F.; Česlová, L.; Dugo, P.; Mondello, L.; Rigano, F. The Phenolic Fraction of Italian Extra Virgin Olive Oils: Elucidation through Combined Liquid Chromatography and NMR Approaches. Food Anal. Methods 2019, 12, 1759-1770.

26. Marín, L.; Miguélez, E.M.; Villar, C.J.; Lombó, F. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. BioMed Res. Int. 2015, 2015, 905215, doi:10.1155/2015/905215.

27. Rowland, I.; Gibson, G.; Heinken, A.; Scott, K.; Swann, J.; Thiele, I.; Tuohy, K. Gut Microbiota Functions: Metabolism of Nutrients and Other Food Components. Eur. J. Nutr. 2018, 57, 1-24, doi:10.1007/s00394-017-1445-8.

28. Selma, M.V.; Espín, J.C.; Tomás-Barberán, F.A. Interaction between Phenolics and Gut Microbiota: Role in Human Health. J. Agric. Food Chem. 2009, 57, 6485-6501, doi:10.1021/jf902107d.

29. Guías Alimentarias Para La Población Española (SENC, Diciembre 2016): La Nueva Pirámide de La Alimentación Saludable. Nutr. Hosp. 2016, 33, 1-48, doi:10.20960/nh.8

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Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to *in Vitro* Digestion-Fermentation

En este trabajo se estudió la capacidad antioxidante de alimentos de origen vegetal con diferentes cocinados tras su digestión y fermentación *in vitro*. Esta última se llevó a cabo con microbiota intestinal procedente de adultos sanos. Así se pudo ver cómo afectaban tanto el tipo de alimento como el cocinado aplicado a la funcionalidad de la microbiota intestinal, en concreto a la capacidad de la misma para generar compuestos con actividad antioxidante. Además, se estudió la contribución del consumo de estos alimentos de origen vegetal a la capacidad antioxidante diaria en la dieta española.

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Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to *in Vitro* Digestion-Fermentation

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Abstract

The antioxidant capacity of foods is essential to complement the body's own endogenous antioxidant systems. The main antioxidant foods in the regular diet are those of plant origin. Although every kind of food has a different antioxidant capacity, thermal processing or cooking methods also play a role. In this work, the antioxidant capacity of 42 foods of vegetable origin was evaluated after *in vitro* digestion and fermentation. All foods were studied both raw and after different thermal processing methods, such as boiling, grilling roasting, frying, toasting and brewing. The cooking methods had an impact on the antioxidant capacity of the digested and fermented fractions, allowing the release and transformation of antioxidant compounds. In general, the fermented fraction accounted for up to 80-98% of the total antioxidant capacity. The most antioxidant foods were cocoa and legumes, which contributed to 20% of the daily antioxidant capacity intake. Finally, it was found that the antioxidant capacity of the studied foods was much higher than those reported by other authors since digestion-fermentation pretreatment allows for a higher extraction of antioxidant compounds and their transformation by the gut microbiota.

Keywords: antioxidant capacity; *in vitro* digestion-fermentation; thermal processing; cooking methods; plant foods.

1. Introduction

According to nutritional epidemiological evidence, the consumption of fruit and vegetables, as well as other plant-derived foods, is associated with a protective effect against several noncommunicable diseases such as cardiovascular disease, diabetes 2, metabolic syndrome, cancer or inflammation, where oxidative stress plays an important role [1]. Phytochemicals, such as phenolic compounds, have been noted as partially responsible for such protective effects against chronic diseases [2]. These compounds are mainly responsible for the antioxidant capacity of plant-derived foods [3], which is related to their culinary treatment. Some vegetables, mostly fruits, are consumed in their raw form, whereas others are cooked before being eaten. There are many different culinary treatments, ranging from those that use water as a cooking medium to those that use oil [4]. Whereas cooking in water may result in the loss of hydrosoluble compounds (mainly vitamins and minerals), cooking in oil could result in an enrichment of phytochemicals, though this will depend on the oil used [5]. Additionally, different cooking methods use different temperatures, ranging from 60 to 70 ºC in steaming to 200 to 220 ºC in grilling. Higher temperatures will have a deeper impact on thermolabile compounds and therefore reduce their availability. However, it has been seen that higher temperatures or intense cooking achieve a deeper breakdown of the plant cell wall, increasing the availability of some compounds, and therefore making them easier to absorb [6]. On the other hand, thermal processing can cause chemical changes in plant foods' composition due to the development of the Maillard reaction, triggered by the interaction of carbonyl compounds with amino groups [7]. Therefore, a long thermal processing time in cooking could result in the generation of potentially toxic chemical species [8] and loss of bioactive compounds [9].

On the other hand, once ingested, foods undergo gastrointestinal digestion. However, due to the lack of enzymes to digest plant cell wall polysaccharides, some structures remain relatively intact upon reaching the large intestine. It is well known that fiber is the main energy source for gut microbes [10] and phenolic compounds are mainly absorbed in the large intestine after extensive metabolization by gut bacteria [11]. Therefore, most antioxidants from vegetables are absorbed in the large intestine.

In order to shed some light on this matter, this paper evaluates the antioxidant capacity of the most representative food items from the vegetable kingdom submitted to different cooking methods (boiling, grilling, roasting, frying and toasting). In addition, these foods were submitted to *in vitro* digestion-fermentation to simulate the activity of the human gut microbiota for a more realistic estimation of the antioxidant capacity of foods. Finally, the contribution of the intake of plant foods to the daily antioxidant capacity of the Spanish diet was determined.

2. Materials and Methods

2.1. Chemicals

2.1.1. *In Vitro* Digestion and Fermentation

Salivary alpha-amylase, pepsin, porcine bile acids (porcine bile extract), sodium dihydrogen phosphate, sodium sulfide, resazurin, tryptone and cysteine were obtained from Sigma-Aldrich (Darmstadt, Germany). Pancreatin (from porcine pancreas) was purchased from Alpha Aesar (Kandel, Germany).

2.1.2. Antioxidant Capacity

2,2 Diphenyl-1-1-picrythydrazul hydrate 95% (DPPH), Trolox ((±)-6-hydroxy-2,5,7,8- tetramethylchromane-2-carboxylic acid), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), sodium acetate, iron (III) chloride hexahydrate, methanol, hydrochloric acid, sodium carbonate, gallic acid and the Folin-Ciocalteu®reagent were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2. Plant Foods and Cooking Conditions

A total of 42 plant foods (**Table 5** in supplemental information) were studied, belonging to the following groups: alcoholic drinks (beer, red wine), cereals (regular biscuits, whole-grain biscuits, bread, whole-grain bread, breakfast cereals, whole-grain breakfast cereals, penne, whole-grain penne, rice, whole-grain rice), cocoa (dark chocolate, Nutella), coffee (regular coffee, instant coffee), fruits (apple, banana, grape, olive, orange, peach, plum), legumes (kidney beans, lentils), nuts (nuts mixture, peanuts), oils (olive oil, sunflower oil), tubers (potato, sweet potato) and vegetables

(cabbage, carrot, cauliflower, eggplant, lettuce, onion, pepper, spinach, tomato, zucchini). Samples were obtained from three different retail stores and stored under refrigeration (fresh vegetables) or at room temperature (according to the manufacturer's instructions) for a maximum of 3 days before cooking. The regular coffee brew was prepared from commercially roasted coffee (100% Arabica) supplied by a national producer. The coffee brew was prepared with a mocha-type domestic coffee pot with 62.5 g of coffee per 1000 mL of water. The soluble coffee brew (100% Arabica from a national producer) was prepared following the manufacturer's instructions (2.0 g of soluble coffee per 100 mL of boiling water).

The samples were submitted to different culinary treatments: boiling, grilling, roasting, frying, and toasting. Brewing was also included as a culinary treatment (as stated above for coffees), although it involves a technological process to obtain some of the liquid foods studied (beer and red wine). In addition, for some samples, the raw food was also investigated as this is the common way to eat those foods. A total of 107 samples were obtained. Fruits, vegetables and tubers were cut in different sizes to achieve the same final texture after the different thermal treatments were applied.

Extra virgin olive oil (EVOO) was used as a cooking medium for grilling and frying. Boiling was performed at 100 ºC for 20 min at a water/food rate of 5:1. Grilling was performed at 220-250 ºC for 3 min on each side at an oil/food rate of 0.5:1. Roasting was performed at 180 ºC for 10 min. Fried foods were obtained at 180 ºC for 8 min at an oil/food rate of 5:1. Toasting was performed in a Grunkel TS140H toaster at the fourth level for 3 min at 900 W following the manufacturer's instructions. Cooking times and food/medium rates were acquired from Ramírez-Anaya et al., [5] and

adapted to our equipment and laboratory conditions. Samples and treatments are listed in **Table 5** (in supplemental information).

The utensils used for sample preparation were the following: stainless steel spoons, forks, and knives; saucepan, frying pan, a portable oven (1500 W), fryer, and toaster. All these utensils were purchased from Centro Hogar Sánchez (Granada, Spain). Samples were homogenized and stored under a nitrogen atmosphere at -80 ºC in order to avoid oxidation. All analyses were performed in duplicate.

2.3. In Vitro Gastrointestinal Digestion and Fermentation

Plant foods, after proper cooking, were submitted to *in vitro* batch digestion-fermentation in order to mimic physiological processes in the human gut, according to a protocol previously described [12]. For each sample, 5 g of food was submitted (in triplicate) to *in vitro* gastrointestinal digestion followed by *in vitro* fermentation. The food was added to falcon tubes along with three phases: oral, gastric and intestinal. The oral digestion phase was performed with α-amylase for 2 min under agitation at 37 ºC. The gastric phase was performed with pepsin for 2 h with agitation at pH 2-3 ay 37 ºC. The gastric phase was performed with bile salts and pancreatin for 2 h under agitation at pH 7 at 37 ºC. *In vitro* fermentation was performed at 37 ºC for 24 h using fecal samples from five healthy donors (mean body mass index = 21.3; no antibiotics taken for three months prior to the assay). The fecal samples were pooled together to reduce interindividual variability. The samples were then centrifuged, and the supernatants were taken for analysis. A control fermentation was performed using only the fecal fermentation solution (inoculum composed of peptone, cysteine, and resazurin).

After *in vitro* gastrointestinal digestion and fermentation, two fractions were obtained: a digested fraction (available for absorption at the small intestine) and a fermented fraction (available for absorption at the large intestine).

2.4. Antioxidant Assays

The antioxidant capacity was evaluated in the two fractions obtained after *in vitro* digestion and fermentation: the supernatant obtained after gastrointestinal digestion (potentially absorbable in the small intestine) and the supernatant obtained after fermentation (potentially absorbable in the large intestine). The sum of the two fractions accounts for the total antioxidant activity that each food could exert within the human body [13]. Three different methods were used to determine antioxidant capacity (DPPH, FRAP and Folin-Ciocalteu). All the antioxidant capacity values of the three methods were corrected for their respective blanks (enzymes, chemicals and inoculum).

$TEAC_{DPPH}$ assay. This method was conducted according to the procedure of Yen and Chen [14] on a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). Briefly, 20 μL of either digestion or fermentation supernatants was added to a 96-well plate in duplicate and mixed with 280 μL of DPPH reagent (74 mg DPPH/L methanol). The antioxidant reaction was monitored at 37 ºC for 60 min. The calibration curve was prepared with Trolox in the range of 0.01-0.4 mg/mL. Results were expressed as mmol Trolox equivalent/kg of food.

Folin-Ciocalteu assay. The method was conducted as described by Moreno-Montoro and colleagues [15] on a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). Briefly, 30 μL of either

digestion or fermentation supernatants was added in duplicate to a 96-well plate and mixed with 15 µL of Folin-Ciocalteu reactive, 190 µL of distilled water and 60 µL of 10% sodium carbonate solution. The antioxidant reaction was monitored at 37 ºC for 30 min. The calibration curve was prepared with gallic acid in the range of 0.1-2.5 mg/mL. Results were expressed as mg gallic acid equivalent/kg of food.

$TEAC_{FRAP}$ assay. The ferric reduction capacity of samples was assessed through the procedure described by Benzie and Strain [16] on a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). Briefly, 20 µL of either digestion or fermentation supernatants was added to a 96-well plate, in duplicate, and mixed with 280 µL of FRAP reagent (freshly prepared each day). The antioxidant reaction was monitored at 37 ºC for 30 min. The calibration curve was prepared with Trolox in the range of 0.01-0.4 mg/mL. Results were expressed as mmol Trolox equivalent/kg of food.

2.5. Calculations of Daily Antioxidant Intake

The individual contribution of each food group to the dietary antioxidant capacity intake was calculated, taking into account their daily consumption and the amount of food per serving [17] as well as the antioxidant capacity previously measured for the samples. The antioxidant capacity of each food referred to the usual serving size in Spain [18] and was compared with the results previously published by Saura-Calixto and Goñi [19].

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 a significant variation ($p < 0.05$). As a factor for ANOVA, we used the type of cooking (boiled, brewed, fried, grilled, raw, roasted and toasted), type of food (alcoholic drinks, cereals, cocoa, coffee, fruits, legumes, nuts, oils, tubers and vegetables) and type of sample (cereals: biscuits, bread, breakfast cereals, penne and rice; fruits: apple, banana, grapes, olives, orange, peach and plum; vegetables: cabbage, carrot, cauliflower, eggplant, lettuce, onion, pepper, spinach, tomato and zucchini). Statistical analysis was performed using raw vegetables and the mean of all food groups as the reference groups. Pearson's correlation coefficient was calculated to show the linear relation between antioxidant capacity at $p < 0.05$. To obtain the significance between the different levels within the same group, Tukey's test was performed. All statistical analyses were performed using Statgraphics Plus software, version 5.1.

3. Results

We tested the potential physiological antioxidant capacity of plant foods after invitro digestion-fermentation with three different methods (DPPH, FRAP and Folin-Ciocalteu). In general, a linear correlation was obtained by the Spearman method between the three methods (**Figure 5** in supplemental information). The significant correlations found *($p < 0.05$)* were positive, with values around Rs = 0.80.

In the following sections, a deeper description of the results obtained by type of cooking, type of food and each group of plant foods will be reported.

3.1. Samples by Type of Cooking

Cooking methods had an impact on the antioxidant capacity of the digested fraction, as will be explained in the following sections.

3.1.1. Gastrointestinal Digested Fraction

Raw foods showed a significantly *($p < 0.05$)* higher antioxidant capacity than boiled and toasted foods for the $TEAC_{DPPH}$ assay (**Table 6** in supplemental information) but lower than fried foods (**Figure 18A**). In the case of the Folin-Ciocalteu method, we observed a significantly higher antioxidant capacity in toasted foods vs. raw foods ($p < 0.05$) as opposed to the previous method; however, raw foods were still more antioxidant than those cooked with the other methods (**Figure 18B**). For the $TEAC_{FRAP}$ method, raw foods were more ($p < 0.05$) antioxidant than boiled, grilled and toasted foodstuffs, as in the other methods (**Figure 18C**).

Comparing the mean antioxidant capacity of digested samples after different cooking treatments, the following significant differences were found (ANOVA paired comparison; $p < 0.05$): for the $TEAC_{DPPH}$ method, fried foods were more antioxidant than boiled; for the Folin-Ciocalteu method, toasting resulted in higher antioxidant values than those of the other cooking methods; for the $TEAC_{FRAP}$ method, raw foods showed higher antioxidant capacity than grilled foods.

3.1.2. Gastrointestinal Fermented Fraction

Microbial fermentation has a deep impact on food antioxidant capacity [12] since the gut microbiota is able to metabolize those undigested nutrients reaching the large intestine and release many metabolites with a potent antioxidant capacity. With the $TEAC_{DPPH}$ method, the antioxidant capacity of the fermented fraction was significantly ($p < 0.05$) lower in boiled and toasted vegetables compared to raw foods (**Figure 18A**). On the other hand, the Folin-Ciocalteu method showed a significantly ($p < 0.05$) lower antioxidant capacity in brews and toasted foods compared to raw foods (**Figure 18B**). The $TEAC_{FRAP}$ results were on the same line (**Figure 18C**).

3.1.3. Total Antioxidant Capacity

In general, the contribution of the digested fraction to the total antioxidant capacity was much lower than that of the fermented fraction since many different bioactive compounds could be released from the food matrix by the microbial activity in addition to a potential generation of new antioxidant metabolites.

Similarly to the fermented fraction, the total antioxidant capacity of boiled and roasted foods was lower ($p < 0.05$) than that of raw foods (**Figure**

18A). Overall, for most of the cooking techniques, the contribution of the digested fraction to the total antioxidant capacity was around 10% (**Figure 19**) but for brewed foods (accounted for 23%) and toasted plant foods (just 1%).

The Folin-Ciocalteu method showed also a significantly ($p < 0.05$) lower total antioxidant capacity for brewed and toasted foods (**Figure 18B**). In this case, however, the digestion fraction had a lower contribution to the total antioxidant capacity for most of the cooking techniques (around 4%), compared to DPPH. The same results were obtained for the FRAP method (**Figure 18C and Figure 19**). For this method, it is noteworthy to mention that although the digested fraction contributed very little to the total reducing capacity (around 3%), in the case of brews, 50% of the antioxidant capacity was obtained for both fractions (**Figure 19**).

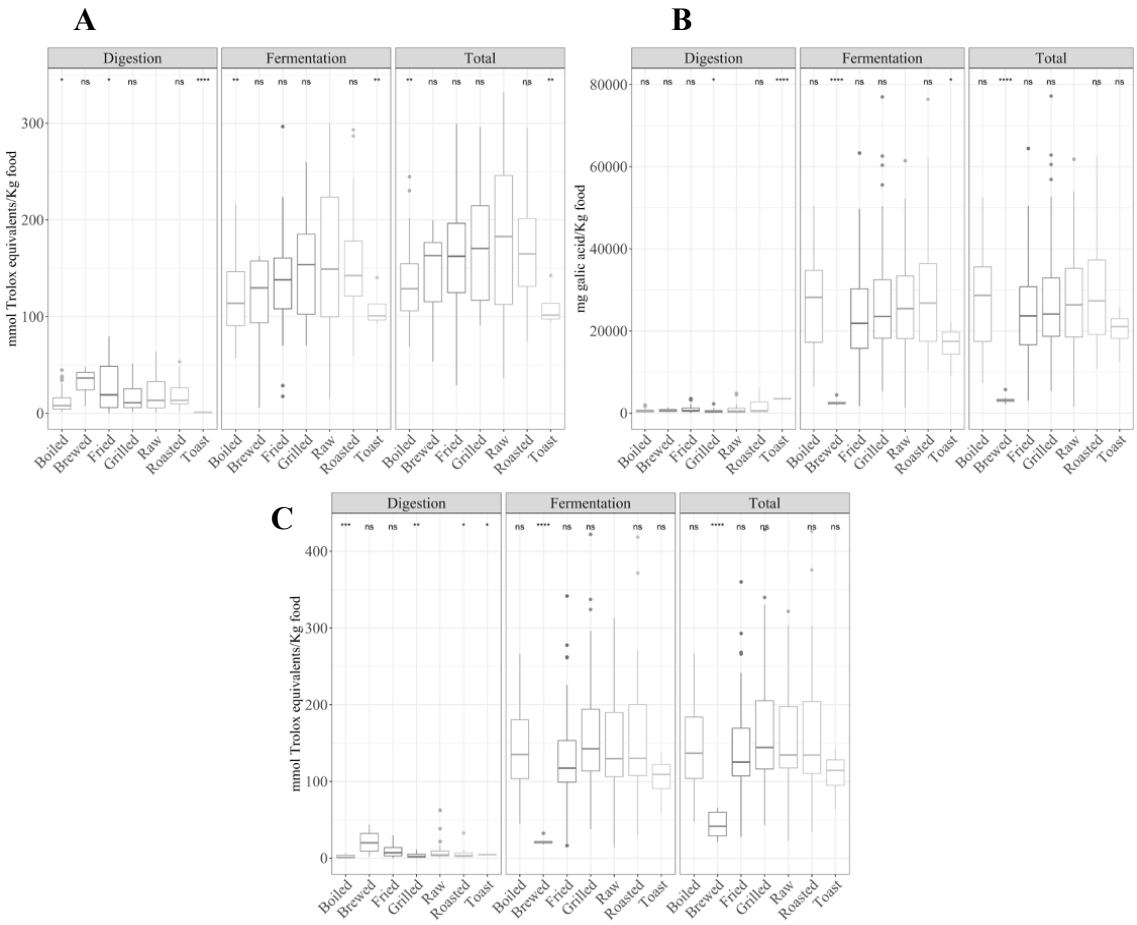

Figure 18. Antioxidant capacity of plant foods (obtained after *in vitro* digestion and fermentation) depending on the cooking technique **(18A** for $TEAC_{DPPH}$, **18B** for Folin-Ciocalteu and **18C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using raw vegetables as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

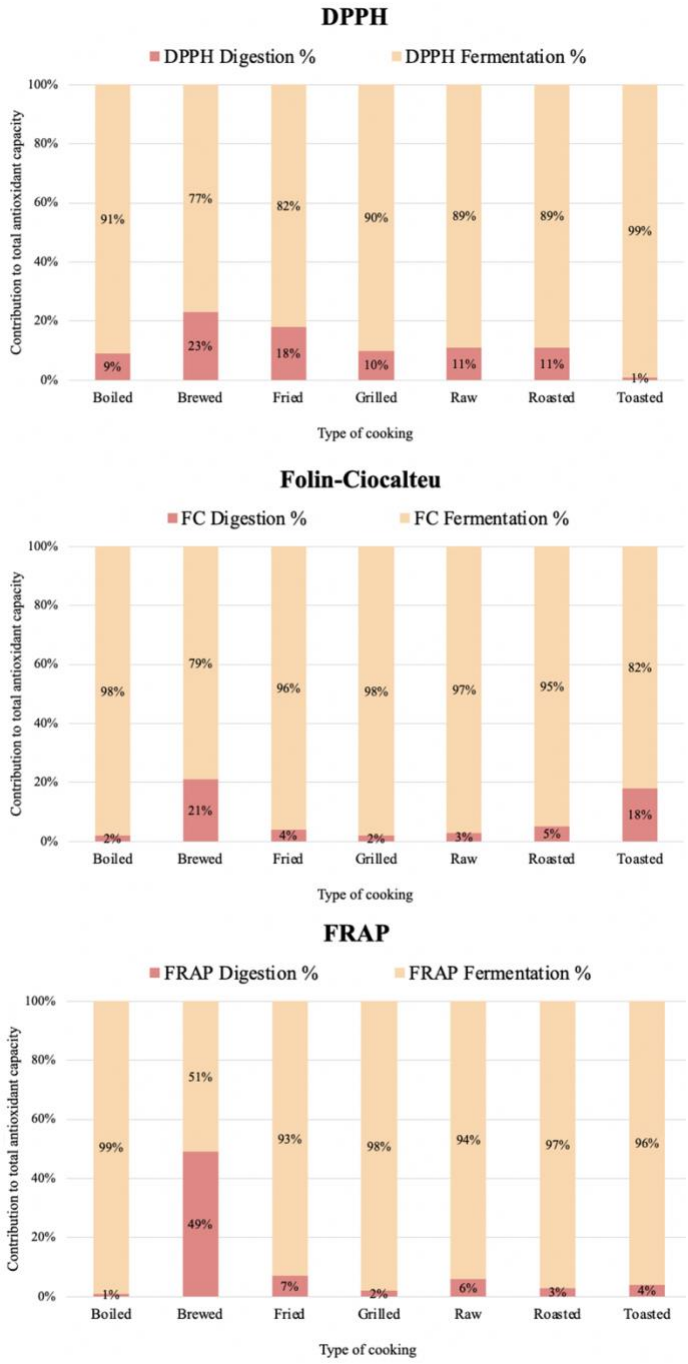

Figure 19. Contribution to the total antioxidant capacity of each fraction depending on the cooking technique.

3.2. Samples by Type of Food

The type of plant had an important effect on the antioxidant capacity since the food matrix and composition are different.

3.2.1. Gastrointestinal Digested Fraction

Compared with the mean antioxidant capacity of all plant foods, coffee, fruits and legumes had a significantly higher antioxidant capacity ($p < 0.05$) for the $TEAC_{DPPH}$ method (**Table 7** in supplemental information); however, lower values were obtained for cereals, nuts and tubers (**Figure 20A).** On the other hand, the Folin-Ciocalteu method showed a higher ($p < 0.05$) antioxidant capacity for nuts, tubers, alcoholic drinks, fruits and vegetables (**Figure 20B**). Regarding reducing capacity ($TEAC_{FRAP}$), it was higher ($p < 0.05$) for coffee but lower for tubers, fruits and vegetables (**Figure 20C**).

When comparisons were made between different types of plant foods (ANOVA paired comparison; $p < 0.05$), the following significant differences were found: for the $TEAC_{DPPH}$ method, fruits had a higher antioxidant capacity than cereals and vegetables, whereas legumes were more antioxidant than cereals, nuts, tubers and vegetables. In the case of the Folin-Ciocalteu method, cocoa and nuts had a higher mean than the rest of the foods (with no significant differences between them), and fruits were more antioxidant than cereals. Finally, for the $TEAC_{FRAP}$ method, cocoa and coffee were more antioxidant than the other groups.

3.2.2. Gastrointestinal Fermented Fraction

Cocoa and legumes had a stronger antioxidant capacity against DPPH radicals ($p < 0.05$) than the other food groups (**Figure 20A**). On the other

hand, Folin-Ciocalteu showed a significantly ($p < 0.05$) higher antioxidant capacity for cocoa, legumes and tubers (**Figure 20B**). The $TEAC_{FRAP}$ results followed the same tendency as the Folin-Ciocalteu method (**Figure 20C**).

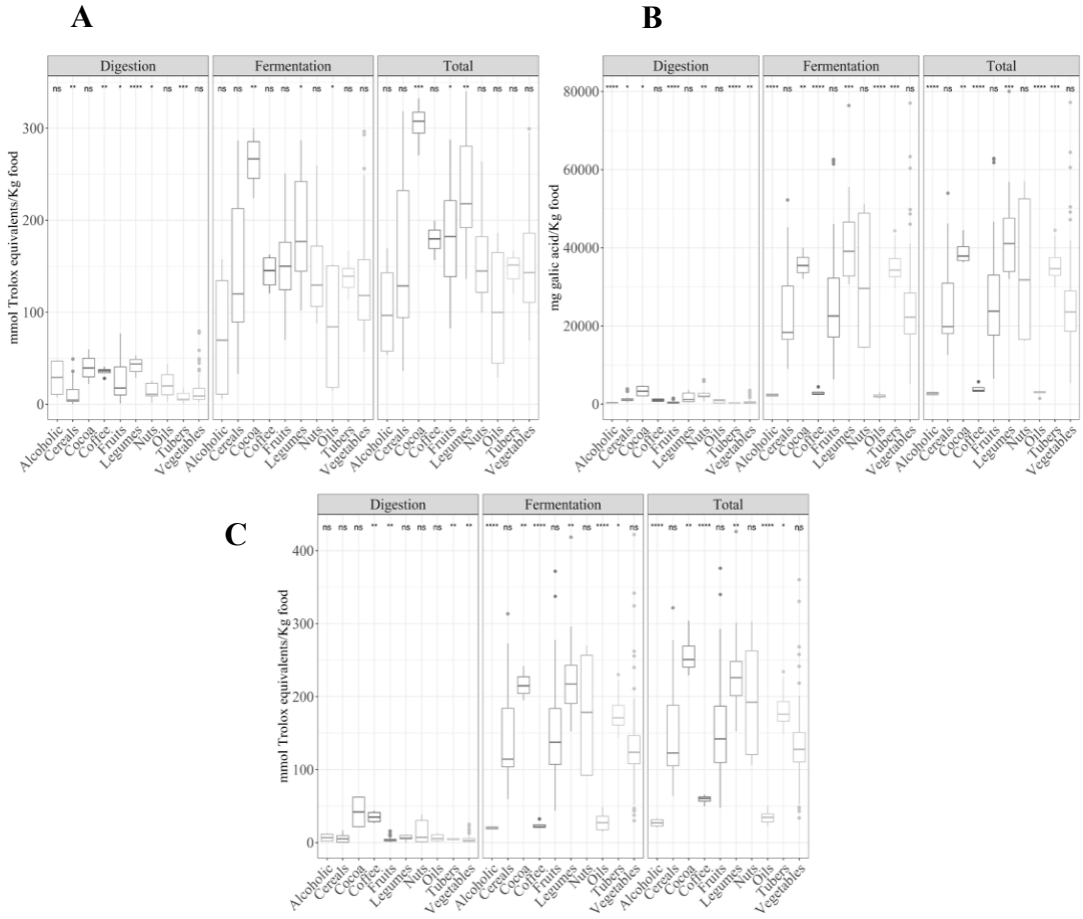

Figure 20. Antioxidant capacity of plant foods (obtained after *in vitro* digestion and fermentation) depending on the food group (**20A** for $TEAC_{DPPH}$, **20B** for Folin-Ciocalteu and **20C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using the mean antioxidant capacity of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant

3.3. Detailed Analysis of Large Food Groups

Data on the digested and fermented fractions, as well as the total antioxidant capacity of those samples from larger groups (cereals, fruits and vegetables), were also analyzed separately.

3.3.1. Cereals

Regarding the effect of cooking methods on the antioxidant capacity of cereals, the mean antioxidant capacity of raw cereals (ANOVA paired comparisons, $p < 0.05$) measured with $TEAC_{DPPH}$ was higher than that of the other cooking methods for both the digested and fermented fractions, resulting in a higher total antioxidant capacity (**Figure 21A**). In the case of the Folin-Ciocalteu method (**Figure 21B**), the antioxidant capacity of the digested fraction decreased as follows: toasted > raw > boiled. However, for the fermented fraction and total antioxidant capacity, raw cereals were the most antioxidant foodstuffs ($p < 0.05$). Finally, the reducing capacity of cooked cereals ($TEAC_{FRAP}$) showed the same behavior as the DPPH method (**Figure 21C**).

On the other hand, when samples were compared, depending on the type of cereal-based food, they behaved similarly irrespective of the antioxidant assay (**Figure 22A-C**). For the digested fraction, biscuits were more antioxidant ($p < 0.05$) than the other foods. However, for the fermented fraction and total antioxidant capacity, the following order was obtained: biscuits = breakfast cereals > bread = rice = pasta. Comparisons were also made between refined and whole-grain cereal products, but no significant differences were observed.

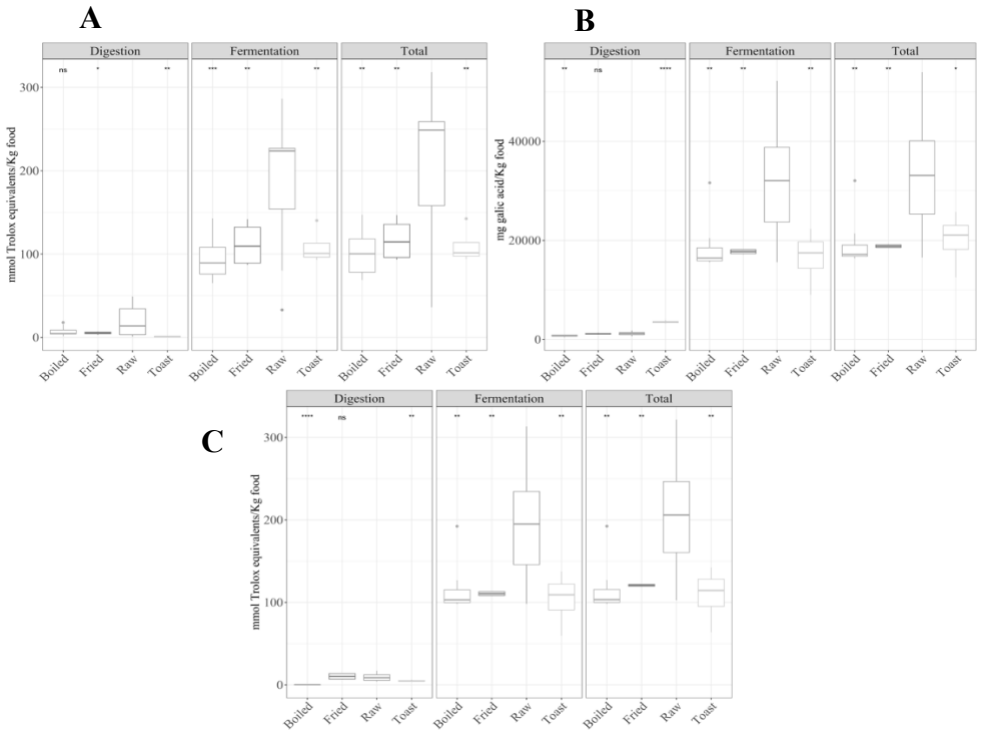

Figure 21. Antioxidant capacity of digested-fermented cereals depending on the type of cooking technique (**21A** for $TEAC_{DPPH}$, **21B** for Folin-Ciocalteu and **21C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using raw cereals as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

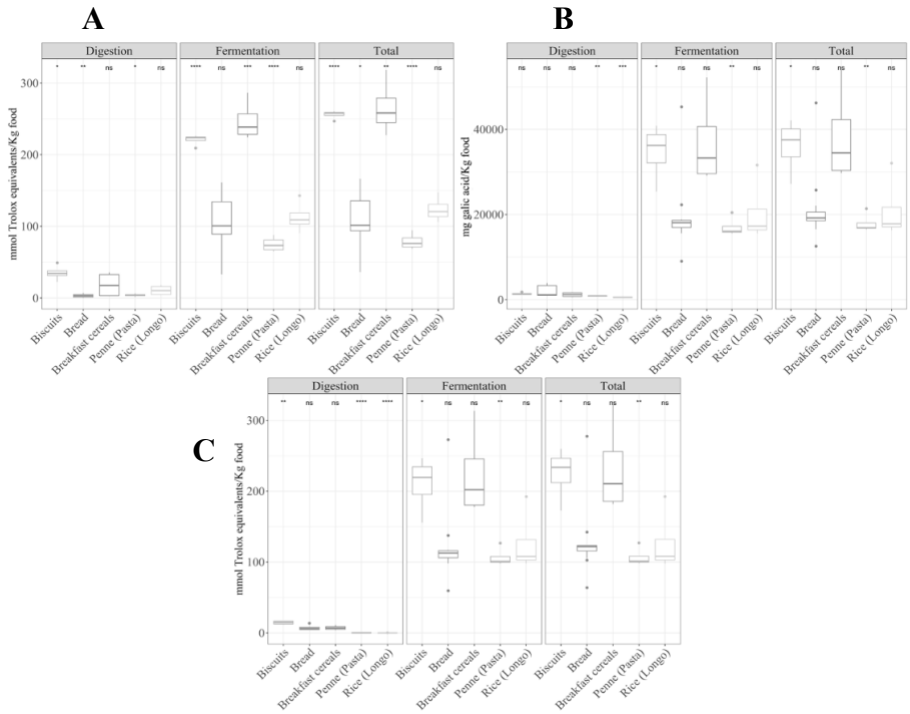

Figure 22. Antioxidant capacity of digested-fermented cereals depending on the sample type (**22A** for $TEAC_{DPPH}$, **22B** for Folin-Ciocalteu and **22C** for $TEAC_{FRAP}$). Statistical analysis was performed through ANOVA using the mean of all food groups as the reference value. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.3.2. Fruits

Although fruits are usually consumed in raw form, they were submitted to different cooking techniques since some are heat-treated, especially for some desserts. In the digested fractions, fried and roasted fruits were more antioxidant ($p < 0.05$) than raw and grilled foods but only for the Folin-Ciocalteu method (**Figure 23B**). No statistically significant differences were observed for the $TEAC_{DPPH}$ (**Figure 23A**) and $TEAC_{FRAP}$ assays (**Figure**

23C). No significant differences among the four cooking techniques were observed for the fermented fraction or total antioxidant capacity.

Seven different fruits (apple, banana, grape, olive, orange, peach and plum) were analyzed. The digested fraction of olives and plum were more antioxidant ($p < 0.05$) than the other fruits but just for the $TEAC_{DPPH}$ method (**Figure 24A**). For the fermented fraction and total antioxidant capacity, the following order was obtained: olives > peach = plum > grape = orange = banana = apple for the $TEAC_{DPPH}$ (**Figure 24A**), Folin-Ciocalteu (**Figure 24B**) and $TEAC_{FRAP}$ (**Figure 24C**) methods.

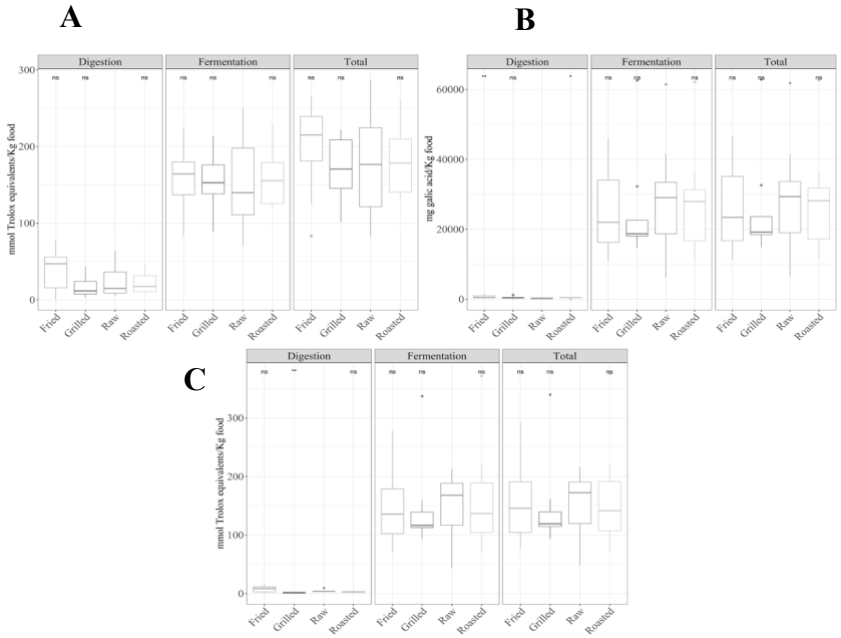

Figure 23. Antioxidant capacity of digested-fermented fruits depending on the type of cooking technique (**23A** for $TEAC_{DPPH}$, **23B** for Folin-Ciocalteu and **23C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using raw fruits as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

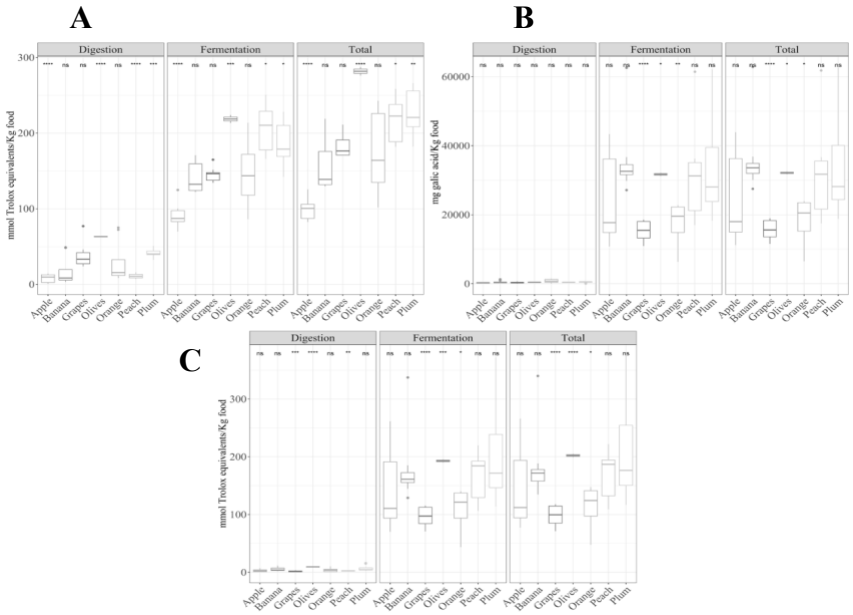

Figure 24. Antioxidant capacity of digested-fermented fruits depending on sample type (**24A** for $TEAC_{DPPH}$, **24B** for Folin-Ciocalteu and **24C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using the mean of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.3.3. Vegetables

The analysis of the effect of cooking techniques on vegetables was interesting since these types of foods can be eaten both raw or heat-treated. In the case of the digestion fraction, for the $TEAC_{DPPH}$ (**Figure 25A**), Folin-Ciocalteu (**Figure 25B**) and $TEAC_{FRAP}$ (**Figure 25C**) methods, fried vegetables were more antioxidant than raw vegetables (ANOVA paired comparisons, $p < 0.05$). No statistically significant differences were observed for either the fermented fraction or total antioxidant capacity, although after-fermentation values were always much higher than those obtained for the digested fraction.

Ten different vegetables were individually studied (cabbage, carrot, cauliflower, eggplant, lettuce, onion, pepper, spinach, tomato and zucchini). Overall, the antioxidant capacity of the digested fractions across vegetables was similar and not significant ($p < 0.05$) differences were found between most, regardless of the antioxidant assay used (**Figure 26A-C**). Only cabbage, carrot and zucchini were less antioxidant ($p < 0.05$) than the others. For the fermented fraction and total antioxidant capacity vegetables behaved differently depending on the antioxidant capacity method assessed, and no clear tendency was observed.

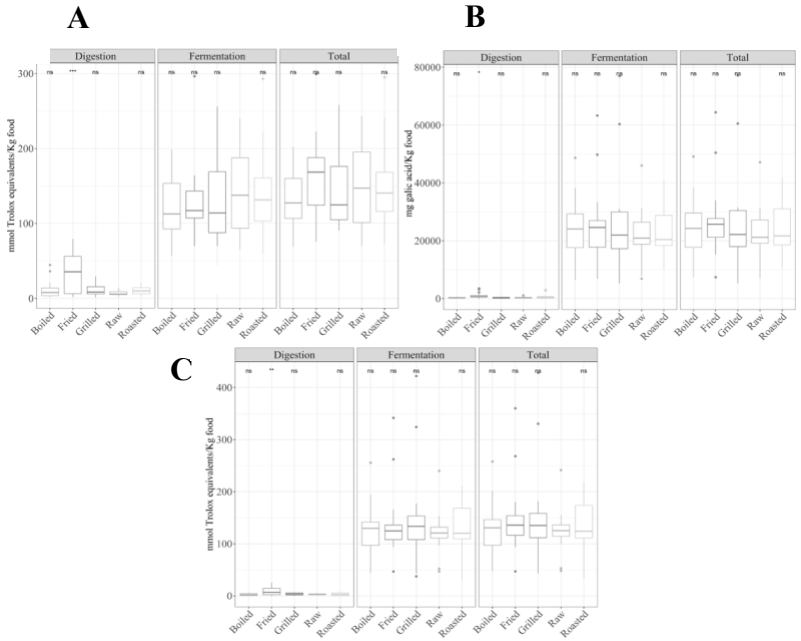

Figure 25. Antioxidant capacity of digested-fermented vegetables depending on the type of cooking technique type (**25A** for $TEAC_{DPPH}$, **25B** for Folin-Ciocalteu and **25C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using the mean of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

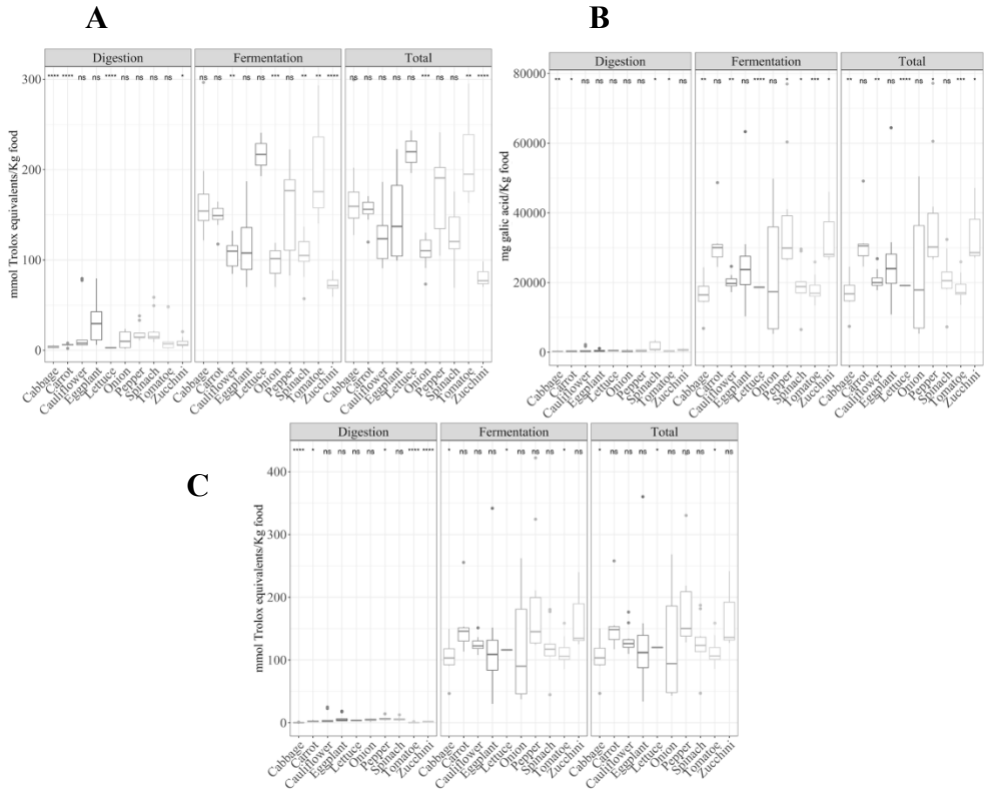

Figure 26. Antioxidant capacity of digested-fermented vegetables depending on sample type (**26A** for $TEAC_{DPPH}$, **26B** for Folin-Ciocalteu and **26C** for $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using the mean of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

4. Discussion

It is known that heat treatment affects the antioxidant capacity of foods [5,6,9]. In our study, we found that the effect of the cooking technique strongly depends on the antioxidant capacity method used, which agrees with previous results [20]. The different cooking techniques (boiling, grilling, roasting frying and toasting) maintained or increased the total antioxidant capacity of the raw plant foods, suggesting that different antioxidant compounds are generated by thermal processing during the Maillard reaction [21-26] or that more antioxidant compounds are released by cell breakage [27-29]. On the other hand, the total antioxidant capacity of raw samples was similar to some thermal processes, suggesting that it is not necessary to cook certain foods to achieve the extraction of their antioxidant substances, as suggested by other authors [21-24].

In general, the antioxidant capacity of the fermented fraction is much higher (from 80 to 98% of the total antioxidant capacity), potentially due to the essential role that the gut microbiota plays in the release of antioxidant compounds from the undigested food matrix [12,29]. In fact, antioxidant capacity values obtained for digested and fermented foods are higher than those of foods not submitted to these processes [30]. This reinforces the beneficial effect of digestion and fermentation in the release and transformation of bioactive compounds.

The highest total antioxidant capacity was found in cocoa and the lowest in alcoholic drinks. Cocoa is rich in phenolic compounds, substances with a high antioxidant capacity [31], which explains the larger values obtained regardless of the assay, corroborating previous studies [13,29]. The antioxidant capacity of alcoholic drinks was very low, but other liquid foods

(coffee and oils) were highly antioxidant, in line with other papers [31-33] even as a source of lipophilic antioxidant compounds [34].

In cereals, biscuits and breakfast cereals were the most antioxidant foods, potentially due to the generation of melanoidins during thermal processing [22,32]. In the case of rice and pasta, they showed low antioxidant capacity but with values similar to those described above [35]. Such a low antioxidant capacity could be related to the loss of antioxidant compounds in cooking water and also to a low-intensity thermal-treatment, which does not favor the development of antioxidant compounds derived from the Maillard reaction and caramelization [29,32]. Frying increased the antioxidant capacity of bread, potentially due to the enrichment with olive oil. However, in fruits and vegetables, the antioxidant capacity was quite homogeneous, with no differences among cooking techniques.

The antioxidant capacity of a given food could be of interest in regard to its shelf life; higher antioxidant capacity means lower oxidative degradation. However, much attention is drawn to human health. Therefore, it is interesting to calculate the contribution of each kind of food to the daily antioxidant intake in a regular diet. Accordingly**,** **Table 4** shows the contribution of plant foods to the daily antioxidant and total phenolic intake in the Spanish diet. Saura-Calixto and Goñi [19] calculated a total antioxidant intake of 6014 μmol Trolox equivalents/d according to the FRAP method, which was used as a reference (100% total antioxidant capacity) to compare our results. Taking into account the daily intake of each food item/group in Spain [17], the daily mean intake ranged from 296 to 22,423 mol Trolox equivalents/d (coffee and cereals, respectively), which in turn is a contribution of 4.92-373% of the total antioxidant capacity of the diet. A more realistic approach can be obtained by using serving size [18] to calculate the

intake of antioxidant capacity per serving (**Table 4**). With such an approach, the daily intake of antioxidant capacity ranges from 348 to 31,253 μmol Trolox equivalents/d for oils and tubers, respectively. In turn, this realistic approach reaches up to 520% of the daily antioxidant capacity intake calculated [19]. Such high daily intake of antioxidant capacity could be explained taking into account that former calculations performed by Saura-Calixto and Goñi were done with the antioxidant extraction method, which does not take into account the effects of digestion and fermentation in the release of antioxidant compounds.

Table 4. Contribution of plant foods consumption to the daily antioxidant capacity (AOX) in the Spanish diet.

Food type	***Analytical assay***	***AOX daily intake[1] (μmol Trolox/day)***	***AOX serving intake[2] (μmol Trolox/serving)***	***Mean contribution to daily antioxidant capacity intake (%)[3]***	***Mean contribution to daily antioxidant capacity intake per serving (%)[3]***
Alcoholic drinks	FRAP	1228	3649	20.4	60.7
Cereals	FRAP	22423	6343	373	106
Cocoa	FRAP	909	6888	15.1	115
Coffee	FRAP	296	1426	4.92	23.7
Fruits	FRAP	19130	22230	318	370
Legumes	FRAP	1250	16655	20.8	277
Nuts	FRAP	1257	5808	20.9	96.6
Oils	FRAP	1157	348	19.2	5.8
Tubers	FRAP	14804	31253	246	520
Vegetables	FRAP	15336	16001	255	266

[1]Considering consumption for a whole year.

[2]Considering the intake of 1 serving.

[3]Considering the data of Saura-Calixto and Goñi [19]

5. Conclusions

In conclusion, this study reinforces the concept that plant foods are a great source of antioxidant compounds for human beings. After *in vitro* digestion and fermentation, cocoa and legumes stand out among all foods for their antioxidant capacity. In the group of fruits, olives and bananas were the most relevant, whereas lettuce and pepper were the most antioxidant foods in the vegetables group. In addition, based on the results included in this paper, the antioxidant capacity of plant foods has been underestimated in the last decades since the key role of the gastrointestinal system on the release and transformation of antioxidant molecules was not taken into consideration. Therefore, future studies should be conducted including this new approach to test the physiological transformation of foods to calculate their contribution to the daily antioxidant capacity intake.

SUPPLEMENTAL INFORMATION

Table 5. Plant foods and cooking conditions.

Group	Sample name	Cooking method
Alcoholic	Beer	Brewed
Alcoholic	Red wine	Brewed
Cereals	Biscuits	Raw
Cereals	Whole grain biscuits	Raw
Cereals	Bread	Fried
Cereals	Bread	Raw
Cereals	Bread	Toasted
Cereals	Whole grain bread	Fried
Cereals	Whole grain bread	Raw
Cereals	Whole grain bread	Toasted
Cereals	Breakfast cereals	Raw
Cereals	Whole grgain breakfast cereals	Raw
Cereals	Penne (Pasta)	Boiled
Cereals	Whole grain penne	Boiled
Cereals	Rice (Longo)	Boiled
Cereals	Whole grain rice (Longo)	Boiled
Cocoa	Dark Chocolate	Raw
Cocoa	Nutella	Raw
Coffee	Coffee	Brewed
Coffee	Instant coffee	Brewed
Fruits	Apple	Fried
Fruits	Apple	Grilled
Fruits	Apple	Raw
Fruits	Banana	Fried
Fruits	Banana	Grilled
Fruits	Banana	Raw
Fruits	Banana	Roasted
Fruits	Grapes	Fried
Fruits	Grapes	Grilled
Fruits	Grapes	Raw
Fruits	Grapes	Roasted

Fruits	Olives	Raw
Fruits	Orange	Fried
Fruits	Orange	Grilled
Fruits	Orange	Raw
Fruits	Orange	Roasted
Fruits	Peach	Fried
Fruits	Peach	Raw
Fruits	Peach	Roasted
Fruits	Plum	Fried
Fruits	Plum	Grilled
Fruits	Plum	Raw
Fruits	Plum	Roasted
Legumes	Beans (Kidney)	Boiled
Legumes	Beans (Kidney)	Grilled
Legumes	Kidney beans	Roasted
Legumes	Lentils	Boiled
Legumes	Lentils	Grilled
Legumes	Lentils	Roasted
Nuts	Nut mix	Roasted
Nuts	Nut mixture	Fried
Nuts	Nut mixture	Raw
Nuts	Peanuts	Boiled
Nuts	Peanuts	Grilled
Nuts	Peanuts	Roasted
Oils	Olive oil	Fried
Oils	Olive oil	Raw
Oils	Sunflower oil	Fried
Oils	Sunflower oil	Raw
Tubers	Potato	Boiled
Tubers	Potato	Fried
Tubers	Sweet potato	Boiled
Tubers	Sweet potato	Fried
Vegetables	Cabbage	Boiled
Vegetables	Cabbage	Fried

Vegetables	Cabbage	Grilled
Vegetables	Cabbage	Roasted
Vegetables	Carrot	Boiled
Vegetables	Carrot	Fried
Vegetables	Carrot	Grilled
Vegetables	Carrot	Raw
Vegetables	Cauliflower	Boiled
Vegetables	Cauliflower	Fried
Vegetables	Cauliflower	Grilled
Vegetables	Cauliflower	Raw
Vegetables	Cauliflower	Roasted
Vegetables	Eggplant	Boiled
Vegetables	Eggplant	Fried
Vegetables	Eggplant	Grilled
Vegetables	Eggplant	Raw
Vegetables	Eggplant	Roasted
Vegetables	Lettuce	Raw
Vegetables	Onion	Boiled
Vegetables	Onion	Fried
Vegetables	Onion	Grilled
Vegetables	Onion	Raw
Vegetables	Pepper	Boiled
Vegetables	Pepper	Fried
Vegetables	Pepper	Grilled
Vegetables	Pepper	Raw
Vegetables	Pepper	Roasted
Vegetables	Spinach	Boiled
Vegetables	Spinach	Fried
Vegetables	Spinach	Grilled
Vegetables	Spinach	Raw
Vegetables	Spinach	Roasted
Vegetables	Tomato	Boiled
Vegetables	Tomato	Fried
Vegetables	Tomato	Grilled

Vegetables	Tomato	Raw
Vegetables	Tomato	Roasted
Vegetables	Zucchini	Boiled
Vegetables	Zucchini	Fried
Vegetables	Zucchini	Raw
Vegetables	Zucchini	Roasted

Table 6. Antioxidant capacity of *in vitro* digested-fermented plant foods depending on the cooking method.

Cooking technique	**$TEAC_{DPPH}$ (µmol Trolox/g)**		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	12.6 ± 11.7^{a}	121 ± 38.4^{a}	134 ± 41.2^{a}
Brewed	$31.9 \pm 15.2^{a,b}$	110 ± 63.9^{a}	$141 \pm 56.0^{a,b}$
Fried	29.3 ± 17.1^{b}	134 ± 49.0^{a}	$163 \pm 58.6^{a,b}$
Grilled	$17.3 \pm 15.2^{a,b}$	155 ± 57.4^{a}	172 ± 59.8^{b}
Raw	$19.5 \pm 17.2^{a,b}$	157 ± 71.2^{a}	$176 \pm 78.4^{a,b}$
Roasted	$19.3 \pm 14.7^{a,b}$	153 ± 55.7^{a}	$173 \pm 62.1^{a,b}$
Toasted	1.09 ± 0.83^{a}	109 ± 21.6^{a}	$110 \pm 22.2^{a,b}$
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	552 ± 432^{a}	27343 ± 12142^{b}	149 ± 29.0^{b}
Brewed	$691 \pm 442^{a,b}$	2639 ± 763^{a}	11.6 ± 4.76^{a}
Fried	$916 \pm 753^{a,b}$	23154 ± 12815^{b}	24070 ± 12762^{b}
Grilled	520 ± 472^{a}	29271 ± 17069^{b}	29791 ± 17227^{b}
Raw	$840 \pm 960^{a,b}$	25149 ± 13037^{b}	25988 ± 13309^{b}
Roasted	1447 ± 1686^{b}	28849 ± 15322^{b}	30295 ± 16180^{b}
Toasted	3537 ± 268^{c}	16574 ± 5626^{b}	20110 ± 5562^{b}
	$TEAC_{FRAP}$ (µmol Trolox/g)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	2.05 ± 2.05^{a}	148 ± 59.3^{b}	150 ± 60.0^{b}
Brewed	21.1 ± 16.5^{b}	22.0 ± 4.49^{a}	43.2 ± 18.1^{a}
Fried	9.19 ± 7.64^{c}	130 ± 65.3^{b}	139 ± 67.3^{b}
Grilled	$3.35 \pm 2.85^{a,c}$	165 ± 88.0^{b}	168 ± 88.7^{b}
Raw	9.19 ± 13.1^{d}	144 ± 66.7^{b}	153 ± 70.9^{b}
Roasted	$5.00 \pm 6.02^{a,c,d}$	162 ± 83.3^{b}	167 ± 86.3^{b}
Toasted	$4.89 \pm 0.96^{a,c,d}$	104 ± 33.1^{b}	109 ± 33.5^{b}

Table 7. Antioxidant capacity of *in vitro* digested-fermented plant foods depending on the group.

Food	$TEAC_{DPPH}$ (µmol Trolox/g)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Alcoholic drinks	28.4 ± 21.8[a,b]	75.7 ± 77.8[a,c]	104 ± 57.0[a,c]
Cereals	11.2 ± 13.6[a]	140 ± 67.3[a,c]	151 ± 76.8[a,c]
Cocoa	40.2 ± 16.4[a,b]	264 ± 33.1[b]	305 ± 25.8[b]
Coffee	35.5 ± 5.31[a,b]	143 ± 20.1[a,c]	179 ± 18.2[a,c]
Fruits	27.0 ± 21.3[b]	156 ± 44.3[a,c]	183 ± 54.8[a,c]
Legumes	19.3 ± 14.7[b]	187 ± 58.4[b]	229 ± 61.5[b]
Nuts	1.09 ± 0.83[a]	143 ± 52.3[a,c]	157 ± 47.1[a,c]
Oils	41.9 ± 8.01[a,b]	84.1 ± 68.9[a]	105 ± 70.5[a]
Tubers	14.2 ± 8.53[a]	140 ± 18.1[a,c]	148 ± 17.3[a,c]
Vegetables	21.4 ± 15.1[a]	132 ± 51.8[a,c]	148 ± 51.7[a,c]
Mean	*24.4 ± 13.5*	*147 ± 49.2*	*171 ± 48.1*
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Alcoholic drinks	360 ± 100[a,c]	2313 ± 358[a,c]	2673 ± 441[a,c]
Cereals	1395 ± 955[a]	23976 ± 10725[c]	25371 ± 10640[c]
Cocoa	3382 ± 1475[b]	35753 ± 3428[b]	39125 ± 3752[b]
Coffee	1020 ± 393[a,c]	2964 ± 974[a,c]	3985 ± 1189[a,c]
Fruits	466 ± 351[a]	26470 ± 13442[c]	26937 ± 13453[c]
Legumes	1655 ± 1222[a,c]	42451 ± 13380[a,c]	44116 ± 13837[a,c]
Nuts	2686 ± 167[b]	31817 ± 17443[b]	34504 ± 18034[b]
Oils	766 ± 498[a,c]	2131 ± 621[a,c]	2898 ± 615[a,c]
Tubers	300 ± 101[a,c]	35755 ± 5265[a,c]	35954 ± 5181[a,c]
Vegetables	570 ± 679[a,c]	24544 ± 12333[a,c]	25115 ± 12375[a,c]
Mean	*1260 ± 745*	*22809 ± 7797*	*24069 ± 7952*
	$TEAC_{FRAP}$ (µmol Trolox/g)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Alcoholic drinks	7.04 ± 5.95[a]	20.1 ± 2.11[a]	27.1 ± 6.03[a]
Cereals	6.27 ± 5.31[a]	147 ± 63.0[a]	154 ± 65.2[a]
Cocoa	42.1 ± 23.5[b]	217 ± 20.2[b]	259 ± 32.3[b]
Coffee	35.2 ± 8.26[b]	23.9 ± 5.72[b]	59.1 ± 6.87[b]
Fruits	4.34 ± 3.83[a]	151 ± 67.7[a]	155 ± 69.0[a]
Legumes	6.54 ± 3.79[a]	231 ± 70.1[a]	238 ± 70.8[a]
Nuts	15.6 ± 16.4[a]	178 ± 85.8[a]	194 ± 78.7[a]
Oils	6.54 ± 4.95[a]	28.2 ± 12.8[a]	34.8 ± 8.81[a]
Tubers	4.80 ± 0.95[a]	179 ± 30.9[a]	184 ± 30.5[a]
Vegetables	4.31 ± 4.85[a]	133 ± 62.8[a]	137 ± 64.2[a]
Mean	*13.3 ± 7.78*	*131 ± 42.1*	*144 ± 43.3*

Table 8. Antioxidant capacity of *in vitro* digested-fermented cereals depending on the cooking method.

Cereals

Cooking technique	DPPH (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	7.38 ± 5.98[a]	94.0 ± 25.7[a]	101 ± 27.5[a]
Fried	5.27 ± 1.77[a]	112 ± 27.7[a]	117 ± 26.3[a]
Raw	19.0 ± 17.4[b]	190 ± 73.9[b]	209 ± 84.8[b]
Toasted	1.09 ± 0.83[a]	109 ± 21.6[a]	110 ± 22.2[a]
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	731 ± 207[a]	18738 ± 5453[a]	19468 ± 5338[a]
Fried	1137 ± 158[a,b,c]	17706 ± 466[a]	18842 ± 332[a]
Raw	1210 ± 368[b]	32026 ± 11394[b]	33236 ± 11529[b]
Toasted	3537 ± 268[c]	16574 ± 5626[a]	20110 ± 5562[a]
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	0.23 ± 0.16[a]	117 ± 32.1[a]	117 ± 32.0[a]
Fried	10.2 ± 4.12[a]	111 ± 2.74[a]	121 ± 1.44[a]
Raw	9.45 ± 4.51[b]	195 ± 67.0[b]	204 ± 67.7[b]
Toasted	4.89 ± 0.96[a]	104 ± 33.1[a]	109 ± 33.5[a]

Table 9. Antioxidant capacity of *in vitro* digested-fermented cereals depending on the cereal type.

Sample	DPPH (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Biscuits	35.1 ± 10.9[b]	221 ± 7.69[b]	256 ± 6.09[b]
Bread	3.21 ± 2.23[a]	108 ± 35.1[a]	111 ± 35.1[a]
Breakfast cereals	18.6 ± 17.7[a]	247 ± 28.0[a]	266 ± 38.5[b]
Penne (Pasta)	4.02 ± 2.05[a]	75.9 ± 10.4[a]	79.0 ± 11.4[a]
Rice (Longo)	10.7 ± 7.04[a]	113 ± 21.8[a]	124 ± 17.5[b]
Mean	*14.3 ± 7.98*	*153 ± 20.6*	*167 ± 21.7*
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Biscuits	1420 ± 264[b]	34688 ± 6731[b]	36108 ± 6487[b]
Bread	1896 ± 1224[a]	19561 ± 8665[a]	21457 ± 8385[a]
Breakfast cereals	1193 ± 554[a]	36987 ± 10737[b]	38180 ± 11221[b]
Penne (Pasta)	884 ± 84.5[a]	17043 ± 2294[a]	17927 ± 2311[a]
Rice (Longo)	578 ± 173[a]	20432 ± 7514[b]	21010 ± 7403[b]
Mean	*1194 ± 460*	*25742 ± 7188*	*26936 ± 7161*
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Biscuits	14.6 ± 2.54[b]	210 ± 39.6[b]	225 ± 37.5[b]
Bread	7.20 ± 3.42[a]	121 ± 51.0[a]	129 ± 50.5[a]
Breakfast cereals	7.26 ± 3.13[a]	224 ± 63.1[b]	231 ± 64.8[b]
Penne (Pasta)	0.31 ± 0.14[a]	107 ± 13.5[a]	107 ± 14.5[a]
Rice (Longo)	0.16 ± 0.17[a]	127 ± 44.2[b]	127 ± 44.2[b]
Mean	*5.90 ± 1.88*	*158 ± 42.3*	*164 ± 42.1*

Table 10. Antioxidant capacity of *in vitro* digested-fermented fruits depending on the cooking method.

Fruits

Cooking technique	DPPH (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Fried	40.8 ± 27.3[a]	163 ± 40.6[a]	204 ± 54.3[a]
Grilled	17.8 ± 14.4[a]	152 ± 37.3[a]	170 ± 43.6[a]
Raw	25.1 ± 20.1[a]	150 ± 58.3[a]	175 ± 68.4[a]
Roasted	22.3 ± 13.5[a]	159 ± 36.4[a]	181 ± 43.5[a]
	FOLIN (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Fried	708 ± 451[b]	25833 ± 12108[a]	26541 ± 12215[a]
Grilled	482 ± 362[a]	24348 ± 14326[a]	34830 ± 14302[a]
Raw	251 ± 192[a]	28021 ± 13916[a]	28272 ± 13958[a]
Roasted	462 ± 184[b]	27187 ± 15418[a]	27648 ± 15104[a]
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Fried	7.79 ± 5.32[a]	152 ± 68.8[a]	160 ± 72.3[a]
Grilled	1.68 ± 1.14[a]	142 ± 71.5[a]	144 ± 71.8[a]
Raw	4.31 ± 2.30[a]	150 ± 51.3[a]	154 ± 51.8[a]
Roasted	2.88 ± 1.87[a]	159 ± 89.7[a]	162 ± 90.5[a]

Table 11. Antioxidant capacity of *in vitro* digested-fermented cereals depending on the fruit type.

Sample	DPPH (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Apple	8.00 ± 6.02[a]	91.9 ± 18.9[a]	99.9 ± 16.1[a]
Banana	17.3 ± 19.5[a]	141 ± 19.5[a]	158 ± 36.4[a]
Grapes	38.3 ± 17.4[a]	146 ± 9.73[a]	184 ± 17.4[a]
Olives	63.2 ± 0.76[b]	219 ± 7.36[b]	282 ± 8.12[b]
Orange	28.6 ± 28.0[a]	145 ± 42.3[a]	174 ± 55.7[a]
Peach	11.0 ± 3.47[a]	207 ± 34.1[c]	218 ± 32.0[c]
Mean	*29.7 ± 11.5*	*162 ± 23.2*	*192 ± 28.3*
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Apple	273 ± 239[a]	24309 ± 14353[a]	24582 ± 14379[a]
Banana	479 ± 408[a]	35935 ± 11123[a]	36414 ± 11044[a]
Grapes	332 ± 233[a]	15222 ± 3123[a]	15554 ± 2983[a]
Olives	438 ± 83.5[b]	31701 ± 620[b]	32139 ± 537[b]
Orange	790 ± 553[a]	17433 ± 6189[a]	18223 ± 6691[a]
Peach	443 ± 64.1[c]	17433 ± 6189[c]	18223 ± 6691[c]
Plum	434 ± 217[c]	33051 ± 14934[c]	33485 ± 14978[c]
Mean	*456 ± 257*	*27171 ± 9499*	*27626 ± 9532*
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Apple	3.02 ± 2.60[a]	143 ± 76.5[a]	146 ± 76.9[a]
Banana	5.55 ± 3.83[a]	181 ± 65.4[a]	186 ± 64.3[a]
Grapes	1.68 ± 1.19[a]	95.9 ± 18.4[a]	97.6 ± 19.3[a]
Olives	9.45 ± 0.23[b]	193 ± 3.64[b]	202 ± 3.87[b]
Orange	4.22 ± 3.86[a]	109 ± 36.4[a]	113 ± 36.9[a]
Peach	2.55 ± 0.55[c]	167 ± 46.3[c]	169 ± 46.0[c]
Mean	*4.77 ± 2.52*	*156 ± 47.8*	*160 ± 48.2*

Table 12. Antioxidant capacity of *in vitro* digested-fermented vegetables depending on the cooking method.

Vegetables

Cooking technique	DPPH (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	12.2 ± 11.9[a]	119 ± 37.0[a]	131 ± 35.2[a]
Fried	36.7 ± 29.3[b]	128 ± 49.3[b]	165 ± 55.8[b]
Grilled	11.8 ± 8.72[a]	136 ± 61.0[a]	148 ± 55.7[a]
Raw	7.17 ± 3.98[a]	139 ± 53.5[a]	146 ± 54.0[a]
Roasted	10.7 ± 5.56[a]	141 ± 60.8[a]	151 ± 58.9[a]
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	338 ± 232[a]	23906 ± 10870[a]	24244 ± 10826[a]
Fried	1058 ± 981[b]	25962 ± 12926[b]	27020 ± 12861[b]
Grilled	325 ± 247[a]	26757 ± 18324[a]	27082 ± 18328[a]
Raw	381 ± 292[a]	22350 ± 8884[a]	22731 ± 9021[a]
Roasted	766 ± 932[a]	23835 ± 9533[a]	24601 ± 9760[a]
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	2.51 ± 2.06[a]	126 ± 50.5[a]	129 ± 51.2[a]
Fried	9.00 ± 8.23[b]	139 ± 65.9[b]	148 ± 69.8[b]
Grilled	3.54 ± 2.35[a]	150 ± 97.4[a]	153 ± 98.1[a]
Raw	2.93 ± 1.51[a]	121 ± 40.7[a]	124 ± 40.8[a]
Roasted	3.25 ± 2.71[a]	131 ± 48.6[a]	134 ± 49.6[a]

Table 13. Antioxidant capacity of *in vitro* digested-fermented cereals depending on the vegetable type.

Sample	DPPH (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Cabbage	3.92 ± 1.50[b]	171 ± 55.2[a]	175 ± 54.6[a]
Carrot	5.83 ± 1.75[b]	148 ± 14.6[a]	154 ± 15.8[a]
Cauliflower	21.8 ± 29.9[a]	107 ± 15.8[a]	129 ± 34.7[a]
Eggplant	33.5 ± 27.1[a]	114 ± 35.4[a]	147 ± 48.9[a]
Lettuce	2.89 ± 0.61[a]	217 ± 33.9[a]	220 ± 33.3[a]
Onion	11.5 ± 9.56[a]	96.8 ± 18.4[a]	108 ± 18.6[a]
Pepper	18.8 ± 9.37[a]	155 ± 50.4[a]	174 ± 45.6[a]
Spinach	22.3 ± 17.1[a]	105 ± 23.7[a]	127 ± 32.4[a]
Tomato	14.0 ± 18.2[a]	195 ± 53.3[a]	209 ± 44.1[a]
Zucchini	8.58 ± 6.02[b]	72.7 ± 8.84[a]	81.3 ± 10.9a
Mean	*14.3 ± 12.1*	*138 ± 31.0*	*152 ± 33.9*
	Folin-Ciocalteu (mg gallic acid/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Cabbage	291 ± 156[a]	16407 ± 5079[a]	16698 ± 4993[a]
Carrot	292 ± 205[a]	31018 ± 7609[a]	31310 ± 7690[a]
Cauliflower	585 ± 749[b]	20246 ± 2048[a]	20831 ± 2681[a]
Eggplant	491 ± 345[b]	26306 ± 14288[a]	26797 ± 14515[a]
Lettuce	496 ± 78.0[b]	18669 ± 169[a]	19165 ± 247[a]
Onion	320 ± 307[b]	22069 ± 17712[a]	22389 ± 17885[a]
Pepper	420 ± 231[b]	37885 ± 17271[a]	38305 ± 17179[a]
Spinach	1623 ± 1315[b]	18409 ± 7686[a]	20032 ± 8119[a]
Tomato	327 ± 236[b]	18111 ± 3664[a]	18438 ± 3621[a]
Zucchini	656 ± 323[b]	32357 ± 7307[a]	33013 ± 7532a
Mean	*550 ± 395*	*24148 ± 8283*	*24698 ± 8446*
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Cabbage	0.36 ± 0.43[a]	103 ± 29.9[a]	103 ± 30.1[a]
Carrot	2.72 ± 1.02[a]	152 ± 44.7[a]	155 ± 44.3[a]
Cauliflower	6.64 ± 9.17[b]	125 ± 84.0[a]	131 ± 84.0[a]
Eggplant	7.04 ± 5.96[b]	124 ± 84.0[a]	131 ± 88.3[a]
Lettuce	3.87 ± 0.11[b]	116 ± 0.99[a]	120 ± 1.11[a]
Onion	4.74 ± 2.35[b]	118 ± 85.9[a]	122 ± 87.2[a]
Pepper	7.34 ± 3.89[b]	192 ± 102[a]	199 ± 101[a]
Spinach	6.17 ± 3.18[b]	115 ± 45.2[a]	121 ± 46.5[a]
Tomato	0.57 ± 0.73[b]	113 ± 21.5[a]	113 ± 21.6[a]
Zucchini	1.77 ± 0.63[a]	160 ± 43.0[a]	161 ± 43.1[a]
Mean	*4.12 ± 2.75*	*132 ± 46.8*	*136 ± 48.7*

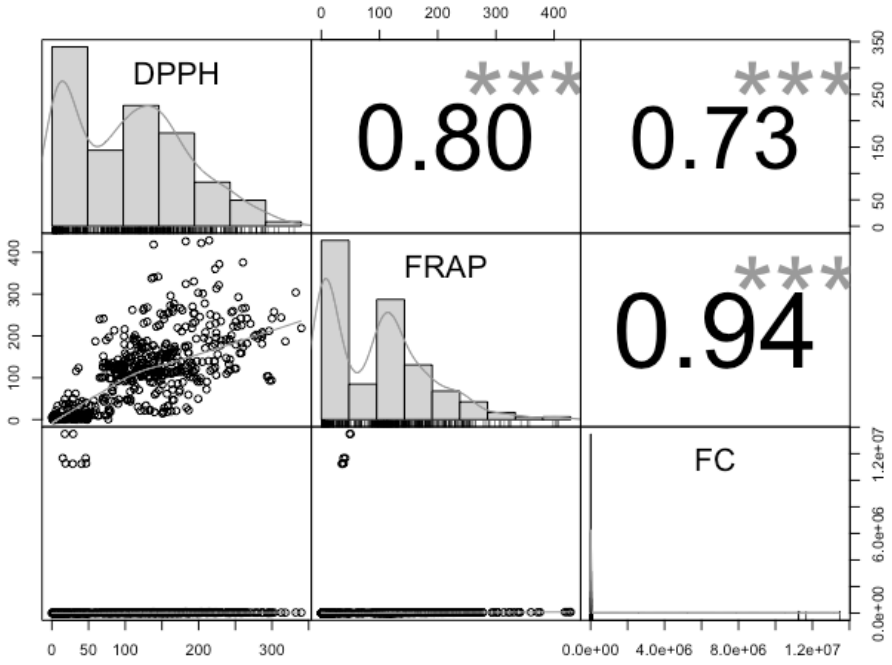

Figure 27. Linear correlations between the antioxidant capacity of plant foods.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Abuajah, C.I.; Ogbonna, A.C.; Osuji, C.M. Functional components and medicinal properties of food: a review. J. Food Sci. Technol. 2015, 52, 2522-2529, doi: 10.1007/s13197-014-1396-5.

2. Leri, M.; Scuto, M.; Ontario, M.L.; Calabrese, V.; Calabrese, E.J.; Bucciantini, M.; Stefani, M. Healthy Effects of Plant Polyphenols: Molecular Mechanisms. Int. J. Mol. Sci. 2020, 21, 1250, doi: 10.3390/ijms21041250.

3. Liu, R.H. Dietary bioactive compounds and their health implications. J. Food Sci. 2013, 78, A18-A25, doi: 10.1111/1750-3841.12101.

4. Bello Gutierrez, J. Los tipos de coccion. In Ciencia y tecnología culinaria, 1st ed.; Bello Gutierrez, J., Ed.; Díaz de Santos S.A., Madrid, Spain, 1998, pp. 129-190.

5. Ramírez-Anaya, J. del P.; Samaniego-Sánchez, C.; Castañeda-Saucedo, Ma.C.; Villalón-Mir, M.; de la Serrana, H.L.-G. Phenols and the antioxidant capacity of Mediterranean vegetables prepared with extra virgin olive oil using different domestic cooking techniques. Food Chem. 2015, 188, 430-438, doi: 10.1016/j.foodchem.2015.04.124.

6. Miglio, C.; Chiavaro, E.; Visconti, A.; Fogliano, V.; Pellegrini, N. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. J. Agric. Food Chem. 2008, 56, 139-147, doi: 10.1021/jf072304b.

7. Rufián-Henares, J.A.; Guerra-Hernández, E.J.; García-Villanova, B. Colour measurement as indicator for controlling the manufacture and storage of enteral formulas. Food Cont. 2006, 17, 489-493, doi: 10.1016/j.foodcont.2005.02.011.

8. Pastoriza de la Cueva, S.; Álvarez, J.; Végvári, Á.; Montilla-Gómez, J.; Cruz-López, O.; Delgado-Andrade, C.; Rufián-Henares, J.A. Relationship between HMF intake and SMF formation in vivo: An animal and human study. Mol. Nutr. Food Res. 2017, 61, 1600773, doi: 10.1002/mnfr.201600773.

9. Rufián-Henares, J.A.; Guerra-Hernández, E.; García-Villanova, B. Effect of red sweet pepper dehydration conditions on Maillard reaction, ascorbic acid and antioxidant activity. J. Food Eng. 2013, 118, 150-156, doi: 10.1016/j.jfoodeng.2013.03.034.

10. Kolodziejczyk, A.A.; Zheng, D.; Elinav, E. Diet-microbiota interactions and personalized nutrition. Nature Rev. Microbiol. 2019, 17, 742-753, doi: 10.1038/s41579-019-0256-8.

11. Rowland, I.; Gibson, G.; Heinken, A.; Scott, K.; Swann, J.; Thiele, I.; Tuohy, K. Gut microbiota functions: metabolism of nutrients and other food components. Eur. J. Nutr. 2018, 57, 1-24, doi: 10.1007/s00394-017-1445-8.

12. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an improved global antioxidant response method (GAR+): Physiological-resembling in vitro digestion-fermentation method. Food Chem. 2018, 239, 1253-1262, doi: 10.1016/j.foodchem.2017.07.024.

13. Pastoriza, S.; Delgado-Andrade, C.; Haro, A.; Rufián-Henares, J.A. A physiologic approach to test the global antioxidant response of foods. The GAR method. Food Chem. 2011, 129, 1926-1932, doi: 10.1016/j.foodchem.2011.06.009.

14. Yen, G.-C.; Chen, H.-Y. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J. Agric. Food Chem. 1995, 43, 27-32, doi: 10.1021/jf00049a007.

15. Moreno-Montoro, M.; Olalla-Herrera, M.; Gimenez-Martinez, R.; Navarro-Alarcon, M.; Rufián-Henares, J.A. Phenolic compounds and antioxidant activity of Spanish commercial grape juices. J. Food Comp. Anal. 2015, 38, 19-26, doi: 10.1016/j.jFolin-Ciocalteua.2014.10.001.

16. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. Anal. Biochem. 1996, 239, 70-76, doi: 10.1006/abio.1996.0292.

17. Mercasa. La alimentación en España. 2020. Available in https://www.mercasa.es/media/publicaciones/281/AEE_2020_web.pdf (accessed on 12 November 2020).

18. Salvador i Castells, G. (2000). Tabla de medidas caseras de alimentos. In J. Salas-Salvadó, A. Bonada, R. Trallero & M.E. Saló (Eds.), Nutrición y Dietética Clínica. (pp. 557-570, Annex 4). Elsevier-Masson, Barcelona.

19. Saura-Calixto, F.; Goñi, I. Antioxidant capacity of the Spanish Mediterranean diet. Food Chem. 2006, 94, 442-447, doi: 10.1016/j.foodchem.2004.11.033.

20. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an improved Global Antioxidant Response method (GAR+): Physiological-resembling in vitro antioxidant capacity methods. Food Chem. 2018, 239, 1263-1272, doi: 10.1016/j.foodchem.2017.07.063.

21. Carvalho, D.O.; Correia, E.; Lopes, L.; Guido, L.F. Further insights into the role of melanoidins on the antioxidant potential of barley malt. Food Chem. 2014, 160, 127-133, doi: 10.1016/j.foodchem.2014.03.074.

22. Pastoriza, S.; Seiquer, I.; Mesías, M.; Rufián Henares, J.A.; Delgado Andrade, C. Evaluation of the availability and antioxidant capacity of Maillard compounds present in bread crust: Studies in Caco-2 cells. Foods 2017, 6, 5, doi: 10.13039/501100004837.

23. Dittrich, R.; Dragonas, C.; Kannenkeril, D.; Hoffmann, I.; Mueller, A.; Beckmann, M.W.; Pischetsrieder, M. A diet rich in Maillard reaction products protects LDL against copper induced oxidation ex vivo, a human intervention trial. Food Res. Int. 2009, 42, 1315-1322, doi: 10.1016/j.foodres.2009.04.007.

24. Martín, M.Á.; Ramos, S.; Mateos, R.; Rufián-Henares, J.A.; Morales, F.J.; Bravo, L.; Goya, L. Biscuit melanoidins of different molecular masses protect human HepG2 cells against oxidative stress. J. Agric. Food Chem. 2009, 57, 7250-7258, doi: 10.1016/j.foodres.2009.04.007.

25. Tagliazucchi, D.; Verzelloni, E.; Conte, A. Antioxidant properties of traditional balsamic vinegar and boiled must model systems. Eur. Food Res. Technol. 2007, 227, 835, doi: 10.1007/s00217-007-0794-6.

26. Ahmad-Qasem, M.H.; Cánovas, C.; Barrajó-Catalá, E.; Carreres, J.E.; Micol, V.; García-Peŕez, J.V. Influence of olive leaf processing on the bioaccessibility of bioactive polyphenols. J. Agric. Food Chem. 2014, 62, 6190-6198, doi: 10.1021/jf501414h.

27. Plengmuankhae, W.; Tantitadapitak, C. Low temperature and water dehydration increase the levels of asiaticoside and madecassoside in Centella asiatica (L.) Urban. South Afric. J. Bot. 2015, 97, 196-203, doi: 10.1016/j.sajb.2015.01.013.

28. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Effect of home cooking on the antioxidant capacity of vegetables: Relationship with Maillard reaction indicators. Food Res. Int. 2019, 121, 514-523, doi: 10.1016/j.foodres.2018.12.007.

29. Bellail, A.A.; Shaltout, O.E.; Youssef, M.M.; Gamal, A.M.A.E. Effect of home-cooking methods on phenolic composition and antioxidant activity of sweetpotato (Ipomoea batatas (L.) Lam.) cultivars grown in Egypt. Food Nutr. Sci. 2012, 3, 720-726, doi: 10.4236/fns.2012.34069.

30. Pimentel, F.A.; Nitzke, J.A.; Klipel, C.B.; Jong, E.V. de Chocolate and red wine - A comparison between flavonoids content. Food Chem. 2010, 120, 109-112, doi: 10.1016/j.foodchem.2009.09.078.

31. Montreau, F.R. Sur le dosage des composés phénoliques totaux dans les vins par la méthode Folin-Ciocalteu. OENO One 1972, 6, 397-404, doi: 10.20870/oeno-one.1972.6.4.2071.

32. Lee, K.W.; Kim, Y.J.; Lee, H.J.; Lee, C.Y. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem. 2003, 51, 7292-7295, doi: 10.1021/jf0344385.

33. Pastoriza, S.; Rufián-Henares, J.A. Contribution of melanoidins to the antioxidant capacity of the Spanish diet. Food Chem. 2014, 164, 438-445, doi: 10.1016/j.foodchem.2014.04.118.

34. Durante, M.; Tufariello, M.; Tomassi, L.; Lenucci M.L.; Bleve, G.; Mita, G. Evaluation of bioactive compounds in black table olives fermented with selected microbial starters. J Sci Food Agric. 2018, 98, 96-103, doi: https://doi.org/10.1002/jsfa.8443

35. Durante, M.; Lenucci, M.S.; Gazza, L.; Taddei, F.; Nocente, F.; De Benedetto, G.E.; De Caroli, M.; Piro, G.; Mita, G. Bioactive composition and sensory evaluation of innovative spaghetti supplemented with free or α-cyclodextrin chlatrated pumpkin oil extracted by supercritical CO_2. Food Chem. 2019, 294, 112-122, doi: https://doi.org/10.1016/j.foodchem.2019.05.032.

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Effect of Cooking Methods on the Antioxidant Capacity of Foods of Animal Origin Submitted to *in Vitro* Digestion-Fermentation

En este trabajo se estudió la capacidad antioxidante de alimentos de origen animal con diferentes cocinados tras su digestión y fermentación *in vitro*. Esta última se llevó a cabo con microbiota intestinal procedente de adultos sanos. Así se pudo ver cómo afectaban tanto el tipo de alimento como el cocinado aplicado a la funcionalidad de la microbiota intestinal, en concreto, a la capacidad de la misma para generar compuestos con actividad antioxidante. Además, se estudió la contribución del consumo de estos alimentos de origen animal a la capacidad antioxidante diaria en la dieta española, teniendo en cuenta los grupos de alimentos y cocinados aplicados.

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Effect of Cooking Methods on the Antioxidant Capacity of Foods of Animal Origin Submitted to *in Vitro* Digestion-Fermentation

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Abstract

The human body is exposed to oxidative damage to cells and though it has some endogenous antioxidant systems, we still need to take antioxidants from diet. The main dietary source of antioxidants are vegetables due to their content in different bioactive molecules. However, there are usually other components of the diet, such as foods of animal origin, that are not often linked to antioxidant capacity. Still, these foods are bound to exert some antioxidant capacity thanks to molecules released during gastrointestinal digestion and gut microbial fermentation. In this work, the antioxidant capacity of 11 foods of animal origin has been studied, submitted to different culinary techniques and to an *in vitro* digestion and gut microbial fermentation. Results have shown how dairy products potentially provide the highest antioxidant capacity, contributing with a 60 % of the daily antioxidant capacity intake. On the other hand, most of the antioxidant capacity was released during gut microbial fermentation (90-98 % of the total antioxidant capacity). Finally, it was found that the antioxidant capacity of the studied foods was much higher than that reported by other authors. A possible explanation is that digestion-fermentation pretreatment allows for a higher extraction of antioxidant compounds and their transformation by the gut microbiota. Therefore, although foods of animal origin cannot be compared to vegetables in the concentration of antioxidant molecules, the processes of digestion and fermentation can provide some, giving animal origin food some qualities, which could have been previously unappreciated.

Keywords: antioxidant capacity; thermal processing; animal origin food; *in vitro* digestion; *in vitro* fermentation; gut microbiota

1. Introduction

Global concern about the increased incidence of chronic diseases such as diabetes, obesity, cancer and cardiovascular disease has led to pay greater attention to lifestyle habits, especially diet [1]. On the other hand, the consumption of animal origin foods has often been linked to the appearance of non-communicable diseases, particularly the consumption of red meat, processed meat and meat derivatives [2,3]. In contrast, the consumption of plant origin foods, such as fruit and vegetables, has been linked to a protective effect against such conditions [4].

Vegetables' content in phytochemicals has been pointed out as one of the reasons behind their beneficial effect against such chronic diseases. Many of these compounds have shown great antioxidant activity and thus the potential to play a beneficial role on oxidative stress related diseases such as cancer, cardiovascular diseases, or type 2 diabetes mellitus [4,5]. At the same time, vegetables' large and diverse content in biochemicals have made this type of food the object of a large variety of studies [4, 5]. In contrast, literature is very limited in relation to bioactive molecules or antioxidant capacity in animal origin foods such as meat, fish, eggs or dairy products, probably due to their lack or low quantities of such molecules, at least in comparison with vegetables. However, we now know that gastrointestinal digestion breaks down food macrostructure and helps to release smaller molecules, some of which could have antioxidant potential [6]. Such is the case of carnosine, a di-peptide with antioxidant activity as well as anti-inflammatory, neuro-protective and anti-ageing properties [7,8]. Therefore, other potentially antioxidant or bioactive molecules are bound to be released during digestion. In addition, other compounds with antioxidant capacity can be found in foods of animal origin, such as taurine [9] and carotenoids from animal feed [10,11].

On the other hand, undigested food passes into the large intestine, where they can be used by the gut microbiota as fermentation substrate; such undigested food can produce compounds with biological and antioxidant activity [12]. Therefore, although food of animal origin is not characterized by a high content of bioactive molecules, it is still possible that after cooking, digestion and fermentation these can be generated. Additionally, cooking methodology will modify in some degree, depending on the temperature and time applied, the chemical composition of foods. Therefore, gastrointestinal digestion and gut microbial fermentation are likely to be affected and so, the molecules released after such processes [13].

Accordingly, the aim of the present paper was to study the antioxidant capacity of animal origin foods, representing the main dietary categories. Different heat treatments were applied, and then they were *in vitro* digested and fermented. Next, the contribution of the consumption of animal origin foods to the daily intake of antioxidant capacity in Spain was calculated. Finally, the overall daily antioxidant capacity intake in Spain was calculated taking also into account the antioxidant capacity of plant foods previously studied [14].

2. Materials and Methods

2.1. Chemicals

2.1.1. *In vitro* digestion and fermentation

Cysteine, sodium di-hydrogen phosphate, sodium sulphide, resazurin, salivary α-amylase and pepsin from porcine bile acids (porcine bile extract) were provided by Sigma-Aldrich (Darmstadt, Germany). Pancreatin from porcine pancreas was provided by Alpha Aesar (United Kingdom).

2.1.2. Antioxidant Capacity

DPPH (2,2 diphenyl-1-1picrythydrazyl), hydrochloric acid, iron (III) chloride hexahydrate, methanol, sodium acetate, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine) and Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were provided by Sigma- Aldrich (Darmstadt, Germany).

2.2. Samples and cooking conditions applied

Eleven animal foods were investigated belonging to the following groups: dairy, egg, fish and meat (**Table 17** in supplemental information). Animal foods were bought in three different supermarkets and stored at room temperature (eggs) or under refrigeration for a maximum of 2 days before cooking.

The foods were submitted to different culinary treatments: boiling, frying, grilling, or roasting (**Table 17** in supplemental information). Some of them (butter, yogurt and salmon) were also analyzed in their raw form (since they are usually consumed as raw) making it a total of 36 samples. Boiling was prepared in a rate of 5:1 (water:food) at 100 ^{o}C for 20 min. Frying and

grilling used Extra virgin olive oil (EVOO) as cooking medium. Frying was prepared in a rate of 5:1 (oil:food) at 180 °C for 8 min. Grilling was prepared in a rate of 0.5:1 (oil:food) at 220-250 °C for 3 min. Roasting was prepared at 180 °C for 10 min. Finally, milk was commercially processed by ultra-high temperature (UHT). Cooking times and food:medium rates were acquired from Olmedilla-Alonso et al., [3] and adapted to our own equipment and laboratory conditions.

The utensils used for sample preparation were the following: a transportable oven (1500 W), fryer, frying pan and saucepan and forks, knives, spoons and stainless steel. All these utensils were purchased from Centro Hogar Sánchez (Granada, Spain). Samples were homogenized and stored under nitrogen atmosphere at -80 °C in order to avoid oxidation. All analyses were carried out in duplicate.

2.3. In vitro digestion and fermentation

Samples were subjected to an *in vitro* gastrointestinal and to an *in vitro* fermentation according to the protocol previously described [15], in triplicate. Food was added to falcon tubes together with simulated salivary fluid (1:1, w/v) composed of salts and α-amylase (75 U/mL). The mix was kept at 37 ºC for 2 min in oscillation. Right after, 10 mL of simulated gastric fluid was added, simulating the gastric juices content in salts and pepsin (2000 U/mL). The mix was kept at 37 ºC for 2 hours, at pH 3 in oscillation. Finally, 20 mL of simulated intestinal fluid was added, simulating the intestinal juices content in salts, bile salts and enzymes (here we used 67.2 mg/mL pancreatine). The mix was kept at 37 ºC for 2 hours, at pH 7, in oscillation. Once the intestinal phase was finished, tubes were kept in ice to stop enzymatic reactions and thereafter centrifuged at 3500 rpm for 10 min. The supernatant, which represents the fraction available for absorption in the

small intestine, was stored in 1 mL tubes at -80 ºC until analysis. The solid pellet, which represents the not digested fraction that goes into the large intestine, was used as *in vitro* fermentation substrate.

The *in vitro* fermentation was carried out using fecal samples from five healthy donors with no previous pathology, who had not taken antibiotics for three months prior to the assay, with a mean (Body Mass Index = 21.3). Individual diets were not assessed since the objective was not to evaluate microbial communities but rather to unravel the potential antioxidant power that average people could extract from animal origin food-stuff. The fecal samples were pooled together to reduced inter-individual variability. The fermentation was carried out at 37 °C for 20 h. Once the *in vitro* fermentation was finished, tubes were kept in ice to stop microbial reactions and thereafter centrifuged at 3500 rpm for 10 min. The supernatant, which represents the fraction available for absorption in the large intestine, was stored in 1 mL tubes at -80 ºC until analysis. The solid pellet, which represents the fraction not fermented and excreted with feces, was appropriately discarded.

Therefore, two fractions were obtained after *in vitro* gastrointestinal digestion and fermentation: digestion supernatant (fraction for absorption in the small intestine), and fermentation supernatant (fraction for absorption in the large intestine). Antioxidant capacity was measured in both fractions, considering as total antioxidant capacity the sum of them.

2.4. Antioxidant Test

Antioxidant capacity of those two fractions was studied. The total antioxidant capacity of the two fractions was taken as the amount of total antioxidant capacity exerted by a given food. [16].

$TEAC_{DPPH}$ assay. The method was based on the protocol of Rapisarda et al., [17] and adjusted to a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Briefly, 280 µL of DPPH reagent (prepared with 74 mg DPPH/L methanol) and 20 µL of digestion-fermentation supernatants were added to a 96-well plate. The antioxidant response was monitored in triplicate for one hour at 37 ◦C. The calibration curve was made up with Trolox at concentrations ranging from 0.01 to 0.4 mg/mL (results expressed as mmol Trolox equivalent/Kg feed).

$TEAC_{FRAP}$ assay. The method followed the protocol of Benzie and Strain [18] to measure the ferric reducing capacity in each sample in a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Briefly, 280 µL of FRAP reagent (prepared daily) and 20 µL of digestion-fermentation supernatants were added to a 96-well plate. The antioxidant reaction was followed in triplicate for 30 min at 37 ºC. A calibration curve was prepared with Trolox (0.01 to 0.4 mg/mL) and the results were expressed as mmol Trolox equivalent/Kg feed.

2.5. Daily Antioxidant Intake Calculations

The contribution of each food group to daily dietary antioxidant capacity intake was calculated based on the amount of food per serving, the daily intake [18] and the anti-oxidant capacity previously measured in the samples. The antioxidant capacity of each food was related to the portion size commonly consumed in Spain [19]. Then, the overall daily antioxidant capacity intake was also studied, including both the consumption of foods of animal and plant origin. The data on antioxidant capacity provided by foods of plant origin were obtained from our previous work [20].

2.6. Statistical analysis

The statistical significance of the results was checked by ANOVA and subsequently by the Duncan test ($p < 0.05$). As issue for ANOVA, it had been used form of cooking (boiled, fried, grilled, raw and roasted), sort of food (dairy, egg, fish and meat) and sort of sample (dairy: butter, cheese, milk and yoghurt; fish: cod fish and salmon; meat: beef, chicken, lamb and pork). Statistical analysis was performed by using boiled or raw foods and mean of all food groups because the reference groups. Pearson parametric statistic was calculated to indicate the lineal relation between anti-oxidant capacity at a *p value* < 0.05. To get the significance between the various levels among an equivalent group, the Tukey test was assigned. All the statistical analyses were performed by using Statgraphics Plus software, version 5.1.

3. Results

For each sample, the antioxidant capacity was measured in the supernatant fraction obtained after gastrointestinal digestion (antioxidant capacity available for absorption in the small intestine) and after fermentation (antioxidant capacity available for absorption in the large intestine). Two different antioxidant assays were applied. All antioxidant capacity values were corrected taking into account the antioxidant capacity provided by enzymes, chemicals and fecal inoculum.

In addition, a linear correlation was obtained by the Spearman method between the two methods. The correlation was significant ($p < 0.005$) with Spearman's rank correlation coefficient (rs) around 0.8.

3.1. Samples by type of cooking

The types of cooking compared were boiled, fried, grilled, roasted and UHT. They were compared with each other as well as with respect to the raw food (**Table 18** in supplemental information).

3.1.1. Gastrointestinal digestion supernatant.

Regarding $TEAC_{DPPH}$, raw foods showed significantly ($p < 0.05$) lower antioxidant capacity than all types of cooking, except for UHT, which was not significant (**Figure 28A**). For $TEAC_{FRAP}$, the antioxidant capacity was significantly ($p < 0.05$) lower in UHT foodstuffs than that of raw foods, but no significance was found for the other types of cooking (**Figure 28B**). In addition, when comparing the means of the different cooking methods, statistically significant differences were found (ANOVA paired comparison; $p < 0.05$; $TEAC_{DPPH}$) for fried foods, being more antioxidant than raw foods.

3.1.2. Fermentation supernatant and total antioxidant capacity.

Regarding $TEAC_{DPPH}$, there were no significant differences (**Figure 28A**). TEACFRAP of UHT showed a significantly ($p < 0.05$) lower antioxidant capacity than raw foods (**Figure 28B**). No other differences with raw foods were found.

In addition, when comparing the means of the different cooking methodologies, the following significant differences were found (ANOVA paired comparison; $p < 0.05$): for $TEAC_{DPPH}$, raw foods were more antioxidant than boiled; for $TEAC_{FRAP}$ UHT were less antioxidant than the rest of cooked foods except roast ones. For both fractions and for the total antioxidant capacity, the significance in ANOVA paired comparison for $TEAC_{FRAP}$, stated that UHT foods were less antioxidant.

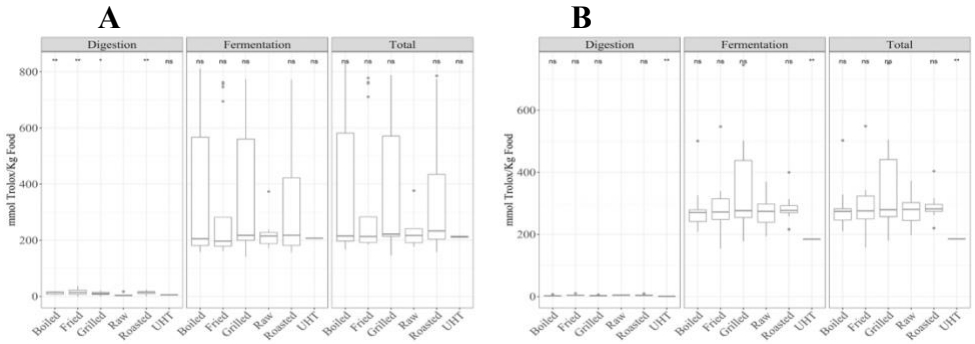

Figure 28. Antioxidant capacity of food of animal origin (butter, cheese, milk, yogurt, egg, cod fish, salmon, beef, chicken, lamb and pork) obtained after *in vitro* digestion and fermentation, depending on the cooking technique ((**28A**) Trolox capacity against DPPH radicals ($TEAC_{DPPH}$), (**28B**) for Trolox equivalent antioxidant capacity referred to reducing capacity ($TEAC_{FRAP}$)). Statistical analysis was performed through ANOVA using raw foods as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.2. Samples by type of food.

The samples to be compared were divided into four groups: dairy products (composed of butter, cheese, milk and yogurt), eggs, meats (including beef, chicken, lamb and pork) and fish, which included salmon and cod fish (**Table 19** in supplemental information).

3.2.1. Gastrointestinal digestion supernatant.

Regarding $TEAC_{DPPH}$, meat showed a significantly ($p < 0.05$) higher antioxidant capacity than the rest of the groups. On the other hand, the antioxidant capacity of dairy products was significantly lower than the average antioxidant capacity of the other food groups (**Figure 29A**). Secondly, for $TEAC_{FRAP}$, the antioxidant capacity of fish was significantly ($p < 0.05$) lower to the other food groups, while that of eggs was the highest (**Figure 29B**).

3.2.2. Fermentation supernatant and total antioxidant capacity.

In the case of $TEAC_{DPPH}$, the fermentation supernatant and total antioxidant capacities were significantly (ANOVA paired comparison; $p < 0.05$) higher in meat, whereas they were lower in dairy products, egg and fish compared with the mean antioxidant capacity of all food groups (**Figure 29A**). For the $TEAC_{FRAP}$ method, there were no significant differences.

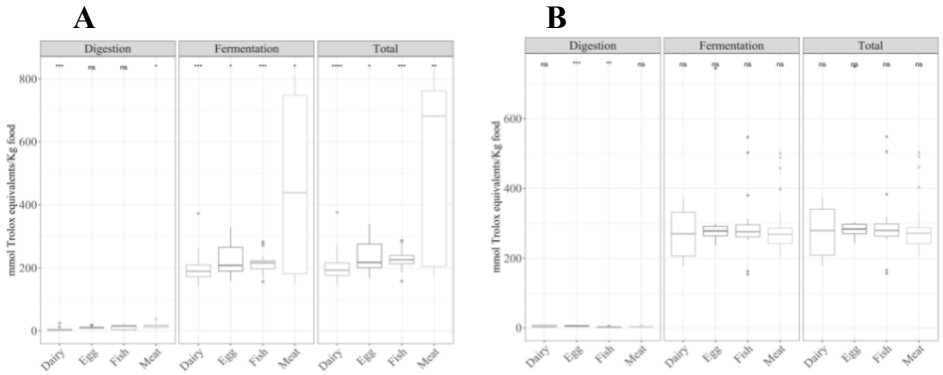

Figure 29. Antioxidant capacity of foods of animal origin (butter, cheese, milk, yogurt, egg, cod fish, salmon, beef, chicken, lamb and pork) obtained after *in vitro* digestion and fermentation, depending on the food group ((**29A**) $TEAC_{DPPH}$ and (**29B**) $TEAC_{FRAP}$). Statistical analysis was performed via ANOVA using the mean antioxidant capacity of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

Figure 30 shows the contribution of each fraction to the total antioxidant capacity. For both methods, the contribution of the digestion fraction was negligible or non-existent, with the fermentation fraction being the most important one.

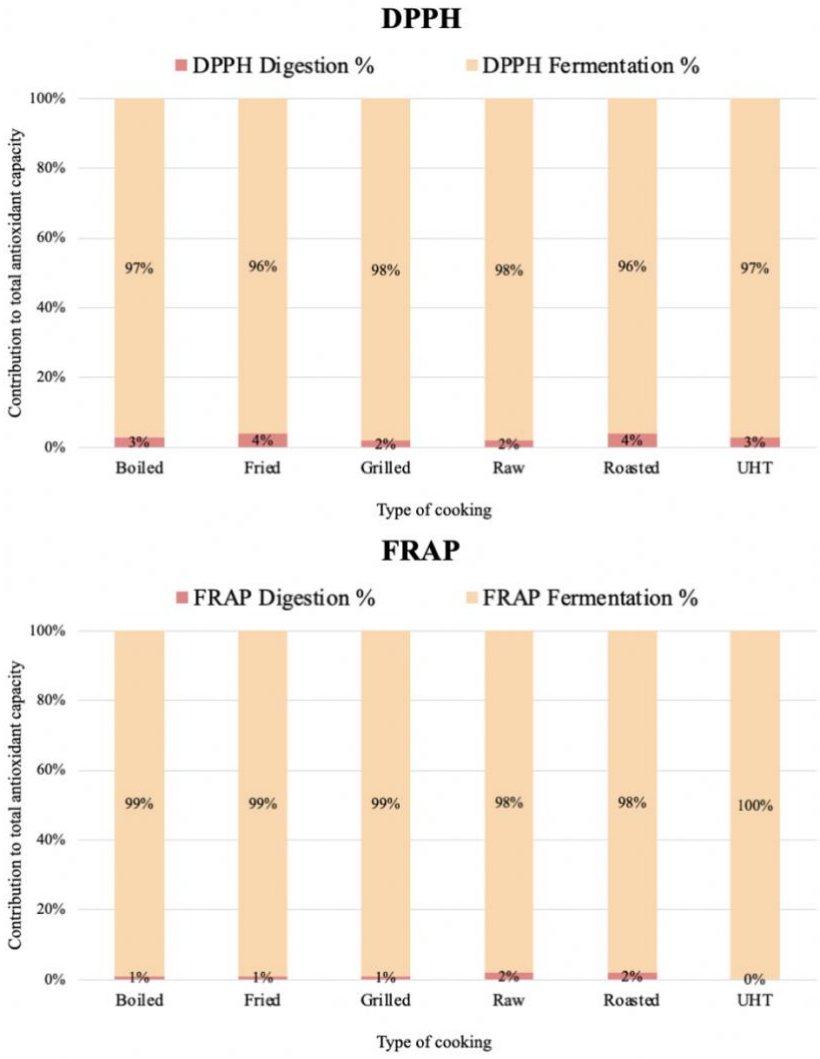

Figure 30. Contribution to the total antioxidant capacity of the fractions obtained after *in vitro* digestion depending of the cooking technique with the two antioxidant assays.

3.3. Specific group analysis.

The antioxidant capacity within each of the above-mentioned food groups (dairy, fish and meat) was also analyzed. Each group was studied by

cooking method and by type of food. Dairy consisted of butter, cheese, milk and yoghurt; fish consisted of cod fish and salmon and meat consisted of beef, chicken, lamb and pork (**Table 17** in supplemental information).

3.3.1. Dairy

By cooking (**Table 20** in supplemental information). Regarding $TEAC_{DPPH}$ (**Figure 31A**), raw dairy products showed higher antioxidant capacity than roasted ones in the digestion fraction. However, raw products showed a significantly ($p < 0.05$) higher antioxidant value than grilled products in the fermentation fraction, as well as a higher total antioxidant capacity. Regarding the $TEAC_{FRAP}$ method (**Figure 31B**), digestion of raw products resulted in a significantly higher antioxidant capacity than UHT, but lower than roasted foods. On the other hand, fer-mentation of raw products released significantly more antioxidant power than UHT, which resulted as well in a higher total antioxidant capacity.

By sample (**Table 21** in supplemental information). In the case of $TEAC_{DPPH}$ (**Figure 31C**), comparing the means of the different dairy products (ANOVA paired comparisons, $p < 0.05$), butter antioxidant capacity was higher than that of cheese in the fermented fraction and total antioxidant capacity; for $TEAC_{FRAP}$ (**Figure 31D**), milk and yogurt were less antioxidant than the other dairy products for the fermented fraction and total antioxidant capacity.

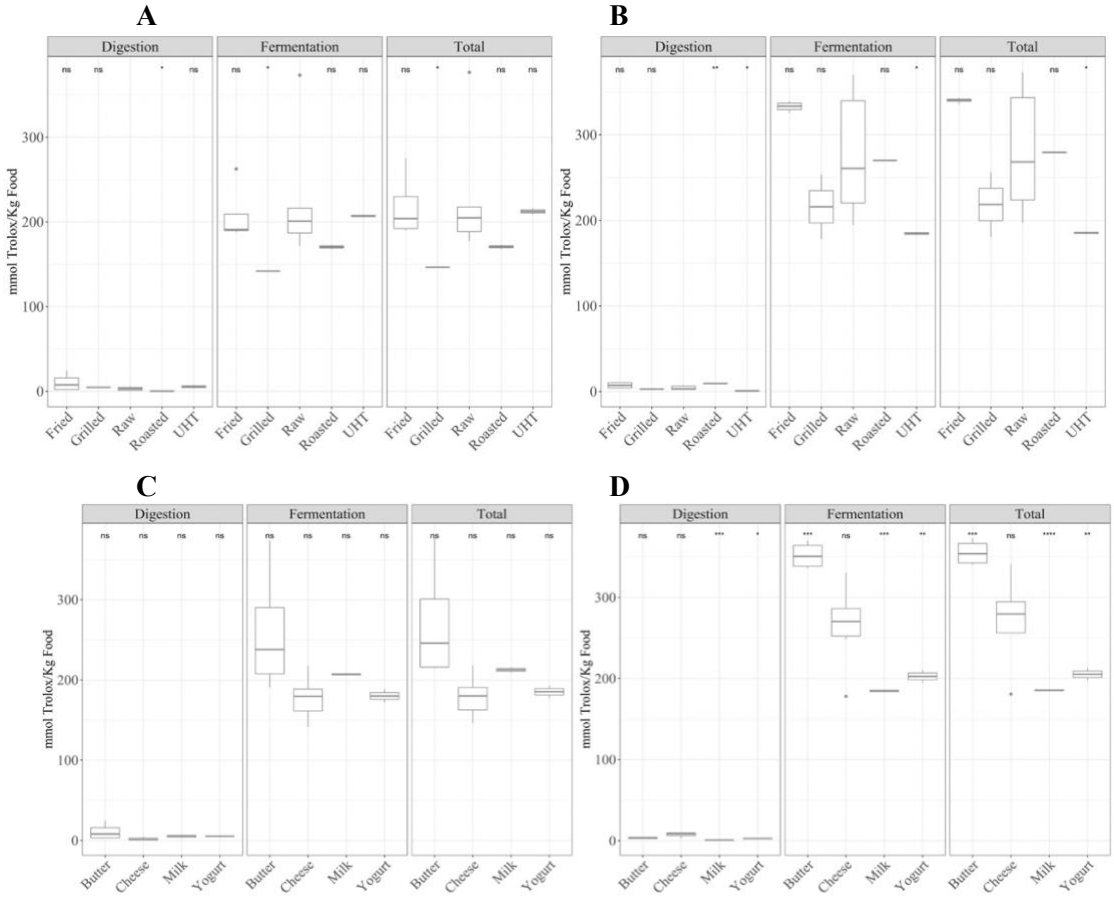

Figure 31. Antioxidant capacity of digested-fermented dairy products (butter, cheese, milk and yogurt) depending on the cooking technique ((**31A**) $TEAC_{DPPH}$, (**31B**) $TEAC_{FRAP}$) and depending on the sample ((**31C**) $TEAC_{DPPH}$, (**31D**) $TEAC_{FRAP}$). Statistical analysis was performed through ANOVA using raw vegetables to figures A and B or mean of all food groups to figures C and D as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.3.2. Fish

By cooking (**Table 22** in supplemental information). No significant differences were found for the $TEAC_{DPPH}$ assay (**Figure 31A**); for $TEAC_{FRAP}$

(**Figure 31B**), the digested fraction of raw fish was more antioxidant than cooked ones when comparing the means of the different samples (ANOVA paired comparisons, $p < 0.05$). In the case of the fermented fraction and total antioxidant capacity, there were no significant differences, only for $TEAC_{DPPH}$, where boiled fish was less antioxidant than raw.

On the other hand, by sample (**Table 23** in supplemental information), in the case of $TEAC_{DPPH}$ (**Figure 31C**), when comparing the means of the different samples (ANOVA paired comparisons, $p < 0.05$), salmon (blue fish) was more antioxidant than cod fish (white fish) after digestion; for the $TEAC_{FRAP}$ method (**Figure 31D**), salmon (blue fish) was the most antioxidant foodstuff when comparing means of different samples (ANOVA paired comparisons, $p < 0.05$).

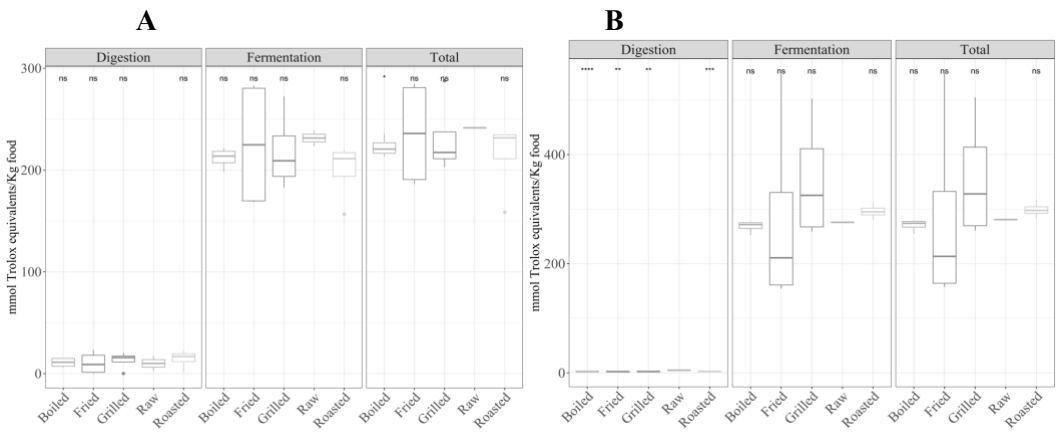

Fig. 32 (Continued)

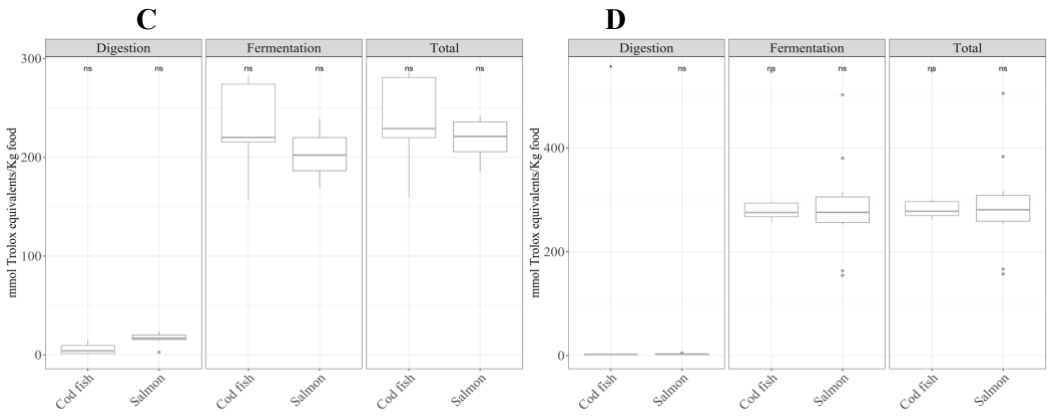

Figure 32. Antioxidant capacity of digested-fermented fish (cod fish and salmon) depending on the cooking technique ((**32A**) $TEAC_{DPPH}$, (**32B**) $TEAC_{FRAP}$) and depending on the sample ((**32C**) $TEAC_{DPPH}$, (**32D**) $TEAC_{FRAP}$). Statistical analysis was performed through ANOVA using raw vegetables or mean of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.3.3. Meat

No significant differences were found in meat by cooking (**Table 24** in supplemental information), either for $TEAC_{DPPH}$ (**Figure 33A**) or for $TEAC_{FRAP}$ (**Figure 33B**). On the other hand, by sample (**Table 25** in supplemental information), for $TEAC_{DPPH}$ (**Figure 33C**) lamb and pork were significantly more antioxidant than beef and chicken after fermentation, as well as the total antioxidant capacity. In the case of $TEAC_{FRAP}$ (**Figure 33D**) the antioxidant capacity of chicken was higher than that of lamb, both total antioxidant capacity and after *in vitro* fermentation. Differences between red and white meat were analyzed (**Table 26** in supplemental information) and not many significant differences were observed (**Figures 33E/F**).

The antioxidant capacities of meats and fish were also compared. In this sense, fish showed significantly lower antioxidant capacity ($TEAC_{DPPH}$) than meat in the fermentation fraction and total antioxidant capacity.

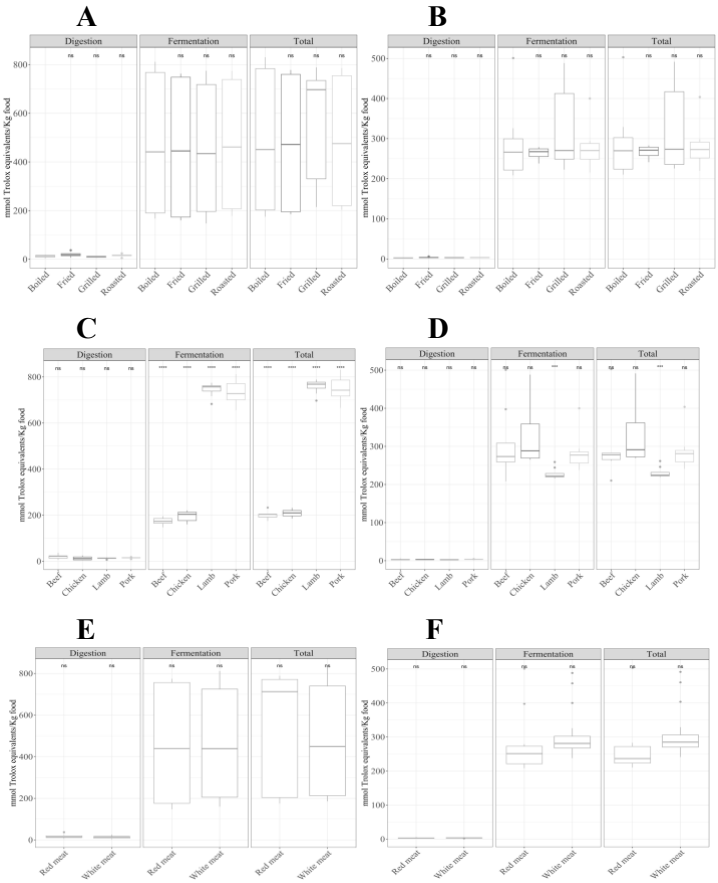

Figure 33. Antioxidant capacity of digested-fermented meat (beef, chicken, lamb and pork) depending on the cooking technique ((**33A**) $TEAC_{DPPH}$, (**33B**) $TEAC_{FRAP}$), depending on the sample ((**33C**) $TEAC_{DPPH}$, (**33D**) $TEAC_{FRAP}$) and depending of the type of meat, red or white ((**33E**) $TEAC_{DPPH}$, (**33F**) $TEAC_{FRAP}$). Statistical analysis was performed through ANOVA using raw vegetables or mean of all food groups as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.4. Daily Antioxidant Intake

We first calculated the contribution of animal food consumption to the daily anti-oxidant capacity intake taking into account just the consumption of food of animal origin (**Tables 14 and 15**), so that their sum reaches 100%. Dairy products showed the highest contribution to the daily antioxidant capacity intake in the Spanish diet, ranging between 56% (DPPH assay) and 66% (FRAP assay) of the antioxidant capacity provided by foods of animal origin. Meats also stood out with a contribution of 35% (DPPH assay) and 23% (FRAP assay). When we considered the antioxidant capacity computed by portion size, fish contributed with a 25% (DPPH assay) and 62% (FRAP assay), whereas meat con-tributed with a 43% (DPPH assay) and 45% (FRAP assay) of the antioxidant capacity (**Table 14**).

Table 14. Contribution of food of animal origin consumption to the daily antioxidant capacity (AOX) intake in the Spanish diet.

Food type	**Analytical assay**	**AOX/daily intake**[1] (μmol Trolox/day)	**AOX/serving intake**[2] (μmol Trolox/serving intake)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Dairy	*DPPH*	49170	23198	56.3	14.1
Egg	*DPPH*	5491	28871	6.29	17.6
Meat	*DPPH*	31308	70944	35.9	43.2
Fish	*DPPH*	1344	41173	1.54	25.1
Food type	**Analytical assay**	**AOX/daily intake**[1] (μmol Trolox/day)	**AOX/serving intake**[2] (μmol Trolox/serving intake)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Dairy	*FRAP*	57643	29660	66.2	34.0
Egg	*FRAP*	7659	40271	8.79	46.2
Meat	*FRAP*	20042	39518	23.0	45.4
Fish	*FRAP*	1765	54028	2.03	62.0

[1]Considering consumption for a whole year

[2]Considering the intake of 1 serving.

Regarding to the cooking method applied (**Table 2**), roasted dairy products contributed a 18% to the daily antioxidant capacity coming from foods of animal origin (DPPH assay), and raw dairy products a 19% (FRAP assay). Taking into account the consumption portion, roasted meat contributed up to a 32% of the daily antioxidant capacity (DPPH assay) derived from an animal source while grilled-roasted fish contributed a 29% (FRAP assay).

Table 15. Contribution of food of animal origin, with different culinary treatments, consumption to the daily antioxidant capacity (AOX) intake in the Spanish diet.

Food type	**Thermal processing**	**Analytical assay**	**AOX/daily intake[1]** (μmol Trolox/day)	**AOX/serving intake[2]** (μmol Trolox/serving)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Dairy	Fried	DPPH	4319	8539	1.69	3.35
Dairy	Raw	DPPH	4670	15299	1.83	6.00
Dairy	Roasted	DPPH	44700	23660	17.5	9.28
Dairy	UHT	DPPH	5973	19564	2.34	7.68
Egg	Boiled	DPPH	35026	46355	13.7	18.2
Egg	Fried	DPPH	5962	31351	2.34	12.3
Egg	Grilled	DPPH	6068	31908	2.38	12.5
Egg	Roasted	DPPH	12030	63257	4.72	24.8
Meat	Boiled	DPPH	6574	34568	2.58	13.6
Meat	Fried	DPPH	32686	72016	12.8	28.3
Meat	Grilled	DPPH	30649	70579	12.0	27.7
Meat	Roasted	DPPH	28329	82381	11.1	32.3
Fish	Boiled	DPPH	31625	71840	12.4	28.2
Fish	Fried	DPPH	1320	40085	0.52	15.7
Fish	Grilled	DPPH	1320	41083	0.52	16.1
Fish	Raw	DPPH	1460	44605	0.57	17.5
Fish	Roasted	DPPH	969	50549	0.38	19.8
Dairy	Fried	FRAP	7552	14410	3.42	6.53
Dairy	Raw	FRAP	41077	23419	18.6	10.6
Dairy	Roasted	FRAP	5973	19564	2.71	8.87
Dairy	UHT	FRAP	35026	46355	15.9	21.0
Egg	Boiled	FRAP	5962	31351	2.70	14.2
Egg	Fried	FRAP	6068	31908	2.75	14.5
Egg	Grilled	FRAP	12030	63257	5.45	28.7
Egg	Roasted	FRAP	6574	34568	2.98	15.7
Meat	Boiled	FRAP	21833	41983	9.90	19.0
Meat	Fried	FRAP	19589	38637	8.88	17.5
Meat	Grilled	FRAP	24088	45616	10.9	20.7
Meat	Roasted	FRAP	22053	40586	10.0	18.4
Fish	Boiled	FRAP	1593	48692	0.72	22.1
Fish	Fried	FRAP	1593	48939	0.72	22.2
Fish	Grilled	FRAP	2191	63983	0.99	29.0
Fish	Raw	FRAP	969	50549	0.44	22.9
Fish	Roasted	FRAP	1770	53802	0.80	24.4

[1]Considering consumption for a whole year

[2]Considering the intake of 1 serving.

The contribution of food consumption to the daily antioxidant intake was also studied taking into account the total diet, including also vegetable

foods (**Table 16**) using for calculations also our results recently published regarding this type of food [14]. Taking into consideration the main food groups of the Spanish diet, it is noteworthy to mention that dairy products (35% in DPPH assay and 28% in FRAP assay) and meat (12% in DPPH assay and 18% in FRAP assay) were the most antioxidant foods when the daily intake was computed. If the serving size were used, the contribution to the daily antioxidant capacity was slightly modified for meat (24% in DPPH assay and 40% in FRAP assay) and fish (32% in DPPH assay and 23% in FRAP assay). Thus, in the case of the DPPH method, the top five food groups contributing to the daily antioxidant intake per serving were fish > egg > meat tubers > fruits. In the case of the FRAP method: meat > fish > egg > fruits > tubers.

Table 16. Antioxidant capacity distributed as a % of each food group in relation to the total diet.

Type of food	***Mean contribution to daily antioxidant capacity intake (%) DPPH assay***	***Mean contribution to daily antioxidant capacity per serving intake (%) DPPH assay***	***Mean contribution to daily antioxidant capacity intake (%) FRAP assay***	***Mean contribution to daily antioxidant capacity per serving intake (%) FRAP assay***
Dairy	35.1	18.1	28.1	13.2
Egg	4.70	24.5	3.10	16.5
Meat	12.2	24.1	17.9	40.5
Fish	1.10	32.9	0.80	23.5
Alcoholic drinks[1]	0.70	2.20	4.40	10.1
Cereals[1]	13.6	3.90	12.7	3.40
Cocoa[1]	0.60	4.20	0.60	4.60
Coffee[1]	0.20	0.90	0.60	2.80
Fruits[1]	11.6	13.5	12.1	15.1
Legumes[1]	0.80	10.1	0.70	9.20
Nuts[1]	0.80	3.50	0.70	2.70
Oils[1]	0.30	0.20	1.10	0.60
Tubers[1]	9.00	19.0	6.50	14.3
Vegetables[1]	9.30	9.70	10.7	9.80

[1]Considering the data of reference [14]

4. Discussion

In most cases, heat treatment positively affects the antioxidant capacity of food [21-23]. In this study, foods subjected to different cooking techniques were compared with their raw form. It was found that cooking generally increased the antioxidant capacity of foods, especially fried foods. Similar results have been found in other studies [24-26] which claim that olive oil used for frying provides a high antioxidant capacity to the preparation. However, some cooking techniques, such as boiling, could result in a loss of hydrosoluble compounds in the cooking water, such as B vitamins, and therefore antioxidant capacity could be reduced [21].

The highest antioxidant capacity was obtained after *in vitro* fermentation of foods (more than 90% of the total antioxidant capacity). This is an important result of our study, since *in vitro* fermentation potentially release-transform bioactive compounds with high antioxidant capacity. Therefore, the gut microbiota seems to play an important role in the release of these compounds from the indigestible matrix of animal-derived foods [24, 25] as in the case of plant-derived foods [14]. Heat treatment catalyze different chemical re-actions such the Maillard reaction [27-29]. In this sense, cooking techniques with a high heat-load (i.e. frying, grilling and roasting) can produce a large amount of melanoproteins [30,31], which are end-products of the Maillard reaction with a high antioxidant capacity [32]. Such melanoidins are hardly digested and reach the colon, where they are metabolized by the gut microbiota [33].

The antioxidant capacity of digested meats (beef, chicken, lamb and pork) ranged from 13.2 to 20.5 mmol Trolox equivalents/Kg meat (**Table 26** in supplemental information) which are in line with those reported by other authors [26]. However, the study reported by Carrillo et al., [26] doesn't

include the antioxidant capacity obtained after *in vitro* fermentation, which is up to a 95% higher, reinforcing the idea that the fermentation step is needed to check the overall antioxidant potential of a given food. Lamb and pork meats were the most antioxidant meats with the DPPH method, while lamb was the lowest one with the FRAP assay (**Table 26** in supplemental information). This could be related to the poor ability of lamb antioxidants to reduce ferric ion to its ferrous form instead of quenching radical species [26]. In addition, although the antioxidant capacity of digested meat and fish was similar (**Table 19** in supplemental information) the final anti-oxidant capacity of meat was higher, since more antioxidant compounds could be released after fermentation. These differences could come from the feed that these animals have. The feeding of meat-producing animals is more controlled than that of fish, and they may have been fed feeds rich in compounds with antioxidant activity [10].

In the group of dairy products, butter stood out as the food with the greatest antioxidant capacity. This could be explained taking into account that some antioxidant compounds in dairy products (such as α-tocopherol, β-carotene, vitamins A and D3 and phospholipids) are found in milk fat, the main component of butter [11].

Among all the foods chosen for this study, meat stood out for its antioxidant capacity, while dairy products and fish had the lowest values, which doesn't mean that their contribution to the antioxidant capacity intake with the diet is also lower. The antioxidant capacity provided by each food was studied, taking into account daily consumption in a regular diet [19], as well as portion sizes [20] (**Table 14**). In **Table 15**, the culinary treatments applied were also taken into account. Dairy products, which are highly

consumed by the Spanish population [19], stood out for their daily intake, as well as roasted meat and grilled fish.

Till now, the efforts on calculating the contribution of the regular diet to the daily antioxidant intake have been centered in plant foods [16,34], since they provide many bioactive antioxidant compounds such as phenolic compounds, vitamins, etc. Thus, our results cannot be compared with other papers on the matter, since there is no scientific literature about the contribution of animal foods to the daily antioxidant capacity. However, foods of animal origin are also a good source of antioxidant compounds like dipeptides (carnosine and anserine), uric acid, polyamines, ascorbic acid, α-tocopherol, B group vitamins, carotenoids, ubiquinone, among others [26]. This is why we calculated the overall contribution of the Spanish diet to the daily antioxidant capacity (**Table 16)** taking into account the intake of animal origin foods (data reported in the paper) and plant foods [14]. The first interesting result is that the Spanish diet provides an average of 175.1 (DPPH) and 164.3 (FRAP) mmol Trolox/day, which is much higher than that previously reported [34] for vegetable products only (6.1 mmol Trolox/day). This could be explained taking into account that that the initials calculations performed by Saura-Calixto and Goñi [33] were computed with the usual extraction method of antioxidant species, avoiding the large effects of digestion and fermentation. In addition, it is noteworthy to mention that the contribution of animal foods was notable (a 49.7% and 53.1% of the total antioxidant capacity intake for DPPH and FRAP methods), reaching 87.1 and 87.3 mmol Trolox/day for DPPH and FRAP assays, respectively. The food groups with a higher contribution to the daily antioxidant capacity intake of the Spanish diet were as follows: dairy > cereals > meat > fruits > vegetables > tubers > egg (DPPH) and dairy > meat > cereals > fruits > vegetables > tubers > egg (FRAP). However, if an increase in antioxidant capacity intake

should be recommended, them the food groups suggested (due to the high antioxidant capacity provided by a portion) will be: fish > egg > meat > tubers > dairy > vegetables (DPPH) and meat > fish > egg > fruits > tubers > dairy.

5. Conclusions

In conclusion, this study reinforces the concept that foods of animal origin could be considered as a good source of antioxidant compounds for humans. This research has demonstrated that though animal origin food may not be rich in bioactive antioxidant components (like plant foods) gastrointestinal digestion and, more importantly, gut microbiota fermentation can improve the antioxidant properties of such foods. Most of the antioxidant power of these foodstuffs was released subsequent to *in vitro* gut microbiota fermentation (around 90%). The food groups with the highest antioxidant capacity were meat and fish. The foods that contributed the most antioxidant capacity to the diet in terms of daily consumption were dairy products, while in terms of portion size, the foods with the highest antioxidant capacity were meat and fish. Therefore, the daily antioxidant capacity intake in the Spanish diet has been revisited, finding that foods of animal origin contribute around a 50% to the daily antioxidant capacity intake. So, further studies on antioxidant capacity involving foods of animal origin after *in vitro* digestion and fermentation should be carried out in the future in order to estimate their contribution to the daily intake of antioxidant capacity.

SUPPLEMENTAL INFORMATION

Table 17. Food of animal origin and cooking conditions.

Group	Sample name	Cooking method
Dairy	Butter	Fried
Dairy	Butter	Raw
Dairy	Cheese (Gouda)	Fried
Dairy	Cheese (Gouda)	Grilled
Dairy	Cheese (Gouda)	Raw
Dairy	Cheese (Gouda)	Roasted
Dairy	Milk	UHT
Dairy	Yogurt	Raw
Egg	Egg	Boiled
Egg	Egg	Fried
Egg	Egg	Grilled
Egg	Egg	Roasted
Fish	Cod fish	Boiled
Fish	Cod fish	Fried
Fish	Cod fish)	Grilled
Fish	Cod fish	Roasted
Fish	Salmon	Boiled
Fish	Salmon	Fried
Fish	Salmon	Grilled
Fish	Salmon	Raw
Fish	Salmon	Roasted
Meat	Beef	Boiled
Meat	Beef	Fried
Meat	Beef	Grilled
Meat	Beef	Roasted
Meat	Chicken	Boiled
Meat	Chicken	Fried
Meat	Chicken	Grilled
Meat	Chicken	Roasted
Meat	Lamb	Boiled
Meat	Lamb	Fried

Meat	Lamb	Grilled
Meat	Lamb	Roasted
Meat	Pork	Bolied
Meat	Pork	Fried
Meat	Pork	Grilled
Meat	Pork	Roasted

Table 18. Antioxidant capacity of *in vitro* digested-fermented foods of animal origin depending on the cooking method.

Cooking technique	**$TEAC_{DPPH}$ (µmol Trolox/Kg food)**		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	$12.5^{a,b} \pm 4.81$	$354^{a} \pm 270$	$368^{a} \pm 272$
UHT	$5.45^{a,b} \pm 2.92$	$207^{a} \pm 2.21$	$213^{a} \pm 5.13$
Fried	$14.5^{b} \pm 10.1$	$322^{a} \pm 233$	$336^{a} \pm 232$
Grilled	$10.2^{a,b} \pm 5.49$	$326^{a} \pm 237$	$361^{a} \pm 59.8$
Raw	$4.76^{a} \pm 5.41$	$227^{a} \pm 63.3$	$231^{a} \pm 63.3$
Roasted	$13.1^{a,b} \pm 7.64$	$347^{a} \pm 240$	$360^{a} \pm 242$
	$TEAC_{FRAP}$ (µmol Trolox/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	$3.07^{a} \pm 1.41$	$277^{a} \pm 71.4$	$280^{a} \pm 71.3$
UHT	$0.77^{a} \pm 0.44$	$185^{a} \pm 2.45$	$185^{a} \pm 2.02$
Fried	$4.29^{a} \pm 2.50$	$282^{a} \pm 83.3$	$287^{a} \pm 83.5$
Grilled	$3.23^{a} \pm 0.95$	$342^{a} \pm 147$	$347^{a} \pm 155$
Raw	$4.41^{a} \pm 2.19$	$276^{a} \pm 63.2$	$281^{a} \pm 62.8$
Roasted	$4.36^{a} \pm 2.37$	$281^{a} \pm 41.1$	$286^{a} \pm 40.9$

Table 19. Antioxidant capacity of *in vitro* digested-fermented foods of animal origin depending on the group.

Food	$TEAC_{DPPH}$ (µmol Trolox/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Dairy	$4.98^{a} \pm 6.12$	$201^{a} \pm 54.5$	$206^{a} \pm 55.2$
Egg	$10.8^{a,b} \pm 2.91$	$230^{a} \pm 66.0$	$241^{a} \pm 65.6$
Fish	$12.0^{b} \pm 8.00$	$216^{a} \pm 36.1$	$228^{a} \pm 33.5$
Meat	$15.2^{b} \pm 6.90$	$463^{b} \pm 284$	$499^{b} \pm 280$
Mean	*8.60 ± 5.98*	*278 ± 110*	*294 ± 109*
	$TEAC_{FRAP}$ (µmol Trolox/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Dairy	$5.03^{a} \pm 3.47$	$271^{a} \pm 67.0$	$276^{a} \pm 68.3$
Egg	$5.68^{a} \pm 0.89$	$330^{a} \pm 169$	$336^{a} \pm 169$
Fish	$2.73^{b} \pm 0.89$	$297^{a} \pm 96.6$	$300^{a} \pm 96.4$
Meat	$3.23^{b} \pm 0.87$	$288^{a} \pm 77.3$	$288^{a} \pm 77.1$
Mean	*4.18 ± 1.53*	*297 ± 102*	*300 ± 103*

Table 20. Antioxidant capacity of *in vitro* digested-fermented dairy foods depending on the cooking method.

Cooking technique	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
UHT	$5.45^{a} \pm 2.92$	$207^{a} \pm 2.21$	$213^{a} \pm 5.13$
Fried	$10.3^{a} \pm 10.8$	$208^{a} \pm 36.4$	$218^{a} \pm 39.7$
Grilled	$4.54^{a} \pm 0.52$	$142^{a} \pm 0.09$	$146^{a} \pm 0.44$
Raw	$3.01^{a} \pm 2.08$	$225^{a} \pm 74.7$	$228^{a} \pm 74.5$
Roasted	$0.22^{a} \pm 0.05$	$170^{a} \pm 3.61$	$171^{a} \pm 3.66$
	FRAP (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
UHT	$0.77^{a} \pm 0.44$	$185^{a} \pm 2.45$	$186^{a} \pm 2.01$
Fried	$7.30^{a} \pm 3.59$	$333^{a} \pm 6.00$	$340^{b} \pm 3.07$
Grilled	$2.75^{a} \pm 0.00$	$216^{a} \pm 53.4$	$219^{a,b} \pm 53.4$
Raw	$4.25^{a} \pm 2.58$	$276^{a} \pm 74.7$	$281^{a,b} \pm 74.3$
Roasted	$9.36^{a} \pm 0.46$	$270^{a} \pm 0.05$	$280^{a,b} \pm 0.51$

Table 21. Antioxidant capacity of in vitro digested-fermented dairy foods depending on the dairy type.

Sample	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Butter	$10.9^{a} \pm 10.1$	$260^{b} \pm 81.4$	$271^{b} \pm 76.0$
Gouda	$1.82^{a} \pm 1.83$	$176^{a} \pm 25.6$	$178^{a} \pm 24.4$
Milk	$5.45^{a} \pm 2.92$	$207^{a,b} \pm 2.21$	$213^{a,b} \pm 5.13$
Yogurt	$5.23^{a} \pm 0.65$	$180^{a,b} \pm 11.9$	$185^{a,b} \pm 11.2$
Mean	*5.85 ± 3.88*	*206 ± 30.3*	*212 ± 29.2*
	FRAP (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Butter	$3.39^{a,b} \pm 0.97$	$352^{b} \pm 16.8$	$355^{b} \pm 15.9$
Gouda	$7.51^{b} \pm 3.14$	$269^{a} \pm 47.7$	$276^{a} \pm 50.3$
Milk	$0.77^{a} \pm 0.44$	$185^{a} \pm 2.45$	$186^{a} \pm 2.01$
Yogurt	$2.62^{a,b} \pm 0.65$	$203^{a} \pm 11.8$	$205^{a} \pm 11.1$
Mean	*3.57 ± 1.3*	*252 ± 19.7*	*256 ± 19.8*

Table 22. Antioxidant capacity of *in vitro* digested-fermented fish depending on the cooking method.

Cooking technique	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	$10.9^{a} \pm 4.69$	$212^{a} \pm 10.1$	$223^{a} \pm 9.96$
Fried	$10.4^{a} \pm 11.3$	$225^{a} \pm 64.6$	$236^{a} \pm 53.8$
Grilled	$13.0^{a} \pm 8.90$	$218^{a} \pm 39.3$	$231^{a} \pm 38.2$
Raw	$10.0^{a} \pm 10.5$	$231^{a} \pm 10.5$	$241^{a} \pm 0.05$
Roasted	$14.5^{a} \pm 8.94$	$200^{a} \pm 29.2$	$214^{a} \pm 37.2$
	FRAP (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	$2.38^{a} \pm 0.09$	$268^{a} \pm 11.1$	$271^{a} \pm 11.2$
Fried	$2.39^{a} \pm 0.84$	$281^{a} \pm 184$	$283^{a} \pm 184$
Grilled	$2.45^{a} \pm 0.44$	$353^{a} \pm 114$	$355^{a} \pm 114$
Raw	$4.89^{b} \pm 0.02$	$276^{a} \pm 0.58$	$281^{a} \pm 0.59$
Roasted	$2.63^{a} \pm 0.18$	$296^{a} \pm 13.8$	$299^{a} \pm 13.9$

Table 23. Antioxidant capacity of *in vitro* digested-fermented fish depending on the fish type.

Sample	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Cod fish	$6.00^{a} \pm 6.26$	$232^{a} \pm 43.3$	$238^{a} \pm 44.1$
Salmon	$16.7^{b} \pm 5.79$	$202^{a} \pm 23.5$	$219^{a} \pm 20.4$
Mean	*11.4 ± 6.03*	*217 ± 33.4*	*229 ± 32.3*
	FRAP (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Cod fish	$2.20^{a} \pm 0.38$	$310^{a} \pm 96.9$	$312^{a} \pm 96.7$
Salmon	$3.16^{b} \pm 0.96$	$287^{a} \pm 100$	$290^{a} \pm 100$
Mean	*2.68 ± 0.67*	*299 ± 98.5*	*301 ± 98.4*

Table 24. Antioxidant capacity of *in vitro* digested-fermented meat depending on the cooking method.

Cooking technique	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	$13.2^{a} \pm 5.11$	$474^{a} \pm 311$	$488^{a} \pm 314$
Fried	$19.8^{a} \pm 9.21$	$457^{a} \pm 303$	$477^{a} \pm 296$
Grilled	$10.6^{a} \pm 3.35$	$450^{a} \pm 288$	$559^{a} \pm 268$
Roasted	$16.1^{a} \pm 5.52$	$470^{a} \pm 291$	$487^{a} \pm 290$
	FRAP (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Boiled	$2.65^{a} \pm 0.68$	$288^{a} \pm 95.0$	$290^{a} \pm 95.0$
Fried	$3.62^{a} \pm 1.21$	$263^{a} \pm 14.9$	$267^{a} \pm 15.7$
Grilled	$3.26^{a} \pm 0.39$	$323^{a} \pm 108$	$325^{a} \pm 119$
Roasted	$3.39^{a} \pm 0.74$	$277^{a} \pm 57.7$	$280^{a} \pm 57.7$

Table 25. Antioxidant capacity of *in vitro* digested-fermented meat depending on the meat type.

Sample	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Beef	$20.5^{a} \pm 10.8$	$175^{a} \pm 16.5$	$201^{a} \pm 19.4$
Chicken	$13.2^{a} \pm 8.94$	$196^{a} \pm 22.1$	$209^{a} \pm 16.2$
Lamb	$13.2^{a} \pm 3.12$	$745^{b} \pm 31.1$	$759^{b} \pm 31.2$
Pork	$15.2^{a} \pm 2.67$	$735^{b} \pm 52.7$	$750^{b} \pm 54.5$
Mean	*15.5 ± 3.4*	*463 ± 320*	*480 ± 317*
	FRAP (mmol Trolox equivalents/Kg)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Beef	$3.31^{a} \pm 1.55$	$306^{a,b} \pm 95.0$	$309^{a,b} \pm 102$
Chicken	$3.12^{a} \pm 0.55$	$331^{b} \pm 89.8$	$335^{b} \pm 89.8$
Lamb	$2.86^{a} \pm 0.80$	$228^{a} \pm 15.3$	$231^{a} \pm 14.9$
Pork	$3.64^{a} \pm 0.38$	$285^{a,b} \pm 49.9$	$289^{a,b} \pm 50.1$
Mean	*3.23 ± 0.33*	*288 ± 43.9*	*291 ± 44.2*

Table 26. Antioxidant capacity of *in vitro* digested-fermented red and white meat.

Other meats	DPPH (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Red meat	$16.3^{a} \pm 7.99$	$460^{a} \pm 296$	$520^{a} \pm 287$
White meat	$14.2^{a} \pm 5.88$	$466^{a} \pm 281$	$480^{a} \pm 282$
Mean	*15.3 ± 1.48*	*463 ± 4.24*	*500 ± 28.3*
	FRAP (mmol Trolox equivalents/Kg food)		
	Digested fraction	**Fermented fraction**	**Total antioxidant capacity**
Red meat	$3.06^{a} \pm 1.15$	$267^{a} \pm 77.1$	$269^{a} \pm 73.8$
White meat	$3.37^{a} \pm 0.53$	$308^{a} \pm 74.2$	$312^{a} \pm 74.1$
Mean	*3.20 ± 0.22*	*288 ± 29.0*	*291 ± 30.4*

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Sotos Prieto, M.; Guillen, M.; Sorlí, J.V.; Asensio, E.M.; Gillem Sáiz, P.; González, J.I.; Corella, D. Consumo de Carne y Pescado En Población Mediterránea Española de Edad Avanzada y Alto Riesgo Cardiovascular. Nutr. Hosp. 2011, 26, 1033-1040, doi:10.3305/nh.2011.26.5.5102.

2. Yip, C.S.C.; Lam, W.; Fielding, R. A Summary of Meat Intakes and Health Burdens. Eur. J. Clin. Nutr. 2018, 72, 18-29, doi:10.1038/ejcn.2017.117.

3. Olmedilla-Alonso, B.; Jiménez-Colmenero, F.; Sánchez-Muniz, F.J. Development and Assessment of Healthy Properties of Meat and Meat Products Designed as Functional Foods. Meat Sci. 2013, 95, 919-930, doi:10.1016/j.meatsci.2013.03.030.

4. Abuajah, C.I.; Ogbonna, A.C.; Osuji, C.M. Functional Components and Medicinal Properties of Food: A Review. J. Food Sci. Technol. 2015, 52, 2522-2529, doi:10.1007/s13197-014-1396-5.

5. Leri, M.; Scuto, M.; Ontario, M.L.; Calabrese, V.; Calabrese, E.J.; Bucciantini, M.; Stefani, M. Healthy Effects of Plant Polyphenols: Molecular Mechanisms. Int. J. Mol. Sci. 2020, 21, 1250, doi:10.3390/ijms21041250.

6. Pimentel, F.A; Nitzke, J.A; Klipel, C.B.; Jong, E.V. de chocolate and red wine—A comparison between flavonoids content. Food Chem. 2010, 120, 109-112. doi:10.1016/j.foodchem.2009.09.078

7. Marcolini, E; Babini, E; Bordoni, A; Di Nunzio, M; Laghi, L; Maczó, A; Picone,G; Szerdahelyi, E; Valli, V; Capozzi, F. Bioaccessibility of the Bioactive Peptide Carnosine during in Vitro Digestion of Cured Beef Meat. J. Agric. Food Chem. 2015, 63, 4973-4978, doi:10.1021/acs.jaFolin-Ciocalteu.5b01157.

8. Xing, L.; Chee, M.E.; Zhang, H.; Zhang, W.; Mine, Y. Carnosine—a Natural Bioactive Dipeptide: Bioaccessibility, Bioavailability and Health Benefits. J. Food Bioact. 2019, 5, 8-17, doi:10.31665/JFB.2019.5174.

9. Radzik-Rant, A.; Rant, W.; Sosnowiec, G.; Świątek, M.; Niżnikowski, R.; Szymańska, Ż. The Effect of Genotype and Muscle Type on the Physico-Chemical Characteristics and Taurine, Carnosine and L-Carnitine Concentration in Lamb Meat. Arch. Anim. Breed. 2020, 63, 423-430, doi:10.5194/aab-63-423-2020.

10. Chen, D.; Wu, M.; Xie, S.; Li, X.; Tao, Y.; Wang, X.; Huang, L.; Pan, Y.; Peng, D.; Yuan, Z. Determination of Tartrazine, Lutein, Capsanthin, Canthaxanthin and β-Carotene in Animal-Derived Foods and Feeds by HPLC Method. J. Chromatogr. Sci. 2019, 57, 462-468, doi:10.1093/chromsci/bmz019.

11. Grażyna, C.; Hanna, C.; Adam, A.; Magdalena, B.M. Natural Antioxidants in Milk and Dairy Products. Int. J. Dairy Technol. 2017, 70, 165-178, doi:10.1111/1471-0307.12359.

12. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an improved global antioxidant response method (GAR+): Physiological-resembling in vitro digestion-fermentation method. Food Chem. 2018, 239, 1253-1262. doi: 10.1016 / j.foodchem.2017.07.024.

13. Carrillo, C.; Barrio, Á.; Del Mar Cavia, M.; Alonso-Torre, S. Global Antioxidant Response of Meat. J. Sci. Food Agric. 2017, 97, 2358-2365, doi:10.1002/jsfa.8047.

14. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to In Vitro Digestion-Fermentation. Antioxidants 2020, 9, 1312, doi:10.3390/antiox9121312.

15. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an Improved Global Antioxidant Response Method (GAR+): Physiological-Resembling in Vitro Digestion-Fermentation Method. Food Chem. 2018, 239, 1253-1262, doi:10.1016/j.foodchem.2017.07.024.

16. Pastoriza, S.; Delgado-Andrade, C.; Haro, A.; Rufián-Henares, J.A. A Physiologic Approach to Test the Global Antioxidant Response of Foods. The GAR Method. Food Chem. 2011, 129, 1926-1932, doi:10.1016/j.foodchem.2011.06.009.

17. Rapisarda P, Tomaino A, Lo Cascio R, Bonina F, De Pasquale A, Saija A. Antioxidant Effectiveness As Influenced by Phenolic Content of Fresh Orange Juices. J Agric Food Chem. 1999;47:4718-23, doi: 10.1021 / jf9901111.

18. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal. Biochem. 1996, 239, 70-76, doi:10.1006/abio.1996.0292.

19. Mercasa. La Alimentación en España. 2020. Available online: https://www.mercasa.es/media/publicaciones/ 281/AEE_2020_web.pdf (accessed on 15 January 2021).

20. Salvador i Castells, G. Tabla de Medidas Caseras de Alimentos. In Nutrición y Dietética Clínica; Salas-Salvadó, J., Bonada, A., Trallero, R., Saló, M.E., Eds.; Elsevier-Masson: Barcelona, Spain, 2000; pp. 557-570.

21. Ramírez-Anaya, J.P.; Samaniego-Sánchez, C.; Castañeda-Saucedo, M.C.; Villalón-Mir, M.; de la Serrana, H.L.-G. Phenols and the antioxidant capacity of Mediterranean vegetables prepared with extra virgin olive oil using different domestic cooking techniques. Food Chem. 2015, 188, 430-438. doi:10.1016/j.foodchem.2015.04.124.

22. Miglio, C.; Chiavaro, E.; Visconti, A.; Fogliano, V.; Pellegrini, N. Effects of different cooking methods on nutritional and physicochemical characteristics of selected vegetables. J. Agric. Food Chem. 2008, 56, 139-147, doi:10.1021/jf072304b.

23. Rufián-Henares, J.A.; Guerra-Hernández, E.; García-Villanova, B. Effect of Red Sweet Pepper Dehydration Conditions on Maillard Reaction, Ascorbic Acid and Antioxidant Activity. J. Food Eng. 2013, 118, 150-156, doi:10.1016/j.jfoodeng.2013.03.034.

24. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Effect of Home Cooking on the Antioxidant Capacity of Vegetables: Relationship with Maillard Reaction Indicators. Int. Food Res. J. 2019, 121, 514-523, doi:10.1016/j.foodres.2018.12.007.

25. Pérez-Burillo, S.; Pastoriza, S.; Jiménez-Hernández, N.; D´Auria, G.; Francino, M.P.; Rufián-Henares, J.A. Effect of Food Thermal Processing on the Composition of Gut Microbiota. J. Agric. Food Chem. 2018, 66, 11500-11509, doi:10.10021/acs.jaFolin-Ciocalteu.8b04077.

26. Carrillo, C.; Barrio, A.; Cavia, M.M.; Alonso-Torre, S. Global antioxidant response of meat. J. Sci. Food Agric. 2017, 97, 2358-2365, doi:10.1002/jsfa.8047.

27. Rufián-Henares, J.A.; Guerra-Hernández, E.; García-Villanova, B. Colour measurement as indicator for controlling the manufacture and storage of enteral formulas. Food Cont. 2006, 17, 489-493, doi:10.1016/j.jfoodcont.2005.02.011.

28. Delgado-Andrade, C.; Rufián-Henares, J.A.; Morales, F.J. Lysine availability is diminished in commercial fibre-enriched breakfast cereals. Food Chem. 2007, 100, 725-731, doi:10.1016/j.foodchem.2005.10.031.

29. Pastoriza de la Cueva, S.; Álvarez, J.; Végvári, Á.; Montilla-Gómez, J.; Cruz-López, O.; Delgado-Andrade, C.; Rufián-Henares, J.A. Relationship between HMF intake and SMF formation in vivo: An animal and human study. Mol. Nutr. Food Res. 2017, 61, 1600773, doi: 10.1002/mnfr.201600773.

30. Zhou, Y.; Xie, F.; zhou, X.; Wang, Y.; Tang, W.; Xiao, Y. Effects of Maillard reaction on flavor and safety of Chinese traditional food: roast duck. J. Sci. Food Agric. 2016, 96, 1915-1922, doi:10.1002/jsfa.7297.

31. Rodríguez, A.; Lema, P.; Bessio, M.I.; Moyna, G.; Panizzolo, L.A.; Ferreira, F. Isolation and Characterization of Melanoidins from Dulce de Leche, A Confectionary Dairy Product. Molecules 2019, 24, 4163, doi:10.3390/molecules24224163.

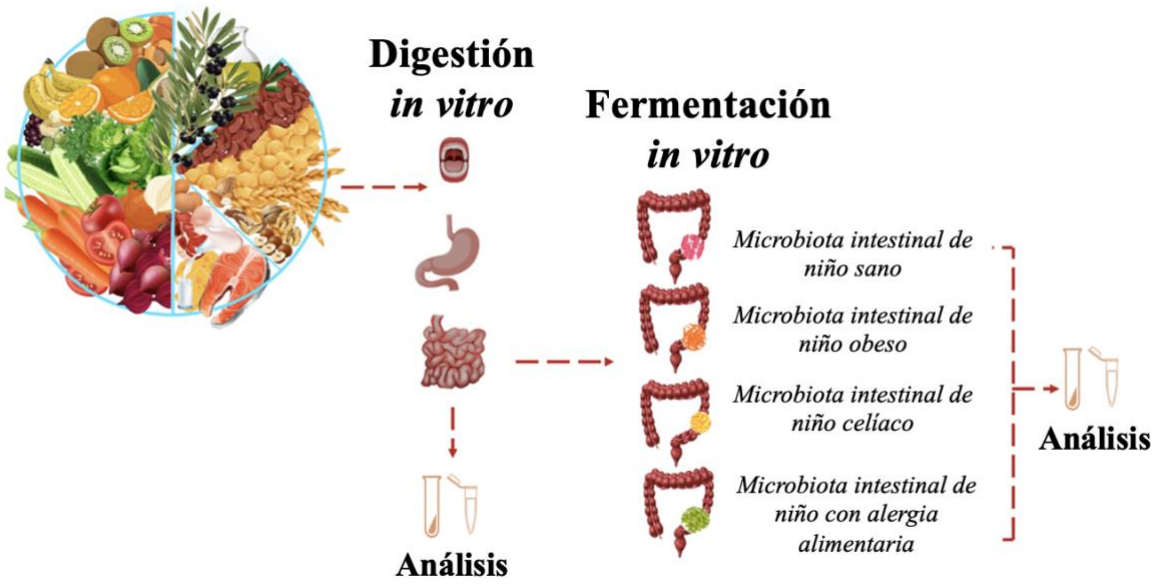
32. Gu, F.L., Kim, J.M.; Abbas, S.; Zhang, X.M., Xia, S.Q., Chen, Z.X. Structure and antioxidant activity of hugh molecular Maillard reaction products from casein-glucose. Food Chem. 2010, 120, 505-511, doi:10.1016/j.foodchem.2009.10.044.

33. Tagliazucchi, D.; Bellesia, A. The gastro-intestinal tract as the major site of biological action of dietary melanoidins. Amino Acids 2015, 47, 1077-1089, doi:10.1007/s00726-015-1951-z.

34. Saura-Calixto, F.; Goñi, I. Antioxidant capacity of the Spanish Mediterranean diet. Food Chem. 2006, 94, 442-447, doi: 10.1016/j.foodchem.2004.11.033.

CAPÍTULO II

Estudio de la funcionalidad de la microbiota intestinal de diferentes grupos de niños



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The Gut Microbiota of Obese Children Releases Lower Antioxidant Capacity from Food than That of Lean Children

En este trabajo se estudió cómo la microbiota intestinal de niños con obesidad extraía una concentración de capacidad antioxidante diferente a la que era capaz de extraer la de niños sin patologías. Para ello, se digirieron y fermentaron *in vitro* diferentes alimentos, tanto de origen vegetal como animal, con material fecal de niños obesos y delgados. Se utilizaron tres métodos diferentes para medir la capacidad antioxidante liberada durante la digestión y la fermentación *in vitro*.

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The Gut Microbiota of Obese Children Releases Lower Antioxidant Capacity from Food than That of Lean Children

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Abstract

Prevalence of obesity has been increasing in children over the last decades, becoming a concern for health professionals and governments. Gut microbial community structure have been found to differ from that of lean subjects for some taxa which could result in different production of microbial metabolites. The aim of the present work was to study whether the gut microbiota from obese children extracts different concentration of antioxidant capacity than the gut microbiota from lean children. For this purpose, different foods were *in vitro* digested and *in vitro* fermented using fecal material from obese and lean children. FRAP, DPPH and Folin-Ciocalteu methods were used to measure the antioxidant capacity released during digestion and fermentation. Overall, when using lean gut microbiota, antioxidant capacity released was higher when measured via DPPH and FRAP. Moreover, according to DPPH results, lean gut microbiota could potentially release more antioxidant power from vegetables than from animal products, while obese gut microbiota did the opposite. On the contrary, with the FRAP method obese gut microbiota released higher levels of antioxidant power from plant products than from animal products, but the final antioxidant capacity was still lower than that released by lean gut microbiota. Therefore, these results reflect that the total antioxidant capacity of foods is influenced by the gut microbiota, although whether that antioxidant capacity is released from plant or animal products can be slightly influenced by the method used for analysis.

Keywords: antioxidant capacity; *in vitro* digestion; *in vitro* fermentation; obesity; children; gut microbiota.

1. Introduction

According to the World Health Organization, obesity is defined as a preventable risk factor that involves an abnormal or excessive accumulation of fat that can be harmful to health and is one of the most serious health problems of the 21st century. In particular, the global prevalence of childhood obesity has markedly increased in recent decades and is considered a global pandemic [1]. It is estimated that 12-36% of European children aged 7-11 years are overweight or obese [2]. In addition to a number of health problems derived from obesity such as cardiovascular disease or diabetes, it is increasingly recognized that gut microbial community structure and functionality change in the context of non-communicable diseases such as obesity [1,3].

The gut microbiota is considered a metabolic organ consisting of a set of microorganisms and their genomes [1]. It is composed of members from different phyla though Firmicutes and Bacteroides are the most dominant. Obese patients have been found to show some disturbances in the community such as lower alpha diversity [4,5]. Additionally, obese patients usually show an increased Firmicutes/Bacteroides ratio [6-11]. There are also some bacteria that is more often found in higher abundance in lean subjects: the family Chistensenellaceae and the genera Methanobacterial, Lactobacillus, Bifidobacteria and Akkermansia [11].

The gut microbiota plays an important role in human metabolism. In fact, it is involved in modulating host nutrition, resulting in vitamin production, fermentation of indigestible dietary components, production of short-chain fatty acids, vitamins and essential amino acids, enrichment of specific lipopolysaccharides, regulation of energy absorption, central appetite, fat storage, chronic inflammation and circadian rhythms [12-14].

Some of the most antioxidant compounds of the diet, phenolics, are actually not absorbed in the small intestine, reaching the colon where they are, in great extent, broken down by gut microbes [15,16]. Therefore, gut microbes can potentially have a great influence on the antioxidant capacity that we are actually obtaining from food. Accordingly, any disturbance in the microbial community could result in an alteration in the concentration of antioxidant capacity extracted from foods.

Different foods of animal origin (e.g. meat, fish, eggs) and most vegetables are consumed after being cooked under different conditions [17]. Heat treatments directly impact the final composition of foods [18,19] due to chemicals modifications such as the Maillard reaction [20]. In this sense, boiling is the less aggressive cooking technique compared to frying, roasting or grilling [21]. Therefore, assessing the effect of mild thermal treatments on the antioxidant capacity is also necessary.

The aim of this work was to study the antioxidant capacity of Mediterranean diet foods after *in vitro* digestion and *in vitro* fermentation. *In vitro* fermentation was performed with gut microbiota obtained from fecal material from obese and lean children.

2. Materials and Methods

2.1. Chemicals

2.1.1. *In vitro* digestion and fermentation

Pancreatin from porcine pancreas was acquired from Alpha Aesar (Kandel, Germany). Tryptone, cysteine, resazurin, sodium dihydrogen phosphate, sodium sulfide, porcine bile acids, pepsin and salivary alpha-amylase were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.1.2. Antioxidant Capacity

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2 diphenyl-1-1picrythydrazul hydrate 95%), sodium acetate, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), iron (III) chloride hexahydrate, gallic acid, Folin-Ciocalteu® reagent and hydrochloric acid. All the reagents for the analysis of antioxidant capacity were obtained from Sigma Aldrich (Darmastad, Germany).

2.2. Samples

A total of 47 foods belonging to the following groups were studied: nuts (nut mix and peanuts), cereals (bread, whole grain bread, pasta, whole grain pasta, rice, whole grain rice, biscuits, whole grain biscuits, breakfast cereals and whole grain breakfast cereals), vegetables (zucchini, pepper, carrot, eggplant, onion, cauliflower, spinach, tomato, cabbage and lettuce), legumes (kidney beans and lentils), fruits (apple, banana, orange, grapes, plum, peach and olives), tubers (potato and sweet potato), cocoa (dark chocolate and cocoa butter), dairy (butter, gouda, milk and yogurt), meat (chicken, beef, lamb and pork) fish (salmon and cod fish) and egg. Samples

were analyzed either in their raw form or submitted to boiling in water for those foods that are usually cooked. Boiling was carried out in ultra-pure water for 20 min at a 5:1 ratio (water:food) on the following foods: pasta, whole grain pasta, rice, whole grain rice, kidney beans, lentils, potato, sweet potato, chicken, beef, lamb, pork, salmon, cod fish, egg, carrot, cauliflower, onion, pepper, spinach and zucchini. The food was acquired from 3 retail shops and stored refrigerated or in a cold room, according to the label or retailer instructions.

2.3. In vitro digestion and fermentation

Every single sample was *in vitro* digested and fermented following previous protocols [22,23]. For each sample, 5 g of sample were weighed (in triplicate) for further *in vitro* gastrointestinal digestion and *in vitro* fermentation. *In vitro* digestion consists of 3 steps: oral, gastric and intestinal. First, samples were weighed into a 50 mL tube. Five milliliters of simulated salivary fluid with 150 U/mL were added and mixed into the 50 mL tube carrying the sample and kept at 37ºC for 2 min. Secondly, 10 mL of simulated gastric fluid with 4000 U/mL of gastric pepsin were added to the mix, the pH lowered to 3 and kept at 37 ºC for 2 hours. Finally, 20 mL of simulated intestinal fluid with 200 U/mL of pancreatin and 20 mM bile salts were added into the tube, the pH increased to 7 and kept at 37ºC for 2 hours. Enzyme activity was halted by immersion in ice for 15 min. Tubes were centrifuged, the supernatant (fraction available for absorption at the small intestine) collected and the pellet (fraction not digested that would reach the colon) used for *in vitro* fermentation.

Fecal samples from 5 lean donors and 5 from obese were used for the *in vitro* fermentation. The common inclusion criteria for both groups of children were age between 8 and 10 years as well as not having taken

antibiotics or probiotics 3 months before the start of the study. Common exclusion criteria were diagnosis of chronic gastrointestinal disorders or any other chronic disease or special diet. Only lean children with a BMI between the 5th and 85th percentile for sex, height and age were considered. For the obese group, BMI had to be greater than the 95th percentile for sex, weight and age. Recruitment of the study participants was done via the pediatric unit at the hospital in Athens (Greece). Parents were given an informed consent as well as information and questionnaires for inclusion/exclusion criteria. The study was approved by ethics committee at the University General Hospital in Athens.

Fecal material was pooled by donor group (lean children and obese children) to account for inter-individual variability. *In vitro* fermentation was carried out at 37 ºC for 20 hours, in oscillation. For this purpose, 0.5 grams of the pellet obtained after *in vitro* gastrointestinal digestion were used, as well as 10% of the supernatant. Fermentation medium composed of peptone (14 g/L, cysteine 312 mg/L, hydrogen sulfide 312 mg/L and resazurin 0.1% v/v) was added to the fermentation tube at a volume of 7.5 mL. A fecal inoculum mas made from fecal material by mixing it with PBS at a concentration of 33%. Two milliliters of inoculum were added to the fermentation tube. Afterwards, nitrogen was bubbled into the tube until reaching anaerobic conditions (transparent solution as opposed to pink when oxygen is dissolved). After 20 hours at 37 ºC, microbial activity was halted by immersion in ice for 15 min and tubes were centrifuged to collect the supernatant (fraction available for absorption at the large intestine), which was stored at -80 ºC until further analysis. Blanks carrying water instead of sample were included in the *in vitro* digestion as well as in the *in vitro* fermentation.

2.4. Antioxidant Assays

Antioxidant capacity was studied in both the fraction obtained from *in vitro* digestion (highly in the small intestine) as well as in the fraction obtained after *in vitro* fermentation (absorbable in the large intestine). The sum of these two fractions would make for the total antioxidant capacity of the sample [24]. Three different methods were used to measure antioxidant capacity since different antioxidant methodologies are usually carried out under different physicochemical conditions such as pH or solvent. In addition, different antioxidant assays can reflect different redox principles or use different chemicals as donors/acceptors of electrons.

Folin-Ciocalteu assay. A previous protocol was followed [25]. It was performed with a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). 30 µL of sample were added in duplicate to each of the 96 wells of a plastic plate. This was mixed with 190 µL of bidistilled water, 15 µL of Folin-Ciocalteu reagent and 60 µL of 10% sodium carbonate solution. The calibration curve was prepared with gallic acid with a concentration that ranged from 0.1 to 2.5 mg/mL. The antioxidant reaction was monitored for 60 min at 37 ºC. The results were expressed as mg gallic acid equivalent/kg of food.

$TEAC_{FRAP}$ assay. The protocol of Benzie and Strain [26] was followed to study the reducing capacity of iron of the different samples. The procedure was carried out in a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). In this case, 20 µL of sample were placed in duplicate in the 96-well microplate and mixed with 280 µL of freshly prepared FRAP reagent (25 mL of 0.3 mM sodium acetate pH 3.6, 2.5 mL of 20mM ferric chloride and 2.5 mL of 40 mM TPTZ). The antioxidant reaction was monitored for 30 min at 37oC and the calibration curve ranged from 0.01-04

mg of Trolox/mL. Results were expressed as mmol Trolox equivalent/kg food.

$TEAC_{DPPH}$ assay. This method was carried out following the protocol of Rapisarda et al., [27]. The procedure was carried out in a microplate reader (FLUOStar Omega, BMG Labtech, Ortenberg, Germany). Twenty μL of sample were added in duplicate to each well of the 96-well plate and mixed with 280 μL of DPPH reagent (74 mg/L methanol prepared daily). The antioxidant reaction was monitored at 37 ºC for 60 min and the calibration curve ranged from 0.01 to 0.4 mg of Trolox/mL. Results were expressed as mmol Trolox equivalent/kg food.

2.5. Statistical analyses

Statistical differences were computed using unpaired Kruskal Wallis test with a 95% confidence comparing the antioxidant capacity of each food group with the base-mean antioxidant capacity (average antioxidant capacity presented by all groups). Therefore, we are showing whether one particular food group exhibits higher or lower antioxidant capacity than the average. A multivariate PCA was carried out to explore differences between groups. Spearman parametric statistic was calculated to show the lineal relation between antioxidant capacity at a $p\ value < 0.05$. Statgraphics Plus software, version 5.1 was used to perform all the statistical analysis.

3. Results

Antioxidant capacity was measured in the supernatant obtained after *in vitro* digestion as well as in the one obtained after *in vitro* fermentation. Both of them account for the total antioxidant capacity of a given food. It is important to bear in mind that antioxidant values obtained after *in vitro* digestion are the same for both type of subjects, lean and obese, since food was only digested once and then fermented with both lean fecal material and obese fecal material. Overall, *in vitro* fermentation accounted for most of the total antioxidant capacity of the samples, as it will be laid out in the following sections. Three different methods (Folin-Ciocalteu, $TEAC_{FRAP}$ and $TEAC_{DPPH}$) were used to measure the antioxidant capacity. All values obtained were corrected for the antioxidant capacity provided by chemicals, enzymes and fecal material.

We checked for linear correlations using Pearson method between the different antioxidant assays. Considering all the values (n = 1472), we obtained significant ($p < 0.05$) correlations for the all the pairs: DPPH-FRAP (coefficient = 0.29), DPPH-Folin-Ciocalteu (coefficient = 0.161) and FRAP-Folin-Ciocalteu (coefficient = 0.632).

3.1. Antioxidant capacity produced during in vitro digestion of foods

DPPH analysis (**Figure 34**) showed that cocoa products, legumes and fruits exerted significantly more antioxidant capacity than the average ($p < 0.05$) whereas dairy presented significantly lower values. The Folin-Ciocalteu assay (**Figure 34**) showed that cocoa products, fish, meat and nuts produced higher antioxidant capacity than the base-mean while fruits and vegetables were on the opposite side ($p < 0.05$). Regarding $TEAC_{FRAP}$ (**Figure 34**), cocoa products again were the group with the highest antioxidant capacity.

However, all the other food groups exhibited similar values with no significant differences versus the base-mean ($p < 0.05$).

As summary, *in vitro* digestion presented cocoa products as the food group with highest antioxidant potential in all three methods whereas opposite results were found for other food groups. There are two situations catching the eye: high Folin-Ciocalteu values achieved by meats and fish and low ones obtained by vegetables and fruits in some assays. This will be discussed in section 4.

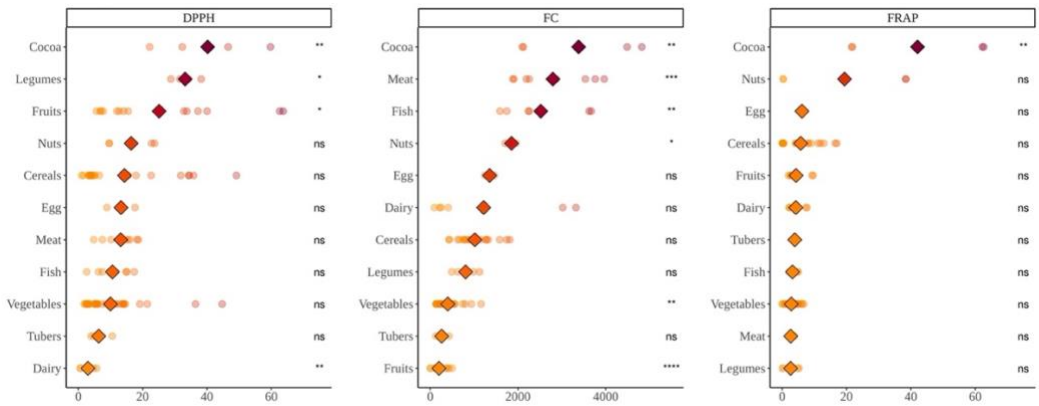


Figure 34. Antioxidant capacity of food obtained after *in vitro* digestion of selected foods. DPPH expressed as mmol Trolox equivalents/kg(L) of food; Folin-Ciocalteu (FC) expressed as mg equivalents of gallic acid/kg(L) of food; FRAP expressed as mmol Trolox equivalents/kg(L) of food. Diamonds represent the mean antioxidant capacity of each group. Dots represent the antioxidant capacity of each food within the group. Statistical analysis was performed via Kruskal Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.2. Study of antioxidant capacity of food fermented with fecal material from lean children

DPPH analysis (**Figure 35**) showed that cereals presented the highest antioxidant values *(*$p < 0.05$), while meat, fruits and fish (this one not significantly) exhibited the lowest values. When we consider total antioxidant capacity (**Figure 36**) (*in vitro* digestion values + *in vitro* fermentation values), results are very similar with cereals at the top and meat at the bottom.

Folin-Ciocalteu assay (**Figure 35**) showed fish as the group with significantly higher antioxidant capacity compared to the average of all food groups ($p < 0.05$). Tubers, on the other hand, showed significantly lower values ($p < 0.05$). Here again, it is surprising to have eggs or meats at similar level than fruits or cocoa, whereas legumes are among the lowest in antioxidant capacity. If we account for the antioxidant capacity released during digestion (**Figure 36**), fish is still the most antioxidant, though now cocoa products are also significantly above the base-mean. Vegetables however, join tubers with significantly lower values.

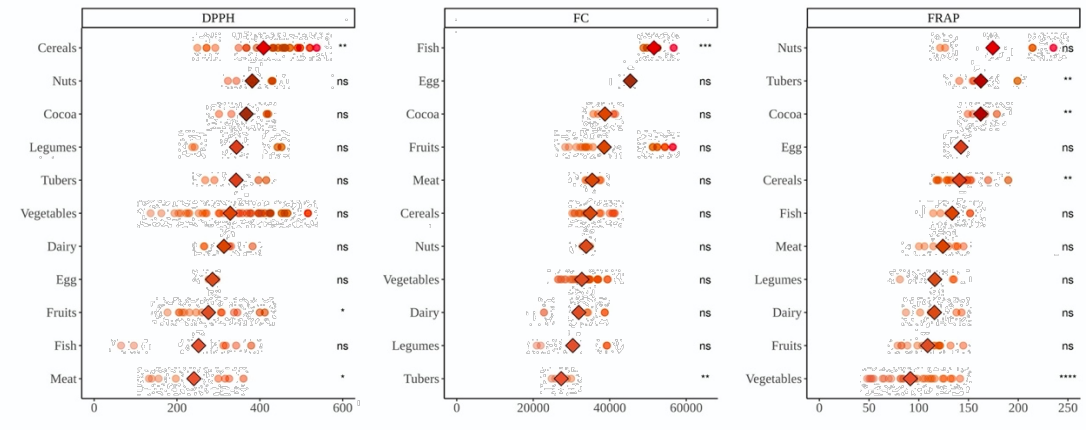


Figure 35. Antioxidant capacity of food obtained after *in vitro* fermentation of selected foods with fecal material from lean children. DPPH expressed as mmol Trolox equivalents/kg (L) of food; Folin-Ciocalteu (FC) expressed as mg equivalents of gallic acid/kg(L) of food; FRAP expressed as mmol Trolox equivalents/kg(L) of food. Diamonds represent the mean antioxidant capacity of each group. Dots represent the antioxidant capacity of each food within the group. Statistical analysis was performed via Kruskal Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

Regarding $TEAC_{FRAP}$ **(Figure 35)**, although nuts, as a group, showed the highest antioxidant capacity, only tubers and cocoa were significantly above the base-mean ($p < 0.05$), due to differences between different types of nuts. Vegetables showed, on the other hand, the lowest values. Similar results were obtained when considering total antioxidant capacity **(Figure 36)**.

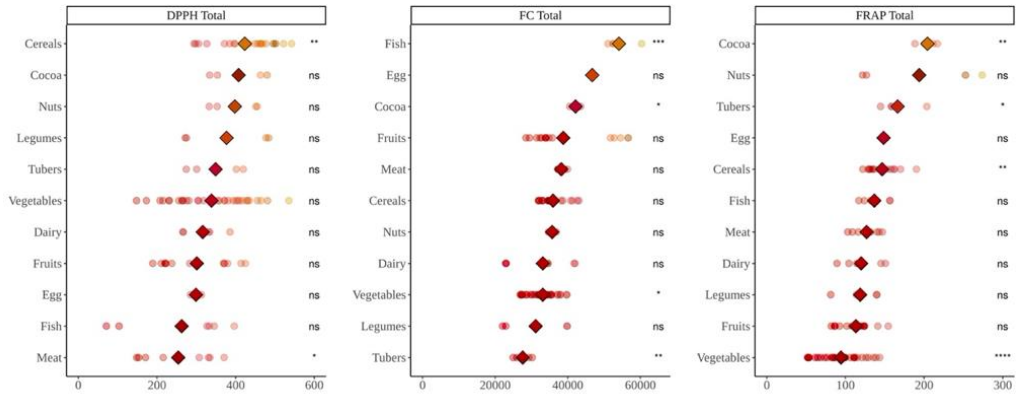


Figure 36. *In vitro* digestion values + *in vitro* fermentation values of food obtained after *in vitro* fermentation of selected foods with fecal material from lean children. DPPH expressed as mmol Trolox equivalents/kg(L) of food; Folin-Ciocalteu (FC) expressed as mg equivalents of gallic acid/kg(L) of food; FRAP expressed as mmol Trolox equivalents/kg(L) of food. Diamonds represent the mean antioxidant capacity of each group. Dots represent the antioxidant capacity of each food within the group. Statistical analysis was performed via Kruskal Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.3. Study of antioxidant capacity of food fermented with obese fecal material

Regarding the DPPH assay (**Figure 37**), cocoa and meat showed the highest antioxidant capacity ($p < 0.05$). On the other hand, no food group showed significantly lower values than the average though tubers and legumes were the ones with lowest antioxidant capacity. Total antioxidant capacity showed similar results (**Figure 38**) though now eggs were second to the lowest.

Regarding the Folin-Ciocalteu assay (**Figure 37**), meats showed the highest antioxidant values whereas tubers exhibited the lowest ones ($p < 0.05$). Cocoa products had a mean antioxidant capacity higher than meat products though probably due to differences between products, there was no significance. However, when considering total antioxidant capacity (**Figure 38**), cocoa was at the top with significantly higher values than the mean, followed by meats.

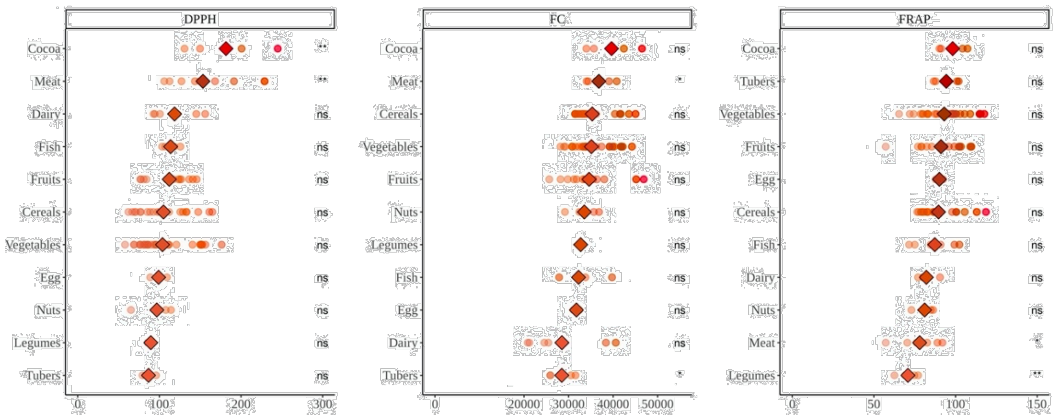


Figure 37. Antioxidant capacity of food obtained after *in vitro* fermentation of selected foods with fecal material from obese children. DPPH expressed as mmol Trolox equivalents/kg(L) of food; Folin-Ciocalteu (FC) expressed as mg equivalents of gallic acid/kg(L) of food; FRAP expressed as mmol Trolox equivalents/kg(L) of food. Diamonds represent the mean antioxidant capacity of each group. Dots represent the antioxidant capacity of each food within the group. Statistical analysis was performed via Kruskal Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant

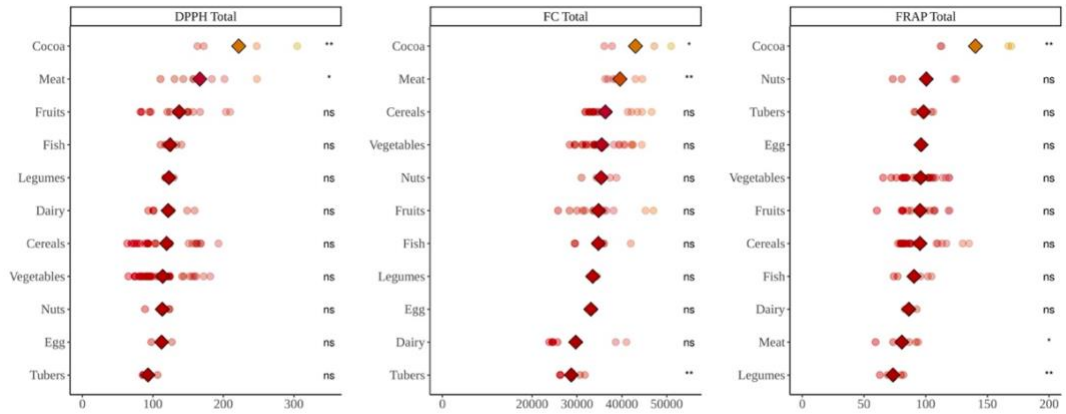


Figure 38. *In vitro* digestion values + *in vitro* fermentation values of food obtained after *in vitro* fermentation of selected foods with fecal material from obese children. DPPH expressed as mmol Trolox equivalents/kg(L) of food; Folin-Ciocalteu (FC) expressed as mg equivalents of gallic acid/kg(L) of food; FRAP expressed as mmol Trolox equivalents/kg(L) of food. Diamonds represent the mean antioxidant capacity of each group. Dots represent the antioxidant capacity of each food within the group. Statistical analysis was performed via Kruskal Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.4. Comparison between lean and obese subjects

A PCA was carried out to study the distribution of the samples. **Figure 39** shows the distribution of the samples in a 2-dimensional plot. PCA represents, on one hand, that antioxidant capacity released *during in vitro* digestion was different than that released during *in vitro* fermentation (see section about contribution of each fraction to total antioxidant capacity). On the other hand, it also showed how samples belonging to each type of children

mostly cluster together. PCA is therefore showing that the ability to release antioxidant capacity from foods could depend on the subject.

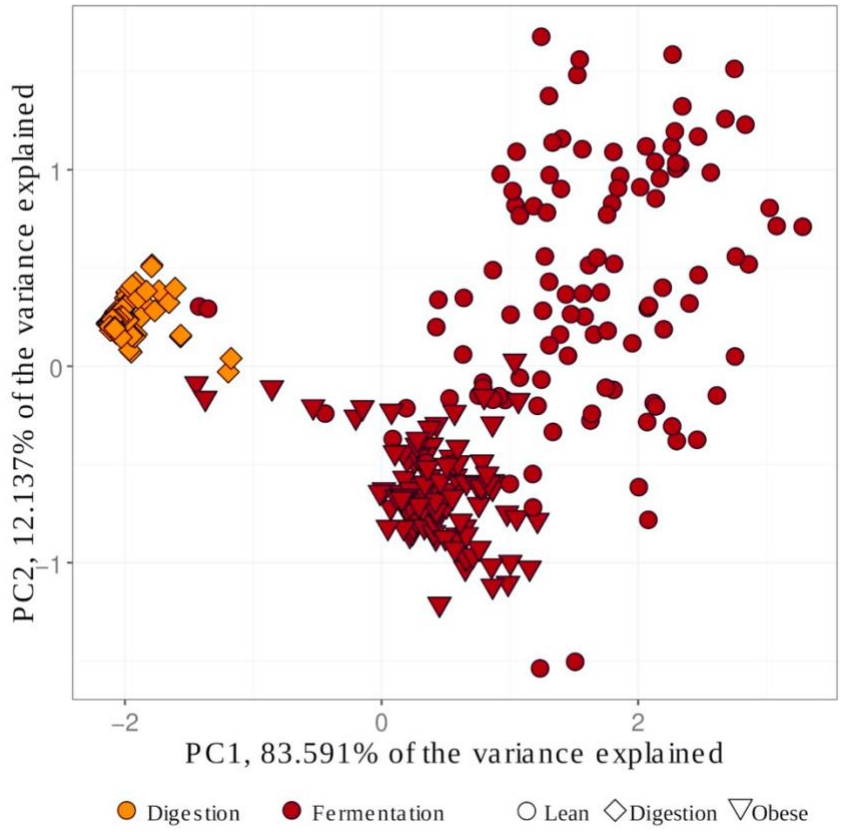


Figure 39. PCA of total antioxidant capacity obtained from different intervention groups with the three methods used (Folin-Ciocalteu, FRAP and DPPH).

Overall, when foods were fermented with fecal material from lean children, the antioxidant capacity released was higher, particularly in DPPH and FRAP assays (**Figure 40**). Folin-Ciocalteu assay presented very similar results for both type of subjects, and significant differences were only found in vegetables and fish. Fecal material from lean children yielded higher ($p < 0.05$) antioxidant capacity from vegetables (DDPH), tubers (DPPH and

FRAP), nuts (DPPH and FRAP), meat (FRAP), legumes (DPPH and FRAP), fruits (DPPH and FRAP), fish (FOLIN-CIOCALTEU and FRAP), dairy products (DPPH and FRAP), cocoa products (DPPH and FRAP) and cereals (DPPH and FRAP). On the other hand, fecal material from obese children only exhibited higher ($p < 0.05$) antioxidant capacity when fermenting vegetables and measured via Folin-Ciocalteu.

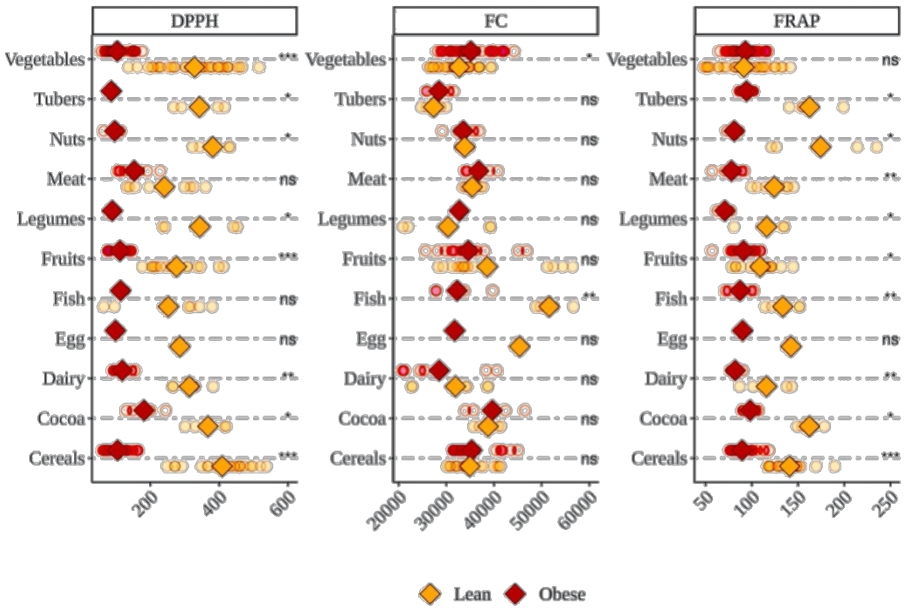


Figure 40. Comparison between the antioxidant capacity of different food groups after *in vitro* fermentation with fecal material from obese and lean children. DPPH expressed as mmol Trolox equivalents/kg(L) of food; Folin-Ciocalteu (FC) expressed as mg equivalents of gallic acid/kg(L) of food; FRAP expressed as mmol Trolox equivalents/kg(L) of food. Diamonds represent the mean antioxidant capacity of each group. Dots represent the antioxidant capacity of each food within the group. Statistical analysis was performed via Kruskal Wallis test. Comparisons were made using "Lean" as the reference group. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

We also looked at how much each of the fractions (digestion and fermentation) contributed to total antioxidant capacity or, in other words, which process was able to extract the highest antioxidant power from the foods under study (**Figure 41A-B**). As we can see, for both, lean and obese, *in vitro* fermentation provided most of the antioxidant capacity. The contribution of digestion to total antioxidant capacity was higher in obese than in lean, especially when measured with DPPH, the reason being that obese fecal material generally yielded less antioxidant power.

A **B**

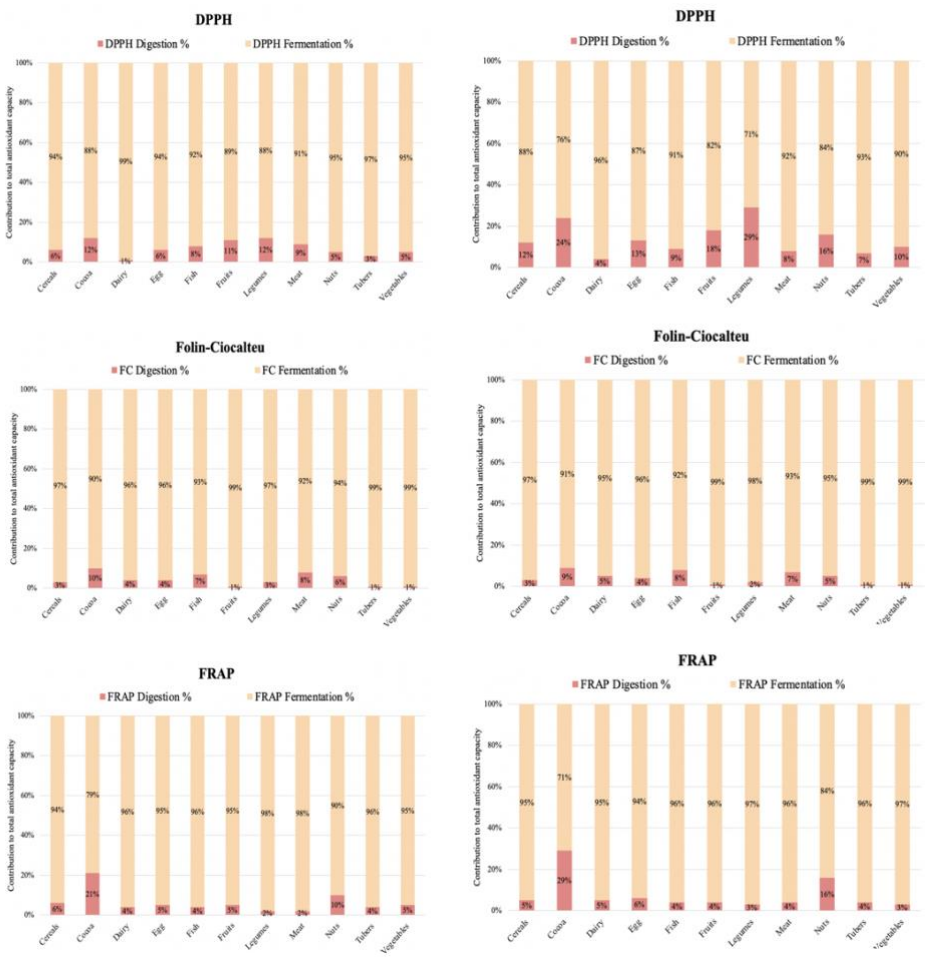


Figure 41. Contribution to the total antioxidant capacity of the fractions obtained after *in vitro* digestion and fermentation for lean and obese. **41A:** Contributions fermenting with fecal material from lean children**. 41B:** Contributions fermenting with fecal material from obese children.

4. Discussion

In this work, a total of 48 different foods of plant and animal origin were submitted to *in vitro* digestion followed by *in vitro* fermentation using fecal material from different donors: healthy children and obese children. Antioxidant capacity (DPPH, FRAP and Folin-Ciocalteu) was measured in the potentially absorbable fraction obtained after *in vitro* digestion as well as after gut microbial fermentation.

Regarding *in vitro* digestion results, cocoa showed the highest antioxidant capacity for all three methodologies. Cocoa is known to possess a high amount of phytochemicals, especially phenolic compounds from the flavan-3-ol family such as catechin or epicatechin [28]. This could, in part, explain why cocoa show the highest antioxidant value as well as why fruits and legumes also showed significantly higher antioxidant capacity than the base-mean with DPPH. Vegetables, on the other hand, was surprisingly found as one of the least antioxidant group of foods. It has been found that due to the nature of vegetable's cell wall, these are hard to break down during digestion which could lead to a reduced release of its content and thus, phytochemicals during gastrointestinal digestion [29]. Some more aggressive cooking methods such as grilling or frying would help making the structure softer so it can more easily be broken down and release more antioxidant capacity during digestion [29]. Similar behavior for vegetables was found across all three methods.

However, meats and fish, followed by nuts, also exhibited significantly higher antioxidant capacity than the base-mean when antioxidant capacity was measured via Folin-Ciocalteu. At the same time, vegetables, legumes and fruits showed lower values than the base-mean, only significant for vegetables and fruits. These results did not fall within

expectations. The Folin-Ciocalteu assay is often used to estimate total polyphenol content [25]. However, it has been found that reducing compounds interact with the reagent regardless of their actual antioxidant power. Among those compounds we can find sugars or amino acids [28]. Therefore, the actual phenolic content could be overestimated [30]. In order to test this possibility, we perform a correlation test between Folin-Ciocalteu values a protein content for all 48 foods, finding it to be large ($r = 0.8339$) and significant ($p < 0.05$).

FRAP, on the other hand, did not show much variation between different food categories with the exception of cocoa and nuts, though the latter did not show significantly higher antioxidant values than the rest. These results would make sense regarding animal products which are not known for their antioxidant potential and would also agree with DPPH.

Regarding *in vitro* fermentation with fecal material from lean children, cocoa was once again among the top three foods with the highest antioxidant capacity, though it was only significantly in the case of FRAP. Nuts were also among those top three when using DPPH and FRAP methods, though only significantly for DPPH. As commented above, cocoa [28] as well as nuts are known carriers of phytochemicals such as phenolics [31]. High protein foods were among the ones with the lowest antioxidant values regarding DPPH whereas vegetables situated half way, probably due to large differences between specific vegetable foods. Fruits also showed significantly lower antioxidant potential than the base-mean although here too there were large differences within the group. Cereals were the food group that showed to be significantly above the base-mean for DPPH method. Comparatively speaking, vegetables are now, after fermentation, producing more antioxidant capacity than during digestion. This could indicate that their microbial

degradation could help releasing antioxidants that otherwise would be inaccessible.

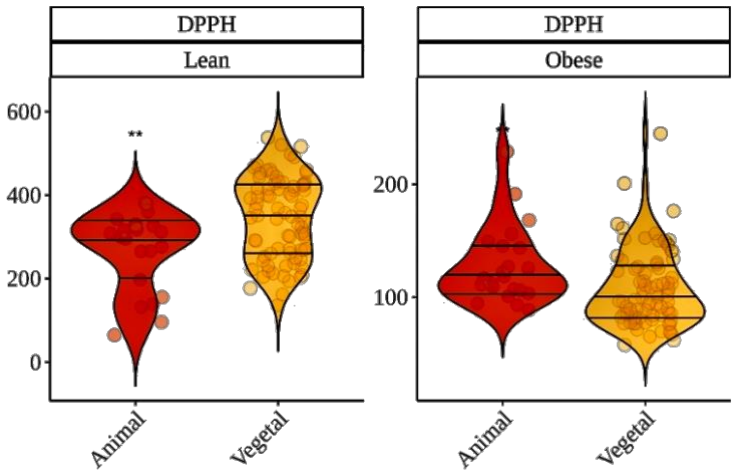
Folin-Ciocalteu showed similar results to those obtained during *in vitro* digestion with high protein foods showing some of the highest antioxidant values, though only fish was statistically significant ($p < 0.05$). Here again, we found a statistically significant correlation between protein content and Folin-Ciocalteu values ($r = 0.3942$; $p < 0.05$). However, here is not clear whether high antioxidant values are due to interactions as described above or to actual antioxidant compounds. It has been proven before that several bacterial species such as different *Bacteroides*, *Eubacterium hallii* or *Clostridium barlettii* can metabolize aromatic amino acids into small phenolic compounds, same as the ones produced in plants [32,33]. These include phenylpropionic acids, phenylacetic acids or 4-hydroxy-phenylacetic acid [32,33]. Therefore, though Folin-Ciocalteu values could indeed be overestimated, some of the antioxidant capacity registered could come from those phenolics. However, low DPPH and FRAP values of animal foods could indicate that in this case, Folin-Ciocalteu is actually overestimating antioxidant power.

As stated above, cocoa was, along with tubers and nuts (this one not significantly) the ones with higher antioxidant power when measured via FRAP. The rest of the groups showed similar antioxidant capacity and only vegetables were significantly below the base-mean. Although there are great differences within group, some vegetables did show low antioxidant values whereas others displayed values comparable to other groups.

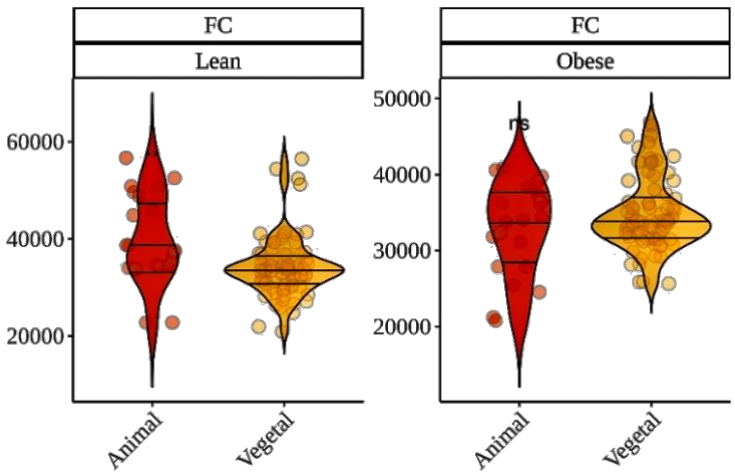
Regarding *in vitro* fermentation with fecal material from obese children, cocoa was once more the most antioxidant group though only significantly in the case of DPPH. We can probably suggest now that, overall,

cocoa was the most antioxidant food. DPPH showed that high protein foods were among the most antioxidant food groups, which was, in part, opposite to what we found using lean fecal material. Since the DPPH reagent has not been reported to react with proteins (as Folin-Ciocalteu), we tried to find another explanation. This finding could be related to differences between gut microbiota from lean and obese. As depicted in **Figure 38**, whereas meat is second to cocoa using obese fecal material, when we use lean gut microbiota the result is opposite and is, in fact, where differences between lean and obese are the lowest (**Figure 38**). Actually, while lean gut microbiota can extract significantly more antioxidant capacity from plat origin foods, obese gut microbiota does the opposite and extracts significantly more from animal products (**Figure 42**). There have been several reports that have consistently found specific features of obese gut microbiota, probably the most apparent is the depletion in Bacteroidetes and higher abundance of Firmicutes [34,35]. This would lead to a reduced ability to ferment plant polysaccharides when compared to a lean microbiota [36]. Therefore, breakdown of vegetable cells would become harder and lower concentrations of phytochemicals would be released. Obesity has also been associated to high protein, high fat and low fiber diets which could suggests that proteolytic pathways in gut microbes are favored [37]. However, this would only explain why lean gut microbiota is extracting more antioxidant capacity from plant origin foods. Riadaura et al., [36] carried out an experiment that involved a fecal transplantation to gnobiotic mice from twins discordant for obesity. These authors found in plasma higher concentrations of branched chain amino acids and some others including phenyalanine and tyrosine which could indicate lower degradation and formation of small phenolics. Then again, it has also been consistently found that obese gut microbiota has an increased production of phenilalanine, tyrosine and tryptophan [34,35]. Therefore, whether these amino acids are coming from food protein degradation or not requires further study.

A



B



(Figure 42 continued)

C

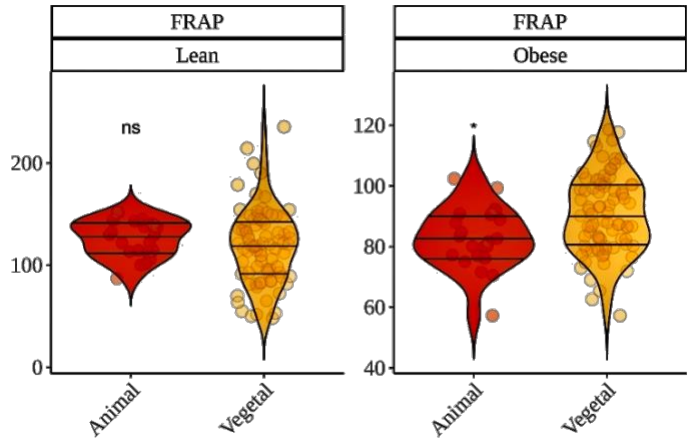


Figure 42. Antioxidant capacity released by lean or obese gut microbiota from plant origin foods and animal products. **(42A)** DPPH; **(42B)** Folin-Ciocalteu; **(42C)** FRAP. Statistical analysis was performed via Kruskal Wallis test. Comparisons were made using "Vegetal" as the reference group. Solid horizontal lines within shapes show 0.25, 0.5, and 0.75 quartiles. Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

However, when antioxidant capacity was measured via FRAP and Folin-Ciocalteu using obese gut microbiota, though meat showed high values via Folin-Ciocalteu, animal foods presented in general lower values than plant origin products. Nevertheless, Folin-Ciocalteu values were similar among samples and only tubers and meats were significantly different than the base-mean. When comparing Folin-Ciocalteu values between lean and obese samples, they were very similar only showing differences for egg and fish, which would require further and deeper research. FRAP on the other hand exhibited higher antioxidant values when fermenting with lean fecal material except for vegetables though fruit values were also close to one another.

Although this coincides with DPPH, the specific behavior of the different food groups was different than that found for DPPH. FRAP showed that lean gut microbiota produced similar antioxidant capacity from animal and plant origin products whereas obese gut microbiota produced higher values from plant products. Whether this behavior is gut microbe related or due to the assays´ chemistry, is hard to conclude and additional experiments with more subjects from each group would be needed. However, regarding the antioxidant assays chemistry, pH could be playing here an important role. Whereas DPPH is carried out at pH 7, FRAP is carried out at pH 3.6. Both assays are based on the same redox principle, transference of electrons from the antioxidant to the radical (DPPH) or to the metal (FRAP) [38]. However, polyphenols should be more antioxidant at pH closer to 7 because deprotonation of phenols increase with pH and, therefore, electron transfer is easier [39]. Accordingly, lean gut microbiota could be releasing more phenolics from vegetables or fruits but they would not be detected, resulting in similar values to those obtained with obese children. That could also be true for small phenolics obtained from amino acid degradation. Regardless, whether this is the reason or it lays on the metabolism of gut microbes would, as commented above, require further investigation with a larger cohort.

5. Conclusions

In conclusion, we evaluated a number of foods grouped in several categories. Cocoa products (dark chocolate and cocoa butter) displayed, overall, the highest antioxidant capacity. Antioxidant capacity of foods is released in a two-step process: first, some of this antioxidant capacity is released during digestion, usually more antioxidant power released from those foods more easily digested whereas others, such as vegetables, release lower concentrations. Regardless, most of the antioxidant capacity is released during gut microbial fermentation, which could be due to two main reasons: for plant origin foods (hard to digest due to lack of enzymes) most phytochemicals could be released only after gut microbes have broken down vegetal cells. Secondly, animal products that lack phytochemicals (they could have some due to animal feeds) could exhibit their antioxidant power after amino acids are fermented into small phenolics but not before as larger peptides. Additionally, large differences were detected depending on whether foods were fermented with lean or obese fecal material. Overall, when using lean gut microbiota, antioxidant capacity released was higher, though this was only true for DPPH and FRAP methods. Moreover, according to DPPH results, lean gut microbiota could potentially release more antioxidant power from vegetables than from animal products, while obese gut microbiota did the opposite. FRAP, on the other hand, showed how it was obese gut microbiota the one that released higher levels of antioxidant power from plants products. Although this could be related to the chemistry of the antioxidant assay, it is not clear and further investigation would help elucidate it. Regardless, different antioxidant assays could show different antioxidant behaviors although Folin-Ciocalteu has showed a potential interaction with proteins or amino acids which would limit its usefulness.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Castañeda Guillot: C.; Castañeda Guillot, C. Microbiota Intestinal y Obesidad En La Infancia. Revista Cubana de Pediatría 2020, 92.

2. Alba-Alba, C.M. Sobrepeso y Obesidad Infantil en Oaxaca ¿Es Relevante Trabajar el Tema? Revista Salud y Administración 2015, 2, 47-49.

3. Gao, X.; Zhu, Y.; Wen, Y.; Liu, G.; Wan, C. Efficacy of probiotics in non-alcoholic fatty liver disease in adult and children: A meta-analysis of randomized controlled trials: Meta-analysis. Hepatol Res 2016, 46, 1226-1233, doi:10.1111/hepr.12671.

4. Turnbaugh, P.J.; Hamady, M.; Yatsunenko, T.; Cantarel, B.L.; Duncan, A.; Ley, R.E.; Sogin, M.L.; Jones, W.J.; Roe, B.A.; Affourtit, J.P.; et al., A Core Gut Microbiome in Obese and Lean Twins. Nature 2009, 457, 480-484, doi:10.1038/nature07540.

5. Huttenhower, C.; Gevers, D.; Knight, R.; Abubucker, S.; Badger, J.H.; Chinwalla, A.T.; Creasy, H.H.; Earl, A.M.; FitzGerald, M.G.; Fulton, R.S.; et al., Structure, Function and Diversity of the Healthy Human Microbiome. Nature 2012, 486, 207-214, doi:10.1038/nature11234.

6. Baothman, O.A.; Zamzami, M.A.; Taher, I.; Abubaker, J.; Abu-Farha, M. The Role of Gut Microbiota in the Development of Obesity and Diabetes. Lipids Health Dis 2016, 15, 108, doi:10.1186/s12944-016-0278-4.

7. Castaner, O.; Goday, A.; Park, Y.-M.; Lee, S.-H.; Magkos, F.; Shiow, S.-A.T.E.; Schröder, H. The Gut Microbiome Profile in Obesity: A Systematic Review. International Journal of Endocrinology 2018, 2018, e4095789, doi:10.1155/2018/4095789.

8. Oliveira, R.C.S.; Coelho, P.M.B. de S.; Lozano Estevan, M. del C. Does Microbiota Influence the Risk of Childhood Obesity? Revista Española de Nutrición Humana y Dietética 2018, 22, 157-168, doi:10.14306/renhyd.22.2.389.

9. Indiani, C.M. dos S.P.; Rizzardi, K.F.; Castelo, P.M.; Ferraz, L.F.C.; Darrieux, M.; Parisotto, T.M. Childhood Obesity and Firmicutes/Bacteroidetes Ratio in the Gut Microbiota: A Systematic Review. Childhood Obesity 2018, 14, 501-509, doi:10.1089/chi.2018.0040.

10. Koliada, A.; Syzenko, G.; Moseiko, V.; Budovska, L.; Puchkov, K.; Perederiy, V.; Gavalko, Y.; Dorofeyev, A.; Romanenko, M.; Tkach, S.; et al., Association between Body Mass Index and Firmicutes/Bacteroidetes Ratio in an Adult Ukrainian Population. BMC Microbiology 2017, 17, 120, doi:10.1186/s12866-017-1027-1.

11. Liu, B.-N.; Liu, X.-T.; Liang, Z.-H.; Wang, J.-H. Gut Microbiota in Obesity. World Journal of Gastroenterology 2021, 27, 3837-3850, doi:10.3748/wjg.v27.i25.3837.

12. Fontané, L.; Benaiges, D.; Goday, A.; Llauradó, G.; Pedro-Botet, J. Influencia de la microbiota y de los probióticos en la obesi-dad. Clínica e Investigación en Arteriosclerosis 2018, 30, 271-279, doi:10.1016/j.arteri.2018.03.004.

13. Fukuda, S.; Ohno, H. Gut Microbiome and Metabolic Diseases. Semin Immunopathol 2014, 36, 103-114, doi:10.1007/s00281-013-0399-z.

14. DiBaise, J.K.; Zhang, H.; Crowell, M.D.; Krajmalnik-Brown, R.; Decker, G.A.; Rittmann, B.E. Gut Microbiota and Its Possible Relationship With Obesity. Mayo Clinic Proceedings 2008, 83, 460-469, doi:10.4065/83.4.460.

15. Kawabata, K.; Yoshioka, Y.; Terao, J. Role of Intestinal Microbiota in the Bioavailability and Physiological Functions of Dietary Polyphenols. Molecules 2019, 24, 370, doi:10.3390/molecules24020370.

16. Pérez-Burillo, S.; Navajas-Porras, B.; López-Maldonado, A.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.A. Green Tea and Its Relation to Human Gut Microbiome. Molecules 2021, 26, 3907, doi:10.3390/molecules26133907.

17. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, A.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.A.. Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to in Vitro Digestion-Fermentation. Molecules 2020, 9, 1312, doi: 10.3390/antiox9121312.

18. Rufián-Henares, J.A.; Guerra-Hernández, E.J.; García-Villanova, B. Colour measurement as indicator for controlling the manufacture and storage of enteral formulas. Food Control 2006, 17, 489-493. doi: 10.1016/j.foodcont.2005.02.011.

19. Delgado-Andrade, C.; Rufián-Henares, J.A.; Morales, F.J. Lysine availability is diminished in commercial fibre-enriched breakfast cereals. Food Chemistry 2007, 100, 725-731. doi: 10.1016/j.foodchem.2005.10.031.

20. Rufián-Henares, J.A.; Guerra-Hernández, E.J.; García-Villanova, B. Maillard reaction in enteral formula processing: furosine, loss of ophthaldialdehyde reactivity, and fluorescence. Food Research International 2002, 35, 527-533. doi: 10.1016/S0963-9969(01)00152-1.

21. Ramírez-Anaya, J.P.; Samaniego-Sánchez, C.; Castañeda-Saucedo, M.C.; Villalón-Mir, M.; de la Serrana, H.L.-G. Phenols and the antioxidant capacity of Mediterranean vegetables prepared with extra virgin olive oil using different domestic cooking techniques. Food Chemistry 2015, 188, 430-438. doi: 10.1016/j.foodchem.2015.04.124.

22. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an Improved Global Antioxidant Response Method (GAR+): Physiological-Resembling in Vitro Digestion-Fermentation Method. Food Chem 2018, 239, 1253-1262, doi:10.1016/j.foodchem.2017.07.024.

23. Pérez-Burillo, S.; Molino, S.; Navajas-Porras, B.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; López-Maldonado, A.; Pastoriza, S.; Rufián-Henares, J.Á. An in Vitro Batch Fermentation Protocol for Studying the Contribution of Food to Gut Microbiota Composition and Functionality. Nat Protoc 2021, 16, 3186-3209, doi:10.1038/s41596-021-00537-x.

24. Pastoriza, S.; Delgado-Andrade, C.; Haro, A.; Rufián-Henares, J.A. A Physiologic Approach to Test the Global Antioxidant Response of Foods. The GAR Method. Food Chemistry 2011, 129, 1926-1932, doi:10.1016/j.foodchem.2011.06.009.

25. Everette, J.D.; Bryant, Q.M.; Green, A.M.; Abbey, Y.A.; Wangila, G.W.; Walker, R.B. Thorough Study of Reactivity of Various Compound Classes toward the Folin-Ciocalteu Reagent. J Agric Food Chem 2010, 58, 8139-8144, doi:10.1021/jf1005935.

26. Benzie, I.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal Biochem 1996, 239, 70-76, doi:10.1006/abio.1996.0292.

27. Rapisarda, P.; Tomaino, A.; Lo Cascio, R.; Bonina, F.; De Pasquale, A.; Saija, A. Antioxidant Effectiveness as Influenced by Phenolic Content of Fresh Orange Juices. J Agric Food Chem 1999, 47, 4718-4723, doi:10.1021/jf9901111.

28. Andújar, I.; Recio, M.C.; Giner, R.M.; Ríos, J.L. Cocoa Polyphenols and Their Potential Benefits for Human Health. Oxidative Medicine and Cellular Longevity 2012, 2012, e906252, doi:10.1155/2012/906252.

29. Miglio, C.; Chiavaro, E.; Visconti, A.; Fogliano, V.; Pellegrini, N. Effects of Different Cooking Methods on Nutritional and Physicochemical Characteristics of Selected Vegetables. J. Agric. Food Chem. 2008, 56, 139-147, doi:10.1021/jf072304b.

30. Muñoz-Bernal, Ó.A.; Torres-Aguirre, G.A.; Núñez-Gastélum, J.A.; Rosa, L.A. de la; Rodrigo-García, J.; Ayala-Zavala, J.F.; Álvarez-Parrilla, E. Nuevo acercamiento a la interacción del reactivo de Folin-Ciocalteu con azúcares durante la cuantificación de polifenoles totales. TIP. Revista especializada en ciencias químico-biológicas 2017, 20, 23-28, doi:10.1016/j.recqb.2017.04.003.

31. Ou, K.; Gu, L. Absorption and Metabolism of Proanthocyanidins. Journal of Functional Foods 2014, 7, 43-53, doi:10.1016/j.jff.2013.08.004.

32. Rowland, I.; Gibson, G.; Heinken, A.; Scott, K.; Swann, J.; Thiele, I.; Tuohy, K. Gut Microbiota Functions: Metabolism of Nutrients and Other Food Components. Eur J Nutr 2018, 57, 1-24, doi:10.1007/s00394-017-1445-8.

33. Russell, W.R.; Duncan, S.H.; Scobbie, L.; Duncan, G.; Cantlay, L.; Calder, A.G.; Anderson, S.E.; Flint, H.J. Major Phenylpropanoid-Derived Metabolites in the Human Gut Can Arise from Microbial Fermentation of Protein. Molecular Nutrition and Food Research 2013, 57, 523-535, doi:10.1002/mnfr.201200594.

34. Liu, R.; Hong, J.; Xu, X.; Feng, Q.; Zhang, D.; Gu, Y.; Shi, J.; Zhao, S.; Liu, W.; Wang, X.; et al., Gut Microbiome and Serum Metabolome Alterations in Obesity and after Weight-Loss Intervention. Nat Med 2017, 23, 859-868, doi:10.1038/nm.4358.

35. Fan, Y.; Pedersen, O. Gut Microbiota in Human Metabolic Health and Disease. Nat Rev Microbiol 2021, 19, 55-71, doi:10.1038/s41579-020-0433-9.

36. Ridaura, V.K.; Faith, J.J.; Rey, F.E.; Cheng, J.; Duncan, A.E.; Kau, A.L.; Griffin, N.W.; Lombardo, V.; Henrissat, B.; bain, james r; et al., Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. Science 2013, 341, 1241214, doi:10.1126/science.1241214.

37. Diether, N.E.; Willing, B.P. Microbial Fermentation of Dietary Protein: An Important Factor in Diet-Microbe-Host Interaction. Microorganisms 2019, 7, 19, doi:10.3390/microorganisms7010019.

38. Huang, D.; Ou, B.; Prior, R.L. The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem. 2005, 53, 1841-1856, doi:10.1021/jf030723c.

39. Lemańska, K.; Szymusiak, H.; Tyrakowska, B.; Zieliński, R.; Soffers, A.E.M.F.; Rietjens, I.M.C.M. The Influence of PH on Anti-oxidant Properties and the Mechanism of Antioxidant Action of Hydroxyflavones. Free Radical Biology and Medicine 2001, 31, 869-881, doi:10.1016/S0891-5849(01)00638-4.

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IF: 6.706 Q1 Nutrition and Dietetics-SCIE 15/90

The Intake of Antioxidant Capacity of Children Depends on Their Health Status

En el presente estudio, se digirieron y fermentaron *in vitro* 48 alimentos diferentes sometidos a distintos tratamientos culinarios utilizando heces de niños sin patologías, niños obesos, niños celíacos y niños con alergia a las proteínas de la leche de vaca. Se evaluó la capacidad antioxidante con los ensayos DPPH y FRAP y se dedujo el porcentaje que cada alimento aportaba a la ingesta diaria de antioxidantes, así como la capacidad antioxidante por tamaño de ración. Así se pudieron ver las diferencias en la capacidad de cada una de las microbiotas intestinales utilizadas de extraer compuestos con actividad antioxidante tras la fermentación.

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The Intake of Antioxidant Capacity of Children Depends on Their Health Status

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Abstract

Gastrointestinal digestion of food and further gut microbial activity render a myriad of different molecules that could be responsible for the biological activities classically assigned to their parent compounds. This has been previously showed for some phytochemicals whose anti-oxidant capacity was either increased or decreased after being metabolized by gut microbes. Whether global antioxidant capacity extracted from food is determined by gut microbial com-munity structure is still not well-described. In the present study, we *in vitro* digested and fermented 48 different foods submitted to different culinary treatments using stools of lean children, obese children, celiac children and children with allergy to cow's milk proteins. The antioxidant capacity was assessed with the DPPH and FRAP assays and the percentage that each food con-tributed to the daily antioxidant intake as well as antioxidant capacity by portion size was inferred. Overall, cereals, fruits and vegetables displayed higher contribution to daily antioxidant intake while tubers, fish and meat exhibited higher antioxidant capacity by serving size. Food fermented with lean and allergic to cow´s milk protein fecal material showed higher antioxidant capacity, which could imply a larger role of the gut microbiota.

Keywords: antioxidant capacity; *in vitro* digestion-fermentation, children; gut microbiota, obesity, celiac disease, protein allergy, antioxidant intake.

1. Introduction

The gut microbiota is the community of living microorganisms that reside and coexist in the gut. In the instance of humans, it consists of trillions of microbial cells and hundreds of different species, making it one of the most densely populated communities. It carries out a whole range of biochemical and physiological functions that influence the metabolism of the host [1] and it is responsible for the fermentation of food components. As a result, the gut microbiota is able to generate different compounds that directly affect human health in relation to nutrition, regulation of immunity and systemic inflammation [2,3]. Among these compounds we find some antioxidants that are transformed by the gut microbiota. This transformation is often essential for their absorption and thus critical for them to exert their biological activity [4-6]. The inter-individual variability and plasticity has hampered the endeavour to define what a 'healthy' microbiota is. Therefore, markers of microbiota stability such as richness, diversity and functionality are often used as indicators of gut health due to their inverse association with certain pathologies. [1].

The gut microbiota has been identified as an influencing factor in the development of obesity by increasing the host capacity for energy harvesting [7]. Some alterations in the gut microbial community structure have been associated to obese people including a decrease in the Bacteroides levels while increasing Firmicutes. It has also been observed that the microbiota of obese individuals has a lower microbial biodiversity than that of lean individuals [8]. Dysbiosis is a risk factor for celiac disease. This inflammation of the small intestine is characterized by a continuous gluten intolerance manifested in individuals with genetic predisposition [9]. Gut microbial dysbiosis and some specific bacteria have been associated with celiac disease,

either by increasing the inflammatory response to gluten or by directly influencing mucosal immune responses [1,10]. Last but not least, food allergies have been also associated with disruptions in the gut microbial community structure [11-14].

Therefore, the objective of this paper is to study how different foods can contribute to the daily antioxidant intake after being fermented with fecal material from different children's populations and whether gut microbial differences play an important role or not.

2. Materials and Methods

2.1. Chemicals

2.1.1. *In vitro* digestion and fermentation

Pancreatin (from Alpha Aesar, UK) was from porcine pancreas. Sodium di-hydrogen phosphate, tryptone, pepsin, porcine bile acids, cysteine, resazurin, sodium sulphide and salivary alpha-amylase were from Sigma-Aldrich (Darmstadt, Germany).

2.1.2. Antioxidant Capacity

DPPH (2,2 diphenyl-1-1picrythydrazul hydrate 95%), Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), iron (III) chloride hexa-hydrate, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), hydrochloric acid. All these reagents were from Sigma Aldrich (Darmstadt, Germany).

2.2. Samples

A total of 48 samples belonging to different food groups have been studied: cereals (biscuits, biscuits whole grain, bread, bread whole grain, breakfast cereals, breakfast cereals whole grain, penne (pasta), penne whole grain, rice and rice whole grain), cocoa (dark chocolate and Nutella), fruits (apple, banana, grapes, olives, orange, peach, plum), legumes (kidney beans, lentils), nuts (nut mixture and peanuts), oils (olive oil, sunflower oil), tubers (potatoe and sweet potatoe), vegetables (cabbage, carrot, cauliflower, egg-plant, lettuce, onion, pepper, spinach, tomatoe and zucchini), dairy products (butter, gouda, milk and yogurt), egg, fish (cod fish and salmon) and meat (beef, chicken, lamb and pork). Food items were purchased from 3 different retail shop and stored at -80 ºC until experimental processing.

2.3. In vitro digestion-fermentation

Samples were submitted to *in vitro* digestion and fermentation following our previous protocols [15,16]. For each sample, 5 g of food (in triplicate) were weighed. *In vitro* digestion is divided into three steps: oral, gastric and intestinal. First, 5 mL of simulated saliva with 150 U/mL salivary alpha-amylase were added to 5 g of sample and blended in a 50 mL tube, keeping them at 37 ºC for 2 min. Subsequently, 10 mL of simulated gastric fluid containing gastric pepsin (4000 U/mL) was added to the mixture, the pH was lowered to 3 and kept at 37 ºC for 2 hours. In the last step of the digestion, simulated intestinal fluid (20 mL with 200 U/mL pancreatin and 20 mM bile salts) was added to the tube, the pH was raised to 7 and maintained at 37 ºC for two hours. The enzymatic activity was stopped by immersion in iced water during 15 min. The tubes were then centrifuged, the supernatant (fraction potentially absorbed in the small intestine) was collected and the pellet (undigested fraction that would reach the colon) was used for *in vitro* fermentation.

Fecal samples from 4 different groups of children were used for *in vitro* fermentation: 5 lean donors, 5 celiac donors, 5 obese donors and 5 donors with allergy to cow's milk proteins. The inclusion criterion for all of them was an age comprised from 8 to 10 years. Children taking probiotics or antibiotics in the previous 3 months were removed from the study. For the lean and obese groups, a common exclusion criterion was the diagnosis of chronic gastrointestinal disorders or any other chronic disease or special diet. The BMI of celiac, lean and milk-allergic children was comprised between the 5th and 85th percentile for their gender, height and age. For the obese group, BMI had to be above the 95th percentile for sex, weight and age. Each stool was collected in a hospital of Athens (Greece) by the pediatric

department. The informed consent document was signed by parents. That form included all information of the study as well as the exclusion and inclusion criteria. The study was approved at the University General Hospital (Athens) by the corresponding ethical committee.

Fecal material was combined by group of donors to consider inter-individual variability. *In vitro* fermentation was performed in oscillation at 37 ºC for 20 hours. For this procedure, 0.5 grams of the solid residue obtained after *in vitro* gastrointestinal digestion, plus a 10% of the *in vitro* supernatant, were used.

The fermentation medium included cysteine 312 mg/L, resazurin 0.1% v/v, peptone 14 g/L and hydrogen sulphide 312 mg/L. 7,5 mL of this fermentation medium were added to the fermentation tube. Inoculum was made from fecal material from each of the groups of children. Each of them was mixed with PBS (at 33% concentration). Two mL of inoculum were added to the fermentation tube (each food sample was fermented 4 times, once for each inoculum). Then, in order to reach anaerobic conditions nitrogen was bubbled, leaving a transparent solution (contrary to the pink color obtained under the presence of oxygen). The microbial activity, after fermentation at 37 ºC for 20 hours, was finished by the immersion of tubes for 15 min in ice; then, centrifugation was used to collect the supernatant (the fraction that could be absorbed in the large intestine), which was finally stored for further analysis at -80 ºC. *In vitro* digestion and *in vitro* fermentation included blanks carrying water instead of the sample.

2.4. Antioxidant Analyses

The fraction obtained from *in vitro* digestion was used to study its antioxidant capacity, since it is potentially absorbable in the small intestine.

The liquid fraction obtained after *in vitro* fermentation was also studied, since it could be absorbed in the large intestine. Both fractions can be summed to constitute the total antioxidant capacity of the sample [17]. Two different assays were used to analyze antioxidant capacity on a microplate reader (FLUOStar Omega, BMG Labtech, Germany).

The $TEAC_{FRAP}$ method was performed following the protocol of Benzie and Strain [18]. With this method, the ability of the samples to reduce iron is studied. Twenty µL of sample were added to a 96-well microplate and mixed with 280 µL of FRAP reagent (freshly prepared). This reagent consists of 25 mL sodium acetate (0.3 mM, pH 3.6), 2.5 mL ferric chloride and 2.5 mL 40 mM TPTZ. The assay was monitored at 37oC for half an hour at 595 nm and the calibration curve ranged from 0.01-04 mg Trolox/mL. All the samples were assessed in duplicate.

The $TEAC_{DPPH}$ method was performed with the method of Brand-Williams et al., [19]. Twenty µL of sample were added to a 96-well plate and blended with 280 µL of DPPH daily solution (at a concentration of 74 mg DPPH salt per L of methanol). The assay was followed at 520 nm for one hour at 37 ºC and the calibration curve ranged from 0.01 to 0.4 mg Trolox/mL. All the samples were assessed in duplicate.

2.5. *Daily Antioxidant Intake Calculations and Mean contribution to Daily Antioxidant per Serving Intake*

The contribution of each group of foods to the daily intake of antioxidant capacity within the diet of children was calculated in two different ways. The first was by using the daily food intake according to EFSA [20] and our results of antioxidant capacity with the following equation:

$$\textit{Daily Antioxidant Intake} = \textit{Food daily consumption (g/day)} * \textit{Antioxidant capacity (µmol/g)}$$

The second way was by calculating the antioxidant capacity released by each group of food in terms of the usual serving size for children, according to a previous work [21] and our results of antioxidant capacity.

2.6. Statistical analyses

Statistically significant differences were calculated with the unpaired Kruskal Wallis test at 95% confidence, comparing the contribution to antioxidant capacity of each food group with the mean baseline antioxidant activity provided. Thus, we show whether a particular food group has a higher or lower contribution to the antioxidant capacity of the diet than the mean. The Statgraphics Plus 5.1 software was used to compute all statistical analyses.

3. Results

In this study, the antioxidant power of foods were assessed in the liquid supernatant which was released after the *in vitro* digestion-fermentation. Thus, the total antioxidant capacity of a particular food is the sum of both of the fractions. Each of the food items was *in vitro* digested once but they were fermented with feces from each of the children. Therefore, the antioxidant capacity that was obtained during digestion was the same for each of the children. From these results, the daily antioxidant intake per food group was calculated, grouping each food into its corresponding group, and taking into account the EFSA data [20]. In the same way, the daily intake was calculated, taking into account the regular serving size for children [21]. The foods were grouped as is described in Section 2.2, and the averages were calculated to perform the calculations per group and for the percentages of the daily intake.

3.1. Daily Antioxidant Intake with the FRAP method

Total daily antioxidant intake was calculated for each children group. According to EFSA [20] and our antioxidant capacity data, daily antioxidant intake was: 111 mmol Trolox/day for healthy lean children, 59.9 mmol Trolox/day for obese children, 72.6 mmol Trolox/day for celiac children and 142 mmol Trolox/day for allergic children. Therefore, allergic children were able to extract the highest antioxidant potential from diet whereas obese children did the opposite. When we consider specific food categories, cereals con-tributed with the largest percentage in all 4 groups of children: 27% for lean children, 22% for obese children, 22% for celiac children and 23% for allergic children. Fruits and vegetables contribution to daily antioxidant intake was only second to that of cereals. In contrast, cocoa and legumes showed the lowest contribution to daily antioxidant intake. Cocoa,

specifically, contributed with 0.5% for lean, obese and celiac children, and 0.2% for allergic children (**Table 27 and Figure 43**).

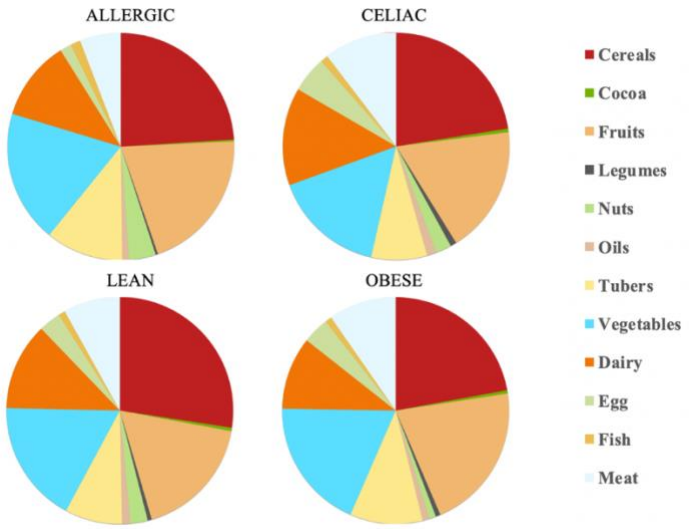


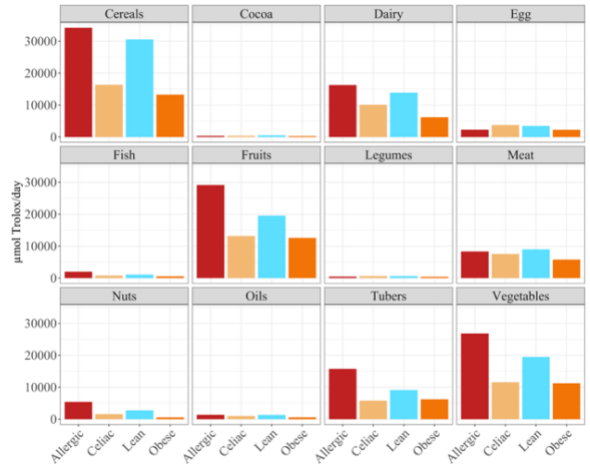
Figure 43. Mean contribution to daily antioxidant capacity intake (%) with FRAP assay in allergic, celiac, lean and obese children.

Overall, the food groups that showed the strongest contribution to daily antioxidant intake for all 4 children population were cereals, dairy, fruits and vegetables (**Figure 44A**). In fact, a statistically significant ($p < 0.05$) higher daily antioxidant intake was obtained for allergic and lean children in cereals and vegetables, while the daily intake of antioxidant capacity was only statistically higher for allergic children in fruits, nuts and tubers. In terms of daily antioxidant intake per serving size, fish was the highest contributing food group (28-44%), while oils contributed the least (0.5-0.7%) (**Figure 44B**). The intake of antioxidant capacity per serving was statistically higher ($p < 0.05$) for allergic children in the case of fish, nuts and tubers. We also observed that the same food group always showed lower contribution to daily antioxidant intake when such food group was fermented using obese fecal

material. No other tendencies such as this one, were found though, as showed by **Figure 44A**, results were heavily influenced by the source of the fecal material used for fermentation. So, in conclusion, with the FRAP method is seems that allergic children are able to extract more antioxidant capacity from food and, on the contrary, obese children do the opposite.

When we studied the antioxidant capacity that each children population was able to extract from a serving size, we observed that as before, antioxidant capacity was lower when food was fermented with obese fecal material (**Figure 44B**). Again, results showed that different fecal materials meant differences in the antioxidant capacity obtained by serving size. The highest antioxidant values were usually obtained when foods were fermented using allergic fecal material although meat, eggs, legumes, cocoa, and cereals exhibited higher antioxidant values when fermented using healthy fecal material (**Figure 44B**).

A



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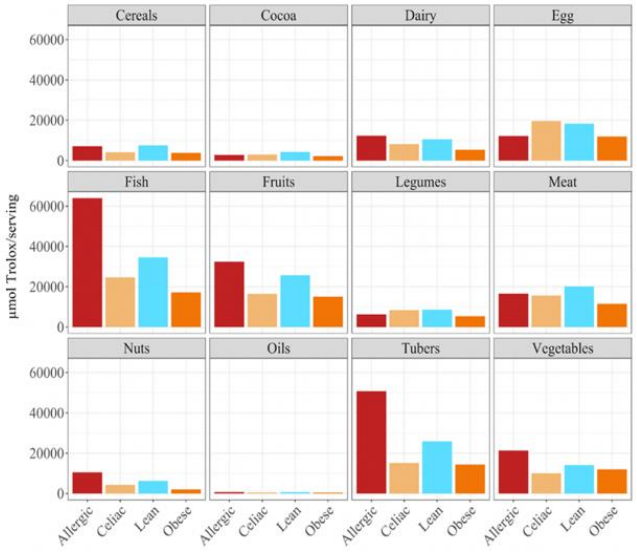


Figure 44. Differences in daily antioxidant intake (**44A**) and per serving intake (**44B**) in different foods groups in allergic, celiac, lean and obese children.

Table 27. Contribution of food consumption to the daily antioxidant capacity (AOX) intake in the children' diet with FRAP assay.

Food goup	**Child**	**AOX/daily intake** (μmol Trolox/day)	**AOX/serving intake**[1] (μmol Trolox/serving)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Cereals	Lean	30,550	7620	27.4	6.84
Cocoa	Lean	537	4284	0.48	3.85
Fruits	Lean	19,544	25,697	17.5	23.1
Legumes	Lean	648	8600	0.58	7.72
Nuts	Lean	2739	6280	2.46	5.64
Oils	Lean	1328	709	1.19	0.64
Tubers	Lean	9105	25,891	8.17	23.2
Vegetables	Lean	19,487	14145	17.5	12.7
Dairy	Lean	13,884	10,583	12.5	9.50
Egg	Lean	3487	18,334	3.13	16.5
Fish	Lean	1089	34,598	0.98	31.1
Meat	Lean	8999	20,143	8.08	18.1
Cereals	Obese	13,264	3799	22.1	6.33
Cocoa	Obese	283	2217	0.47	3.70
Fruits	Obese	12,576	15,136	21.0	25.2
Legumes	Obese	405	5363	0.67	8.94
Nuts	Obese	569	2029	0.95	3.38
Oils	Obese	592	408	0.99	0.68
Tubers	Obese	6210	14,305	10.4	23.9
Vegetables	Obese	11,243	12,004	18.7	20.0
Dairy	Obese	6212	5365	10.4	8.95
Egg	Obese	2260	11,883	3.77	19.8
Fish	Obese	566	17,219	0.94	28.7
Meat	Obese	5788	11,555	9.65	19.3

Food goup	**Child**	**AOX/daily intake** (μmol Trolox/day)	**AOX/serving intake**[1] (μmol Trolox/serving)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Cereals	Celiac	16,337	4159	22.5	5.72
Cocoa	Celiac	375	2980	0.52	4.10
Fruits	Celiac	13,179	16,514	18.1	22.7
Legumes	Celiac	633	8397	0.87	11.6
Nuts	Celiac	1606	4344	2.21	5.98
Oils	Celiac	996	531	1.37	0.73
Tubers	Celiac	5787	15,207	7.96	20.9
Vegetables	Celiac	11,566	10,063	15.9	13.8
Dairy	Celiac	10,105	8228	13.9	11.3
Egg	Celiac	3738	19,656	5.14	27.0
Fish	Celiac	792	24,705	1.09	34.0
Meat	Celiac	7559	15604	10.4	21.5
Cereals	Allergic	34,182	7153	24.0	5.02
Cocoa	Allergic	340	2813	0.24	1.98
Fruits	Allergic	29,123	32,369	20.5	22.7
Legumes	Allergic	475	6310	0.33	4.43
Nuts	Allergic	54,078	10,517	3.80	7.38
Oils	Allergic	1367	724	0.96	0.51
Tubers	Allergic	15,758	50,781	11.1	35.7
Vegetables	Allergic	26,811	21,372	18.8	15.0
Dairy	Allergic	16,272	12,268	11.4	8.61
Egg	Allergic	2316	12,176	1.63	8.55
Fish	Allergic	2028	63,938	1.42	44.9
Meat	Allergic	8332	16,568	5.85	11.6

[1] Considering the intake of 1 serving.

3.2. Daily Antioxidant Intake with the DPPH method

Calculations showed a daily antioxidant intake of 245 mmol Trolox/day for healthy children, 80.3 mmol Trolox/day for obese children whereas for celiac children was 81.3 mmol Trolox/day and for allergic children 97.7 mmol Trolox/day. Therefore, healthy children were the ones able to extract higher antioxidant capacity values, at least daily. Again, fecal material source determined which food group was responsible for most of the daily antioxidant capacity. Thus, while healthy and allergic children were able to scavenge more antioxidant capacity from cereals, obese and celiac children used fruits as their main antioxidant source. Regardless of the fecal material used for fermentation, cereals, fruits and vegetables were always on the top 3 which is in the same line as results obtained with FRAP assay. In contrast, cocoa and oil were the groups with a lower contribution to the daily intake of antioxidant capacity (**Table 28 and Figure 45**).

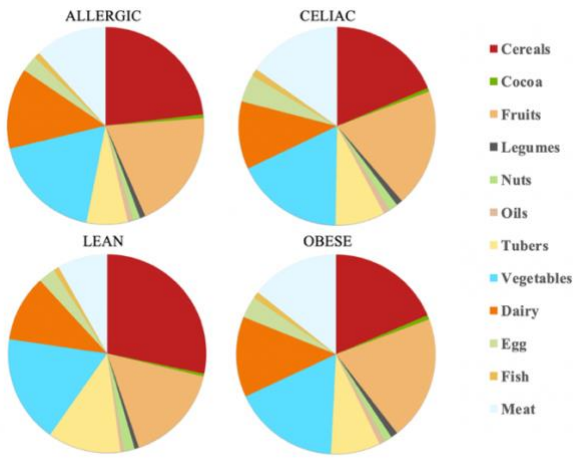
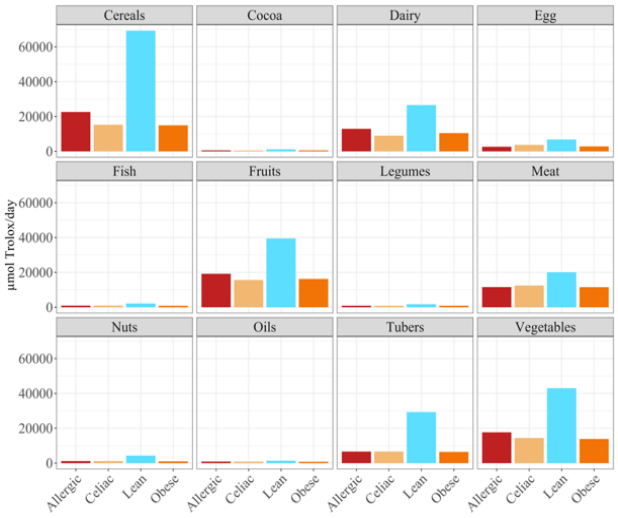


Figure 45. Mean contribution to daily antioxidant capacity intake (%) with DPPH method.

As FRAP assay showed before, obese fecal material also extracted the lowest antioxidant values according to the DPPH assay, except for dairy products and tubers (**Figure 46A**). However, the main difference from FRAP assay is that here, the feces from healthy lean children was the one able to extract the highest antioxidant capacity ($p < 0.05$) either daily or by serving size (**Figure 46A**) for cereals, dairy, fruits, tubers and vegetables.

In terms of the contribution to daily antioxidant intake per serving, in all four groups of children, fish was the highest contributing food group (25-38%), while oils contributed the least (0.3-0.6%), as in FRAP assay (**Figure 46B**). Regarding the differences between the groups of children, the group of lean healthy children had the highest antioxidant capacity per serving in all food groups, being statistically significant ($p < 0.05$) for cereals, dairy, fish, fruits, legumes nuts, tubers and vegetables. This was followed by the group of allergic children, except for fish, meat and nuts, where the second place was occupied by celiac children. The group of obese children ranked last in almost all food groups, except for fruits, cocoa, tubers and vegetables, where celiac children had the lowest antioxidant capacity per serving. (**Figure 46B**).

A



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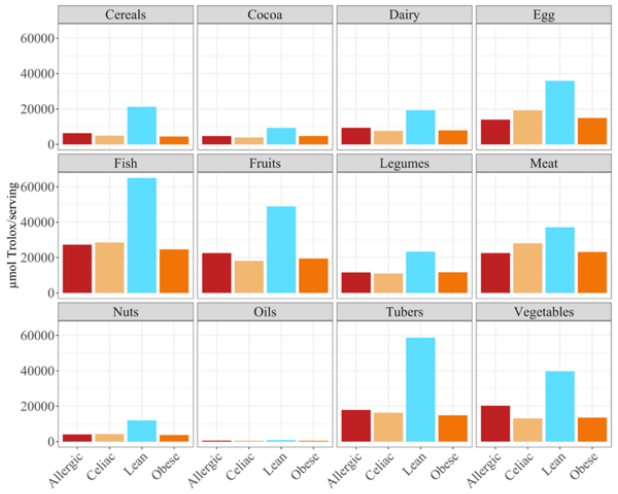

Figure 46. Differences in daily antioxidant intake (**46A**) and per serving intake (**46B**) in different foods groups in allergic, celiac, lean and obese children.

Table 28. Contribution of food consumption to the daily antioxidant capacity (AOX) intake in the children' diet with FRAP assay.

Food goup	**Child**	**AOX/daily intake** (μmol Trolox/day)	**AOX/serving intake**[1] (μmol Trolox/serving)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Cereals	Lean	69,285	21,222	28.3	8.66
Cocoa	Lean	1131	9290	0.46	3.79
Fruits	Lean	39,462	48,910	16.1	20.0
Legumes	Lean	1751	23,332	0.71	9.52
Nuts	Lean	4277	11,966	1.74	4.88
Oils	Lean	1376	844	0.56	0.34
Tubers	Lean	29,273	58,727	11.9	24.0
Vegetables	Lean	43,011	39,670	17.5	16.2
Dairy	Lean	26,546	19,352	10.8	7.89
Egg	Lean	6830	35,911	2.79	14.6
Fish	Lean	2161	65,047	0.88	26.5
Meat	Lean	8999	20,143	8.08	18.1
Cereals	Obese	14,937	4387	18.6	5.46
Cocoa	Obese	560	4750	0.70	5.92
Fruits	Obese	16,215	19,408	20.2	24.2
Legumes	Obese	882	11,714	1.10	14.6
Nuts	Obese	1001	3720	1.25	4.63
Oils	Obese	751	444	0.94	0.55
Tubers	Obese	6445	14801	8.0	18.4
Vegetables	Obese	13,848	13,540	17.2	16.9
Dairy	Obese	10,473	7958	13.0	9.91
Egg	Obese	2839	14929	3.54	18.6
Fish	Obese	828	24599	1.03	30.6
Meat	Obese	11,521	23,155	14.35	28.8

Food goup	**Child**	**AOX/daily intake** (μmol Trolox/day)	**AOX/serving intake**[1] (μmol Trolox/serving)	**Mean contribution to daily antioxidant intake** (%)	**Mean contribution to daily antioxidant per serving intake** (%)
Cereals	Celiac	15,222	4917	18.7	6.04
Cocoa	Celiac	490	3936	0.60	4.84
Fruits	Celiac	15,667	18,125	19.3	22.3
Legumes	Celiac	824	10,942	1.01	13.5
Nuts	Celiac	1196	4218	1.47	5.19
Oils	Celiac	832	487	1.02	0.60
Tubers	Celiac	6648	16,274	8.20	20.0
Vegetables	Celiac	14,410	13,117	17.7	16.1
Dairy	Celiac	8985	7675	11.0	9.44
Egg	Celiac	3659	19,242	4.50	23.7
Fish	Celiac	958	28,529	1.18	35.1
Meat	Celiac	12,456	28,102	15.31	34.5
Cereals	Allergic	22,607	6289	23.1	6.44
Cocoa	Allergic	586	4729	0.60	4.84
Fruits	Allergic	19,192	22,530	19.7	23.1
Legumes	Allergic	873	11,623	0.89	11.9
Nuts	Allergic	1147	4039	1.17	4.14
Oils	Allergic	864	527	0.89	0.54
Tubers	Allergic	6622	17,876	6.78	18.3
Vegetables	Allergic	17,708	20,284	18.1	20.8
Dairy	Allergic	12,923	9343	13.2	9.57
Egg	Allergic	2658	13,974	2.72	14.3
Fish	Allergic	910	27,299	0.93	28.0
Meat	Allergic	11,566	22,532	11.8	23.1

[1] Considering the intake of 1 serving.

4. Discussion

At present, there is not much scientific literature on antioxidant capacity intake. Saura-Calixto and Goñi [22] studied it for the adult population, and did not take into account the physiological processes that food undergoes, such as digestion and fermentation. These processes considerably increase the antioxidants ingested by degrading compounds and releasing others with higher antioxidant capacity [15,22]. Furthermore, they only studied foods of plant origin, when in fact, foods of animal source also provide a high quantity of compounds with antioxidant capacity, such as dipeptides, polyamines, uric acid, B vitamins, among others [23]. In our previous work [24,25], foods of animal and vegetable origin were taken into account for the intake of compounds with antioxidant activity in the Spanish diet for the adult population. In the results for adults, the foods that contributed most to the daily intake of antioxidant capacity were dairy products and meats, while for children it was cereals and vegetables, except in the group of obese children where fruit made a greater contribution than cereals. Overall, cereals contributed between 22-27% for the FRAP method in all four groups of children and between 19-28% for the DPPH trial (except in obese children, where fruit contributed 20% and cereals only 17%). Vegetables contributed between 16-19% for the FRAP method and between 17-18% for the DPPH method.

For adults, the foods that contributed the most when using serving size were fish and meat. For children, these food groups were tubers and fish (30-45%) for the FRAP method, except in children with celiac disease, where the foods that contributed the most anti-oxidant capacity per serving were fish and eggs (27%). For the DPPH method, the results coincided with those of adults, with meat (23-34%) and fish (27-35%) being the food groups, except

in lean children, where, as in FRAP, fish and tubers (24%) were the groups of food with the strongest antioxidant capacity per serving.

Differences in the estimated daily food intake as well as in the serving size between adult and children population could be behind these disagreements regarding antioxidant capacity. Furthermore, while for the adult population Spanish reference intakes were used [26,27] for the children population we decided to use references obtained for children since fecal material was obtained precisely from Greek children [20,21]. However, focusing on the serving size, both studies agree with the high antioxidant capacity found in meat and fish which could be explained by those compounds mentioned earlier as well as by the feeding of the animals themselves [28].

DPPH antioxidant values were usually higher than those obtained via FRAP assay: lean children (245 mmol Trolox/day in DPPH > 111 mmol Trolox/day in FRAP), obese (80.3 mmol Trolox/day for DPPH vs. > 59.9 mmol Trolox/day for FRAP) and celiac (81.3 mmol Trolox/day for DPPH > 72.6 mmol Trolox/day for FRAP). However, the opposite was true for allergic children (97.6 mmol Trolox/day for DPPH < 142 mmol Trolox/day for FRAP).

5. Conclusions

In the current paper we studied the antioxidant intake per day and per serving in three groups of children with different pathologies, as well as in lean healthy children. The differences between the groups suggest that the gut microbiota has a fundamental role in the release of compounds with antioxidant capacity when fermentation takes place at the colonic level. However, since no further investigation of gut microbial community structure was performed, via 16 rRNA or any other, we cannot ensure that our results are actually due to different microbial community structure. For the FRAP method, the group that was able to produce (and therefore ingest more antioxidant compounds) were the children allergic to cow's milk protein, which could mean that their microbiota generates compounds with a greater capacity to reduce iron than the other groups. Lean children were in second place, and celiac and obese children were in last place. In the case of DPPH, it was lean children who ingested the most compounds with antioxidant capacity daily. Cereals, vegetables, and fruits stood out for their contribution to daily antioxidant intake. On the other hand, tubers, fish, and meats stood out for their contribution to antioxidant intake per serving size. Few authors study daily antioxidant intake, and if they do, they do not consider the process of digestion and fermentation of foods during which many antioxidant compounds are generated through the metabolization of others. Foods of animal source are also often neglected in such scientific studies. These conclusions highlight the need for further research in this area, as the scientific literature is scarce and incomplete.

Funding

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Gentile, C.L.; Weir, T.L. The Gut Microbiota at the Intersection of Diet and Human Health. Science 2018, 362, 776-780, doi:10.1126/science.aau5812.

2. Icaza-Chávez, M.E. Gut Microbiota in Health and Disease. Rev. Gastroenterol. México 2013, 78, 240-248, doi:10.1016/j.rgmxen.2014.02.009.

3. Tremaroli, V.; Bäckhed, F. Functional Interactions between the Gut Microbiota and Host Metabolism. Nature 2012, 489, 242-249, doi:10.1038/nature11552.

4. Xu, X.; Harris, K.S.; Wang, H.J.; Murphy, P.A.; Hendrich, S. Bioavailability of Soybean Isoflavones Depends upon Gut Microflora in Women. J. Nutr. 1995, 125, 2307-2315, doi:10.1093/jn/125.9.2307.

5. Setchell, K.D.R.; Brown, N.M.; Lydeking-Olsen, E. The Clinical Importance of the Metabolite Equol-a Clue to the Effective-ness of Soy and Its Isoflavones. J. Nutr. 2002, 132, 3577-3584, doi:10.1093/jn/132.12.3577.

6. Pérez-Burillo, S.; Rajakaruna, S.; Pastoriza, S.; Paliy, O.; Rufián-Henares, J.A. Bioactivity of food melanoidins is mediated by gut microbiota. Food Chem. 2020, 316, 126309, doi: 10.1016/j.foodchem.2020.126309.

7. Sanmiguel, C.; Gupta, A.; Mayer, E.A. Gut Microbiome and Obesity: A Plausible Explanation for Obesity. Curr. Obes. Rep. 2015, 4, 250-261, doi:10.1007/s13679-015-0152-0.

8. Zamudio-Vázquez, V.P.; Ramírez-Mayans, J.A.; Toro-Monjaraz, E.M.; Cervantes-Bustamante, R.; Zárate-Mondragón, F.; Montijo-Barrios, E.; Cadena-León, J.F.; Cázares-Méndez, J.M.; Zamudio-Vázquez, V.P.; Ramírez-Mayans, J.A.; et al., Importancia de la microbiota gastrointestinal en pediatría. Acta Pediátrica México 2017, 38, 49-62, doi:10.18233/apm1no1pp49-621323.

9. Green, P.H.R.; Cellier, C. Celiac Disease. N. Engl. J. Med. 2007, 357, 1731-1743, doi:10.1056/NEJMra071600.

10. Sjöberg, V.; Sandström, O.; Hedberg, M.; Hammarström, S.; Hernell, O.; Hammarström, M.-L. Intestinal T-Cell Responses in Celiac Disease - Impact of Celiac Disease Associated Bacteria. PLOS ONE 2013, 8, e53414, doi:10.1371/journal.pone.0053414.

11. Shu, S.-A.; Yuen, A.W.T.; Woo, E.; Chu, K.-H.; Kwan, H.-S.; Yang, G.-X.; Yang, Y.; Leung, P.S.C. Microbiota and Food Allergy. Clin. Rev. Allergy Immunol. 2019, 57, 83-97, doi:10.1007/s12016-018-8723-y.

12. Clarke, K.; Chintanaboina, J. Allergic and Immunologic Perspectives of Inflammatory Bowel Disease. Clin. Rev. Allergy Immunol. 2019, 57, 179-193, doi:10.1007/s12016-018-8690-3.

13. Hartono, S.; Ippoliti, M.R.; Mastroianni, M.; Torres, R.; Rider, N.L. Gastrointestinal Disorders Associated with Primary Immunodeficiency Diseases. Clin. Rev. Allergy Immunol. 2019, 57, 145-165, doi:10.1007/s12016-018-8689-9.

14. Chen, B.; Sun, L.; Zhang, X. Integration of Microbiome and Epigenome to Decipher the Pathogenesis of Autoimmune Diseases. J. Autoimmun. 2017, 83, 31-42, doi:10.1016/j.jaut.2017.03.009.

15. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an Improved Global Antioxidant Response Method (GAR+): Physiological-Resembling in Vitro Digestion-Fermentation Method. Food Chem. 2018, 239, 1253-1262, doi:10.1016/j.foodchem.2017.07.024.

16. Pérez-Burillo, S.; Molino, S.; Navajas-Porras, B.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; López-Maldonado, A.; Pas-toriza, S.; Rufián-Henares, J.Á. An in Vitro Batch Fermentation Protocol for Studying the Contribution of Food to Gut Microbiota Composition and Functionality. Nat. Protoc. 2021, 16, 3186-3209, doi:10.1038/s41596-021-00537-x.

17. Pastoriza, S.; Delgado-Andrade, C.; Haro, A.; Rufián-Henares, J.A. A Physiologic Approach to Test the Global Antioxidant Response of Foods. The GAR Method. Food Chem. 2011, 129, 1926-1932, doi:10.1016/j.foodchem.2011.06.009.

18. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. Anal. Biochem. 1996, 239, 70-76, doi:10.1006/abio.1996.0292.

19. Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a Free Radical Method to Evaluate Antioxidant Activity. LWT - Food Sci. Technol. 1995, 28, 25-30, doi:10.1016/S0023-6438(95)80008-5.

20. European Food Safety Authority (EFSA). Use of the EFSA Comprehensive European Food Consumption Database in Expo-sure Assessment. EFSA J. 2011, 9, 2097, doi:10.2903/j.efsa.2011.2097.

21. Lyons, J.; Walton, J.; Flynn, A. Development of an Online Database of Typical Food Portion Sizes in Irish Population Groups. J. Nutr. Sci. 2013, 2, e25, doi:10.1017/jns.2013.14.

22. Saura-Calixto, F.; Goñi, I. Antioxidant Capacity of the Spanish Mediterranean Diet. Food Chem. 2006, 94, 442-447, doi:10.1016/j.foodchem.2004.11.033.

23. Carrillo, C.; Barrio, Á.; del Mar Cavia, M.; Alonso-Torre, S. Global Antioxidant Response of Meat. J. Sci. Food Agric. 2017, 97, 2358-2365, doi:10.1002/jsfa.8047.

24. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to In Vitro Digestion-Fermentation. Antioxidants 2020, 9, 1312, doi:10.3390/antiox9121312.

25. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Foods of Animal Origin Submitted to In Vitro Digestion-Fermentation. Anti-oxidants 2021, 10, 445, doi:10.3390/antiox10030445.

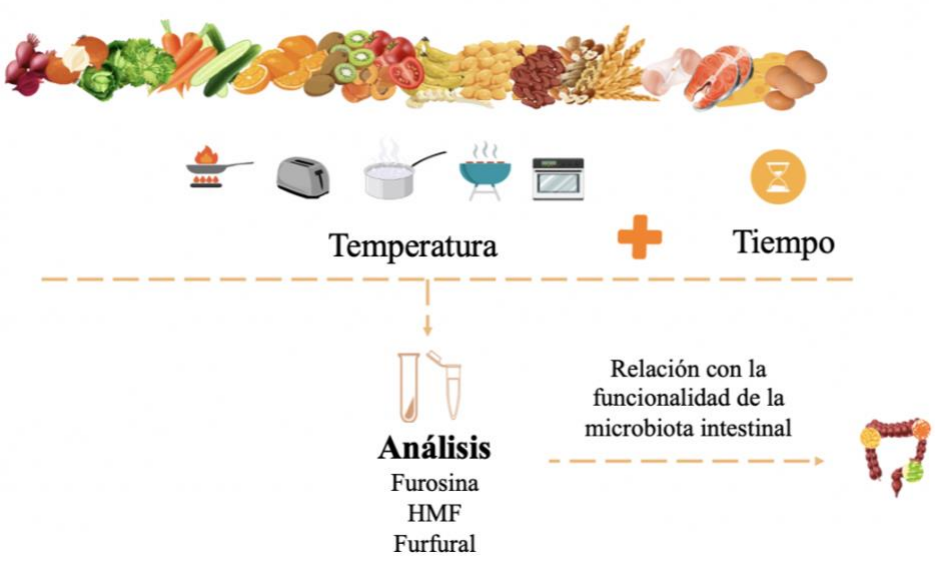
26. Salvadó, J.S.; Sanjaume, A.B.I; Casañas, R.T.; Solà, M.E.S.I; Peláez, R.B. Nutrición y Dietética Clínica; Elsevier: Amsterdam, The Netherlands, 2019.

27. Mercasa. La Alimentación en España. 2020. Available online: https://www.mercasa.es/media/publicaciones/ 281/AEE_2020_web.pdf (accessed on 15 August 2021).

28. Chen, D.; Wu, M.; Xie, S.; Li, X.; Tao, Y.; Wang, X.; Huang, L.; Pan, Y.; Peng, D.; Yuan, Z. Determination of Tartrazine, Lutein, Capsanthin, Canthaxanthin and β-Carotene in Animal-Derived Foods and Feeds by HPLC Method. J. Chromatogr. Sci. 2019, 57, 462-468, doi:10.1093/chromsci/bmz019.

CAPÍTULO III

Estudio del daño térmico en los alimentos y relación con la funcionalidad de la microbiota intestinal



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IF: 7.675 Q1 Food Science and Technology 24/165

Relationship of Thermal Treatment and Antioxidant Capacity in Cooked Foods

En el presente trabajo se estudió la producción de furosina, HMF y furfural en alimentos (tanto de origen animal como vegetal) que se sometieron a diferentes técnicas de cocinado. Además, se investigaron las correlaciones entre dichos marcadores de daño térmico con la capacidad antioxidante producida tras la digestión y fermentación *in vitro*. Con este trabajo se cumplirían los dos últimos objetivos de la presente tesis doctoral.

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Relationship of Thermal Treatment and Antioxidant Capacity in Cooked Foods

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Abstract

Most of the foods we eat undergo a cooking process before they are eaten. During such process, the non-enzymatic browning occurs which generates compounds such as furosine, 5-hydroxymethylfurfural (HMF) and furfural. These are considered markers of cookedness and can therefore be used as quality indicators. In this work, we study the production of these compounds in different foods (both of plant and animal origin) that are cooked with different techniques. Additionally, we investigate correlations between the production of these markers of cookedness and the antioxidant capacity produced after in vitro digestion and fermentation. We observe that, in general, cereals and vegetables are more thermally damaged. Toasting and frying produce the highest concentrations of Maillard compounds whereas boiling the lowest. Furosine content shows a significant positive correlation with in vitro digestion data in fried foods, and with fermentation in roasted foods. Furfural content shows a significant positive correlation with in vitro digestion results in roasted foods, specifically in the Folin-Ciocalteu method.

Keywords: Maillard reaction, furosine, HMF, furfural, cooking, antioxidant activity.

1. Introduction

Cooking a food is an operation that can modify its characteristics to improve the organoleptic properties, digestibility and hygienic conditions. In addition, cooking most frequently involves a heat source in order to raise its temperature. As consequence, food undergoes physical, chemical, or biological changes. Although cooking usually improves taste, flavor or make food safe to be consumed, it can also have a negative impact on the food chemical composition and by extension on human health [1]. There are several types of cooking that can be classified according to how heat is transmitted onto the food. For example, frying or grilling use fats as the medium to transfer heat to the food [2] whereas boiling uses water. On the other hand, others (like roasting) use the air to transfer heat to the food surface.

Heating favors a plethora of chemical changes within the food, some of those as consequence of the non-enzymatic browning, including the Maillard reaction [3]. This reaction involves a set of chemical chain reactions that is favored when food is subjected to moderate heat and gives rise to a plethora of molecules responsible for new colors, smells, tastes and textures that are usually pleasing to the consumer [4,5], although undesirable aromatic substances and brown compounds may also be produced [6]. In order to allow the reaction takes place, a free carbonyl group is needed (such as those from reducing sugars, oxidized lipids or B group vitamins) as well as free amino groups from an amino acid, peptide or protein [7]. The Maillard reaction is divided into three stages; during the early stage, while it is still reversible and browning has not yet occurred, sugars and amino acids begin to degrade [8,9]. Furosine appear during this stage [10]. It was one of the first products to be identified for the Maillard reaction. The concentration of this compound has

been shown to increase as a function of the heat treatment applied and is another marker of heat damage [11,12]. Secondly, the intermediate stage, involves dehydration of sugars by enolic isomerization, giving rise to furfural and 5-hydroxymethylfurfural (HMF) among other compounds. Furfural content of foods correlates with undesirable flavors and is therefore a good quality indicator [13]. HMF also allows to monitor intermediate stages of the Maillard reaction and it is an indicator commonly used by the food industry to assess heat damage in plant food products [14,15]. The final stage involves polymerization and formation of high molecular weight-colored substances called melanoidins [16].

It is remarkable that water-soluble compounds generated during MR have shown the ability to neutralize free radicals [17]. Such antioxidant capacity is proportional to the degree of browning [18] and has a close correlation with the compounds generated from the intermediate and late stages, as well as with the type of sugar involved in the reaction [19]. Despite the partial loss of natural compounds with antioxidant activity that may occur during food processing, antioxidant properties could be maintained and even increased due to the formation of new compounds through the development of the Maillard reaction [20] or release by cell breakage [21]. In previous studies we found that cooking techniques strongly modify the antioxidant capacity of plant [22] and animal foods [23]. Therefore, the aim of this study was to unravel the potential contribution of the development of non-enzymatic browning to the antioxidant capacity of foods. To do that, 23 of the most commonly consumed foods in Spain were submitted to common cooking techniques (including frying, roasting, toasting, boiling and grilling). Furosine, HMF and furfural concentrations were analyzed as indicators of non-enzymatic browning, related with the cookedness of foods. In addition, correlation studies were carried out between these indicators and antioxidant

capacity in the same foods with the same cooking, both after in vitro digestion and colonic fermentation stages.

2. Materials and Methods

2.1 Chemicals

Furosine was purchased from NeoMPS (Strasbourg, France). Furfural, 5-(hydroxymethyl)furfural, hydrochloric acid, methanol, and acetonitrile (HPLC grade) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Alpha Aesar provided the pancreatin in the porcine pancreas (UK). The remaining chemicals, which included analytical-grade salts and enzymes for in vitro digestion and fermentation as well as chemicals and solvents for the determination of antioxidant capacity, were bought from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Foods and cooking conditions

A total of 20 foods were studied, included in these groups: cereals (bread, bread whole grain, penne, penne whole grain, rice, rice whole grain), egg, fish (cod fish and salmon), fruits (apple and banana), legumes (beans and lentils), meat (pork, beef, chicken and lamb), tubers (potato) and vegetables (capsicum, carrot, cauliflower, onion and tomato). Different thermal processes were applied to the samples: boiling, frying, grilling, roasting and toasting). Fruits, tubers and vegetables were cut into different sizes so that the same texture was achieved after the different cooking processes (**Table 33** in supplemental information).

For grilling and frying, extra virgin olive oil (EVOO) was used as a cooking medium. Boiling was performed at a water/food rate of 5:1, for 20 min at 100 °C. Grilling was carried out at an oil/food rate of 0.5:1, for 3 min on each side, at 220-250 °C. Fried foods were obtained at an oil/food rate of 5:1, at 180 °C for 8 min. Roasting was performed for 10 min at 180 °C.

Toasting was carried out for 3 min at 900 W, in a Grunkel TS140H toaster at the fourth level following the manufacturer's instructions. Cooking times and food/average rates were acquired from previous work [2].

The utensils used for foods preparation were forks and knives, stainless steel spoons; frying pan, saucepan, fryer, a portable oven (1500 W), and toaster. These utensils were acquired at Centro Hogar Sánchez (Granada, Spain). Cooked foods were homogenized and stored at -80 °C under a nitrogen atmosphere. All analyses were performed in duplicate.

2.3. Furosine, HMF and Furfural Assays

Furosine assay was carried out following the method of Delgado-Andrade et al., [24]. Samples were hydrolyzed for 23 hours at 120 °C with 7.95 M HCL. The hydrolysate was purified with a Sep-pack C18 cartridge (Millipore, MA), and the resulting solution was analyzed by ion pair RP-HPLC. The analysis was performed in duplicate, and the data are mean values expressed as μg/g food and μg/g of protein. Protein in each food was estimated from a database [25].

HMF and furfural were determined following a previously described protocol [14]. Ground samples were suspended in deionized water, clarified with Carrez I (potassium ferrocyanide, 15% *w/v)* and Carrez II (zinc acetate 30% w/v) solutions. The resulting solution was analyzed by RP-HPLC. The analysis was performed in duplicate, and the data are mean values expressed in μg per g of food.

2.4. In vitro gastrointestinal digestion and fermentation and antioxidant capacity

All samples were submitted to in vitro digestion-fermentation according to the protocol previously described [26,27]. Five g of each food were subjected to in vitro gastrointestinal digestion followed by in vitro fermentation, in triplicate. The in vitro fermentation was carried out using fecal material from five healthy donors (with a mean Body Mass Index = 21.3, and who had not taken antibiotics for three months prior to the assay). All fecal samples were pooled together to restricted inter-individual variability. The fermentation was carried out for 24 hours, at 37 °C. After the in vitro gastrointestinal digestion and fermentation, two fractions were obtained: digestion supernatant, which is available for absorption at the small intestine, and fermentation supernatant which is available for absorption at the large intestine. A control fermentation was carried out, using only the fecal fermentation solution (inoculum composed of peptone, cysteine, and resazurin).

The antioxidant capacity was evaluated in the two supernatant fractions obtained after in vitro digestion and fermentation, which represent the total antioxidant capacity that each food could exert in the human body [28]. Three different methods were used to determine the antioxidant capacity (DPPH, FRAP and Folin-Ciocalteu). The results of such analyses were described in previous work for plant [22] and animal foods [23].

2.5. Statistical Analysis

Statistical differences were calculated using the unpaired Kruskal Wallis test with 95% confidence, comparing the amount of furosine, HMF and furfural in each of the food groups, as well as within each group, the

comparison was made by cooking. Thus, we show whether a particular food group has a higher or lower amount of these indicators of thermal damage. Pearson's parametric statistic was calculated to show the linear relationship between the heat damage markers and between these and the antioxidant capacity produced in the same foods with the same thermal processing at *p-value* < 0.05. Correlations were made for both antioxidant capacity after in vitro digestion of foods and after in vitro fermentation with healthy adult microbiota. The correlations were based on the cooking methods used for the different food groups. The Statgraphics Plus software (version 5.1) was used to perform all statistical analyses.

3. Results

3.1. Furosine Content of Cooked Foods

For the furosine assay, the food group with the highest concentration after thermal processing was cereals, followed by vegetables, meat, legumes, fish, eggs, fruits and tubers. The values for cereals and vegetables were significantly ($p < 0.05$) higher than the mean of the other food groups (**Figure 47A**). When we consider cooking techniques, toasting and frying gave the highest ($p < 0.05$) concentrations. Grilling on the other hand, showed significantly ($p < 0.05$) lower concentrations than the rest (**Figure 47B**).

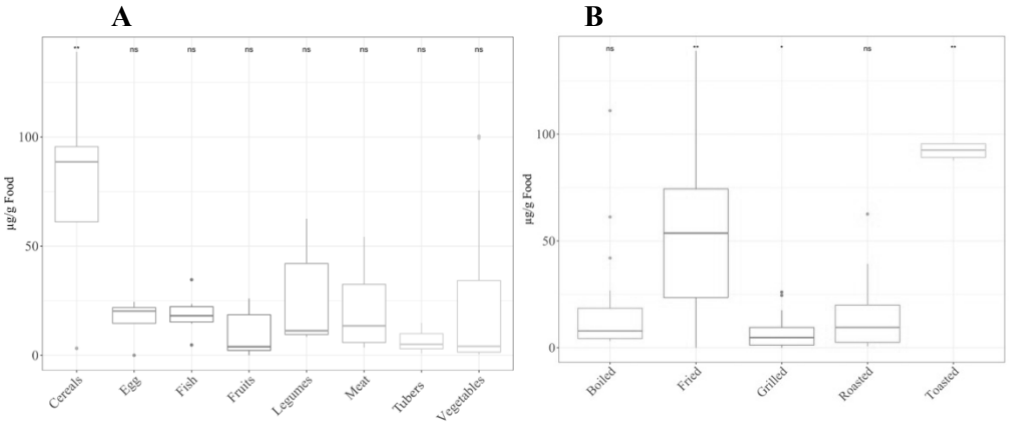

Figure 47. 47A: Furosine levels in different food groups. Statical analysis was performed via Kruskal-Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$. **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant. **47B:** Furosine levels depending on the cooking applied. Statical analysis was performed via Kruskal-Wallis test. Each group was compared to the average of all of them (i.e. base-mean). Statistic labels: **: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.1.1.1 Furosine content by specific foods and cooking method.

Table 29 shows the furosine content of each food depending on the kind of thermal treatment used for cooking. In the cereals group, the highest furosine value was reached with frying (138.9 μg/g), while in the case of eggs it was obtained for grilled eggs (24.5 μg/g). In the fish group, again fried foods had the highest furosine content (fried salmon, 34.6 μg/g) but for fruits, was boiled banana (42.0 μg/g). In legumes, roasted kidney beans demonstrated a high reactivity (furosine values of 62.6 μg/g), but were meat and veg-etables the groups with the highest levels of furosine: 412.5 μg/g for fried cauliflower and 183.6 μg/g for fried meat. Thus, in general, frying (followed by roasting) was the cooking method that produced the highest levels of furosine.

Furosine is a good indicator of the thermal damage suffered by proteins during heat treatment [3,5,10] since it is correlates with the loss of available lysine. Thus, furosine can be also expressed in mg/100 g of protein to show the thermal damage (or heat load) of the food. In this sense, the highest thermal damage was suffered by vegetables (fried cau-liflower and carrot with values surrounding 800 mg furosine/100 g of protein) closely followed by fruits (fried banana with a value of 620 mg furosine/100 g of protein) and cereals (fried bread, 163.4 mg furosine/100 g of protein). Again, frying was the heat treatment with the highest thermal damage, while boiling and grilling were the milder cooking option.

Table 29. Furosine values depending on the cooking method applied to the food.

Food group	Food	Boiled		Fried		Grilled		Roasted		Toasted	
		µg/g food	mg/100 g protein	µg/g food	mg/100 g protein	µg/g food	mg/100 g protein	µg/g food	mg/100 g protein	µg/g food	mg/100 g protein
Cereals	Bread	-	-	138.9	163.4	-	-	-	-	95.4	127.2
	Penne	3.1	5.8	-	-	-	-	-	-	-	-
	Rice	19.5	84.8	-	-	-	-	-	-	-	-
Egg	Egg	14.5	7.1	n.d.	n.d.	24.5	11.5	21.0	8.6	-	-
Fish	Cod fish	-	-	23.5	8.9	4.7	2.6	-	-	-	-
	Salmon	-	-	34.6	8.9	17.6	7.9	18.6	7.2	-	-
Fruits	Apple	-	-	18.6	620.0	n.d.	n.d.	2.2	73.3	-	-
	Banana	42.0	323.1	-	-	26.0	123.8	3.9	32.5	-	-
Legumes	Beans (Kidney)	8.5	14.9	-	-	-	-	62.6	88.2	-	-
	Lentils	40.5	61.4	-	-	11.2	14.7	9.5	11.7	-	-
Meat	Beef	72.4	29.7	183.6	67.0	5.6	1.9	39.2	19.2	-	-
	Chicken	26.8	9.6	54.2	18.4	7.7	3.0	28.9	12.6	-	-
	Pork	59.6	22.7	30.2	9.7	3.6	2.0	8.0	3.7	-	-
	Lamb	-	-	45.4	18.5	5.4	3.6	19.0	11.7	-	-
Tubers	Potatoe	5.0	0.010	14.8	0.062	0.9	0.002	-	-	-	-
Vegetables	Capsicum	3.4	37.8	53.2	110.8	0.5	10.0	1.4	15.6	-	-
	Carrot	14.6	162.2	99.4	764.6	4.1	41.0	0.9	5.6	-	-
	Cauliflower	6.5	31.0	412.5	808.8	1.5	7.5	13.7	52.7	-	-
	Onion	3.9	32.5	74.0	528.6	0.8	5.7	2.8	4.4	-	-

n.d. = not detected. The sign - denotes that such cooking method was not used for that particular food.

3.2 HMF Content of Cooked Foods.

Regarding HMF content, the food group that presented the highest amount of this compound after the different thermal processes was cereals, followed by vegetables, fish, fruit, tubers, meat, legumes and eggs (**Figure 48A**). As for cooking techniques, toasting generated the highest concentrations ($p < 0.05$). Frying, roasting, grilling and boiling followed toasting in HMF production, though only boiling produced significantly lower levels ($p < 0.05$) than the rest (**Figure 48B**).

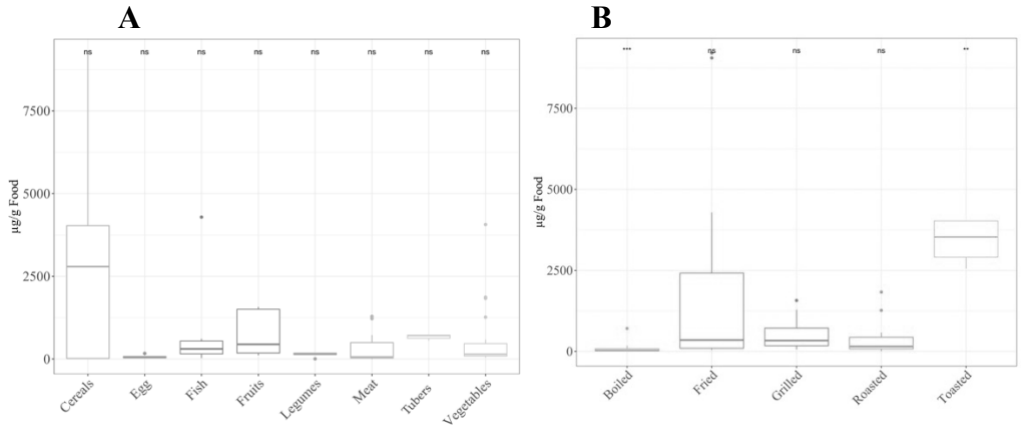

Figure 48. **48A**: HMF levels in different food groups. Statical analysis was performed via Kruskal-Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: ns: not significant. **48B**: HMF levels depending on the cooking applied. Statical analysis was performed via Kruskal-Wallis test. Each group was compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant.

3.2.1 HMF content by specific foods and cooking methods.

The levels of HMF in each food (**Table 30**) were also used to study the effect of the cooking method in the development of non-enzymatic browning. As expected, the heat treatment of cereals produced the highest HMF concentration, up to 10305 µg/g for toasted bread and five times lower for fried bread. Eggs and legumes where not too much affected by cooking, being grilling the most damaging cooking method (169 and 179 µg/g for grilled egg and lentils). Meats showed a higher content than fish after cooking, being grilling again the most harmful thermal treatment (1287 and 613 µg/g for pork and salmon, respectively). In addition, cooking potatoes generated large amounts of HMF, ranging from 550 µg/g during frying till 737 µg/g after grilling. On the other hand, fruits and vegetables were highly

reactive during cooking, showing high HMF levels for grilled banana and fried apple (around 1500 μg/g) and close to 4000 and 2000 μg/g for fried onion and cauliflower, respectively (**Table 30**). Opposite to furosine, in the case of HMF generation there was not a single cooking method with a higher thermal damage, since frying, grilling, roasting and toasting produce large amounts of HMF, depending on the food matrix.

Table 30. HMF values (expressed in μg HMF/g of food) depending on the cooking method applied to the food.

Food group	Food	Boiled	Fried	Grilled	Roasted	Toasted
Cereals	Bread	-	2057.0	-	-	10304.9
	Penne	6.7	-	-	-	-
	Rice	15.3	-	-	-	-
Egg	Egg	47.4	43.8	168.9	26.5	-
Fish	Cod fish	30.8	429.5	336.0	-	-
	Salmon	-	275.2	613.2	115.3	-
Fruits	Apple	-	1505.6	114.1	444.0	-
	Banana	-	n.d.	1572.8	179.4	-
Legumes	Beans (Kidney)	175.8	-	-	154.1	-
	Lentils	5.0	-	178.5	134.5	-
Meat	Beef	13.3	1222.5	720.8	52.9	-
	Chicken	33.5	351.8	720.9	333.0	-
	Pork	4.5	63.7	1286.9	423.3	-
	Lamb	2.6	52.0	61.7	6.2	-
Tubers	Potatoe	708.0	550.4	737.4	-	-
Vegetables	Capsicum	88.0	71.7	352.6	63.2	-
	Carrot	55.1	104.1	223.3	89.5	-
	Cauliflower	48.9	1868.4	89.8	581.1	-
	Onion	127.6	4065.5	237.0	1832.1	-

n.d. = not detected. The sign - denotes that such cooking method was not used for that particular food.

3.3 Furfural Content of Cooked Foods.

Regarding furfural, tubers showed the largest levels ($p < 0.05$), followed by cereals, fruits, vegetables, meat, fish, legumes and eggs (**Figure 49A**). Toasting generated the highest concentrations ($p < 0.05$). After

toasting, in decreasing order of furfural content, we found frying, grilling, roasting and finally boiling. Only the latter showed significantly ($p < 0.05$) lower concentrations than the average of the rest (**Figure 49B**).

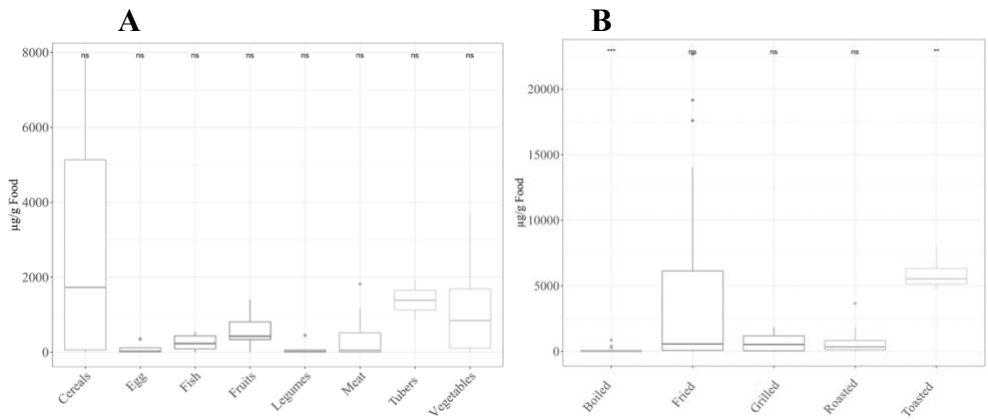

Figure 49. 49A: Furfural levels in different food groups. Statical analysis was performed via Kruskal-Wallis test. Each of the groups were compared to the average of all of them (i.e. base-mean). Statistic labels: ns: not significant. **49B:** Furfural levels depending on the cooking applied. Statical analysis was performed via Kruskal-Wallis test. Each group was compared to the average of all of them (i.e. base-mean). Statistic labels: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: not significant

3.3.1 Furfural content by specific food groups and cooking methods.

Furfural was another furanic compound used as indicator of thermal treatment (**Table 31**). Boiling produced low levels of furfural in cereals such as penne or rice, but roasted and fried bread generated large amounts of this furanic compound (7859 and 1192 µg/g, respectively). In the case of protein-rich foods, eggs and salmon had a relatively high furfural content (from 352 till 545 µg/g), but were fried meats (pork and chicken) those with higher values (over 1100 µg/g in both cases). The highest furfural levels were obtained in cooked tubers and vegetables, reaching very high furfural values:

around 17600 and 19200 μg/g for fried onion and potatoes, respectively (**Table 31**). As in the case of HMF, there was not a single cooking method producing the largest furfural contents, since frying, grilling, roasting and toasting produced high furfural levels depending on the food. It is noteworthy to mention that boiling was the less aggressive heat treatment, giving rise to low furfural levels or even not detected in meats and legumes.

Table 31. Furfural values (expressed in μg Furfural/g of food) depending on the cooking method applied to the food.

Food group	Food	Boiled	Fried	Grilled	Roasted	Toasted
Cereals	Bread	-	1191.6	-	-	7858.6
	Penne	47.3	-	-	-	-
	Rice	98.2	-	-	-	-
Egg	Egg	42.9	n.d.	13.1	351.8	-
Fish	Cod fish	268.0	55.5	n.d.	-	-
	Salmon	-	494.3	544.5	191.1	-
Fruits	Apple	-	14028.8	1.8	810.3	-
	Banana	-		425.4	338.3	-
Legumes	Beans (Kidney)	n.d.		-	449.0	-
	Lentils	n.d.		61.5	27.4	-
Meat	Beef	n.d.	613.6	131.5	90.3	-
	Chicken	n.d.	514.7	1168.0	533.9	-
	Pork	n.d.	54.6	1821.1	41.7	-
	Lamb	n.d.	n.d.	18.5	20.0	-
Tubers	Potatoe	864.2	19164.0	1386.5	-	-
Vegetables	Capsicum	3.8	232.7	1748.4	1844.1	-
	Carrot	83.7	76.2	844.3	139.7	-
	Cauliflower	3.3	3496.7	1199.2	851.8	-
	Onion	396.4	17596.9	524.5	3672.6	-

n.d. = not detected. The sign - denotes that such cooking method was not used for that particular food.

3.4 Correlation of heat damage markers with antioxidant capacity of cooking foods after digestion and fermentation.

The results of antioxidant capacity are deeply described in **Table 32** previous work for plant [22] and animal foods [23]. In general, it was found that intense cooking methods, such as frying, increase the antioxidant capacity of foods. In the case of animal foods, meat was the group with the highest antioxidant capacity [23], while cocoa and legumes were the most antioxidant plant foods [22]. Taking all these information into account, we generated correlations between heat damage markers (furosine, HMF and furfural) and antioxidant capacity obtained after *in vitro* digestion and fermentation (**Figure 50**).

Table 32. Antioxidant capacity measured via Folin-Ciocalteu (mg gallic acid equivalents/kg of food), FRAP (mmol Trolox equivalents/kg of food) and DPPH (mmol Trolox equivalents/kg of food) depending on the type of cooking applied.

AOX method	*In vitro* pre-treatment	Boiled	Fried	Grilled	Roasted	Toasted
Folin-Ciocalteu	Digestion	1259 ± 1144	1368 ± 1018	1409 ± 1374	2262 ± 1609	3536 ± 268
	Fermentation	33396 ± 12455	38221 ± 19990	43498 ± 21926	43837 ± 18024	16573 ± 5625
FRAP	Digestion	2.3 ± 2.0	6.8 ± 5.7	4.1 ± 2.4	4.6 ± 3.0	4.9 ± 1.0
	Fermentation	179 ± 66.9	202 ± 115	239 ± 118	243 ± 98.4	97.4 ± 26.0
DPPH	Digestion	13.2 ± 9.6	22.3 ± 24.1	12.1 ± 8.7	18.0 ± 13.8	1.1 ± 0.8
	Fermentation	213 ± 219	222 ± 206.9	253 ± 199	290 ± 215	108 ± 21.5

Fried foods. Furosine correlated positively with Folin-Ciocalteu, FRAP and DPPH obtained after *in vitro* digestion, though only the last two were statistically significant ($p < 0.05$). HMF and furfural content correlated negatively with all antioxidant capacity results obtained, except with FRAP for digestion (**Figure 50**).

Boiled foods. Although there were no statistically significant correlations, furosine content was positively correlated with Folin-Ciocalteu results for digestion, and FRAP and DPPH for fermentation (**Figure 50**). Furosine correlated negatively with the rest. In the case of HMF content, it was correlated negatively with all antioxidant capacity results except for DPPH obtained from the digestion fraction. Finally, furfural content correlated positively with antioxidant capacity results obtained via Folin-Ciocalteu for fermentation and FRAP for both digestion and fermentation. The rest of the correlations were negative.

Roasted foods. Furosine correlated positively and significantly ($p < 0.05$) with Folin-Ciocalteu, FRAP for fermentation, and the correlation was statistically significant. It was also positively correlated with Folin-Ciocalteu, FRAP and DPPH for digestion, and negatively correlated with DPPH for fermentation (**Figure 50**). HMF content was negatively correlated with all antioxidant capacity results. As for furfural content, it correlated negatively with all results except with Folin-Ciocalteu for digestion, which correlated positively and, moreover, in a statistically significant manner ($p < 0.05$).

Grilled foods. No statistically significant correlations were found, but furosine content was positively correlated with Folin-Ciocalteu for digestion and fermentation, FRAP for fermentation and DPPH for digestion (**Figure 50**). With the others it correlated negatively. HMF content was positively correlated with Folin-Ciocalteu results for digestion and fermentation and FRAP and DPPH for fermentation. With the rest the correlation was negative. Furfural content for grilled foods was positively correlated with all antioxidant capacity results except FRAP and DPPH for digestion, with which it was negatively correlated.

Toasted foods. Furosine content correlated negatively with Folin-Ciocalteu antioxidant capacity results for digestion and fermentation, although FRAP and DPPH in both digestion and fermentation correlated positively. The same was true for HMF and furfural content.

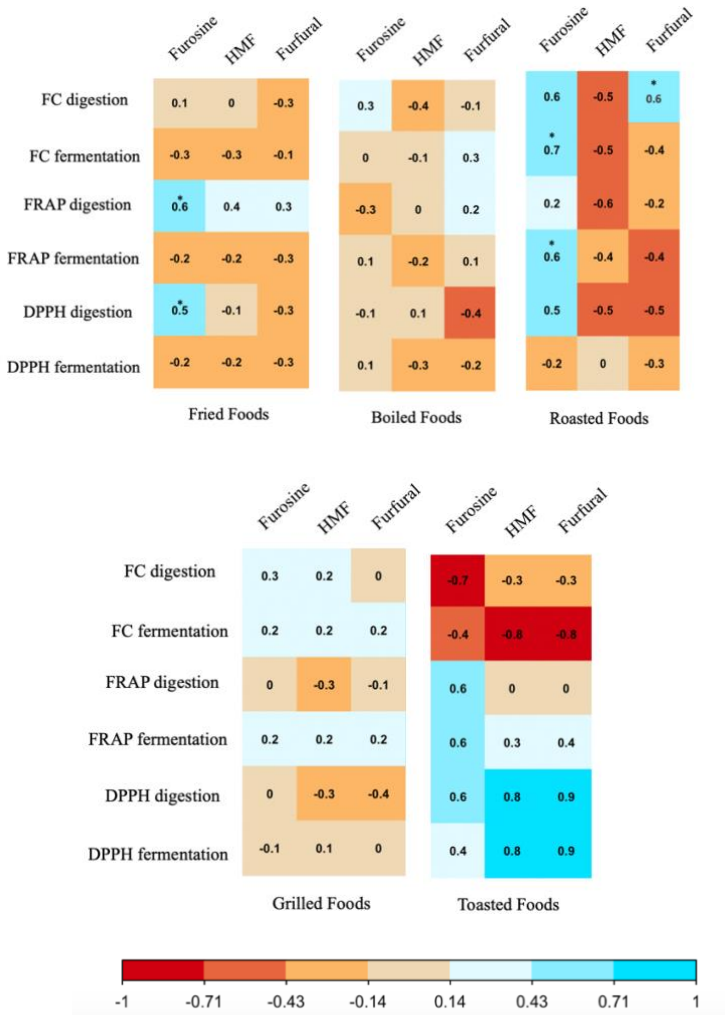

Figure 50. Correlations between heat damage markers (furosine, HMF and furfural) and antioxidant capacity measured via Folin-Ciocalteu (FC) (mg gallic acid equivalents/kg of food), FRAP (mmol Trolox equivalents/kg of food) and DPPH (mmol Trolox equivalents/kg of food) depending on the type of cooking applied. Statistic labels: *: $p < 0.05$.

4. Discussion

Non-enzymatic browning products present both benefits [29-31] and risks [32,33] and research must find a balance between both of them [1]. Furosine is formed along the Maillard reaction from Amadori compounds, and originates before sensory changes take place. Therefore, it is considered a very sensitive indicator for the early detection of quality changes [34]. HMF and furfural are formed both at the intermediate stages of the Maillard reaction and also under acidic conditions involving degradation of sugars at high temperature, known as caramelization, indicating a higher degree of cookedness [14,35]. So, these furanic compounds are indicators of non-enzymatic browning.

In our assays, cereals and vegetables stood out for their high furosine and HMF content. Particularly, cauliflower and bread showed the highest levels. On the other hand, tubers and cereals showed the highest concentration of furfural, particularly bread, agreeing with furosine and HMF levels. These results are in agreement with other previous studies [1] where cereals and tubers in combined dishes considerably increase the amount of furosine, HMF and furfural. Specifically, bread is a food that produces high levels of furosine due to its composition [36]. Eggs exhibited the lowest values of the three indicators, which may be due to a lower level of sugars and amino acids ready to react [1,36,37].

Toasted and fried foods showed higher levels of furosine, HMF and furfural than the rest. Previous studies have shown that toasting and frying generate a considerable concentration of cookedness markers [1,31], and even different nutritional studies advise against the consumption of toasted foods [32]. Cooking plays an important role in the appearance of Maillard reaction products; both the time and the temperature applied are decisive. In the case

of frying, in addition to the heat applied, the composition of the frying oil plays a role, in which, compounds derived from oil decomposition can be formed [38-40]. The cooking that contributed the least amount of HMF and furfural to the food was boiling, being furfural not detected in many boiled foods (beans, lentils, chicken, beef, pork, lamb and tomatoes). This may be due to the temperature used for boiling (100 °C) which is a low temperature compared to other cooking temperatures, including also a dilution effect of the boiling water, which impede the reaction among carbohydrates and amino acids. Grilling was carried out at a higher temperature (250 °C), but for a very short time, including also a small amount of oil. Frying was carried out at a lower temperature (180 °C) but for a longer time, and the amount of oil used was much higher than in grilling. Finally, toasting was only carried out for 3 minutes, but at very high intensity and on foods rich in sugars. All these cooking characteristics could explain the results found.

In addition to the content of furosine, HMF and furfural, in this work we studied the correlations between the indicators of cookedness in foods with the antioxidant capacity of the same foods, once digested and fermented *in vitro*. We mainly focused on the classification according to the type of cooking applied (**Figure 50**), but correlations were also carried out taking into account the different food groups (**Table 34** in supplemental information). **Figure 50** shows that statistically significant correlations were positive: Furosine content and anti-oxidant capacity obtained from FRAP and DPPH digestion for fried foods, furosine content and antioxidant capacity obtained from Folin-Ciocalteu and FRAP digestion, and furfural content with the antioxidant capacity obtained from the Folin-Ciocalteu assay of digested foods for the roasted treatment. All these correlations could argue that aggressive treatment (such as frying and roasting) could improve the availability of some molecules to react through non-enzymatic browning

[1,40,41], while the lack of correlations in milder cooking techniques (i.e. boiling or grilling) could indicate that cellular breakdown and reactants release could be the responsible in antioxidant differences [15,22,23]. Such positive correlations have also been described in other studies. Seiquer et al., [42] reported that a diet rich in processed foods (with a high concentration of Maillard reaction products) was able to suppress lipid peroxidation and increase plasma antioxidant activity, but without modifying the antioxidant activity of enzymes (superoxide dismutase, glutathione peroxidase and catalase). In another study carried out by Manzocco et al., [43], the data on color changes due to browning reactions and their relationship with the formation of antioxidant capacity components were analyzed, finding that the Maillard reaction promotes positive variations in the antioxidant properties of foods, which are directly proportional to the development of browning. Furthermore, a linear correlation (R^2) was found with values of 0.80 - 0.99, which confirms the positive correlation between color and antioxidant activities. In another study, the antioxidant capacity of barley increased with heating intensity, in parallel with color formation [44]. The baking process of barley malt could induce the formation of water-soluble Maillard reaction products, which exerted a strong free radical scavenging capacity [45].

5. Conclusions

This study has shown that foods with a high sugar content (such as bread or some vegetables) generate more Maillard reaction compounds than foods whose composition is mainly proteical (such as eggs or lamb). Another point to consider is the cooking technique applied, in particular roasting and frying induced a stronger thermal damage and a higher development of non-enzymatic browning. The Maillard reaction produces sensory changes in the food that are considered positive (e.g. taste or smell). Thus, in this case it is important to consider the production of furosine, HMF and furfural as good indicators of cookedness, even when the organoleptic characteristics of the food have not yet changed (as in the case of furosine). As for the correlations of the indicators of cookedness with antioxidant capacity, the furosine and furfural content stood out for their statistically positive correlations with the antioxidant capacity of the samples in fried and roasted foods.

SUPPLEMENTAL INFORMATION

Table 33. Samples: foods and applied thermal processing.

Food Group	Food	Cooking Method
Cereals	Bread	Fried
Cereals	Bread	Toasted
Cereals	Penne	Boiled
Cereals	Rice	Boiled
Egg	Egg	Boiled
Egg	Egg	Grilled
Egg	Egg	Fried
Egg	Egg	Roasted
Fish	Cod fish	Boiled
Fish	Cod fish	Grilled
Fish	Cod fish	Fried
Fish	Salmon	Grilled
Fish	Salmon	Fried
Fish	Salmon	Roasted
Fruits	Apple	Grilled
Fruits	Apple	Fried
Fruits	Apple	Roasted
Fruits	Banana	Grilled
Fruits	Banana	Roasted
Legumes	Beans (Kidney)	Boiled
Legumes	Beans (Kidney)	Roasted
Legumes	Lentils	Boiled
Legumes	Lentils	Grilled
Legumes	Lentils	Roasted
Meat	Beef	Boiled
Meat	Beef	Grilled
Meat	Beef	Fried
Meat	Beef	Roasted
Meat	Chicken	Boiled
Meat	Chicken	Grilled
Meat	Chicken	Fried

Meat	Chicken	Roasted
Meat	Lamb	Boiled
Meat	Lamb	Grilled
Meat	Lamb	Fried
Meat	Lamb	Roasted
Meat	Pork	Boiled
Meat	Pork	Grilled
Meat	Pork	Fried
Meat	Potk	Roasted
Tubers	Potatoe	Boiled
Tubers	Potatoe	Grilled
Tubers	Potatoe	Frieded
Vegetables	Capsicum	Boiled
Vegetables	Capsicum	Grilled
Vegetables	Capsicum	Fried
Vegetables	Capsicum	Roasted
Vegetables	Carrot	Boiled
Vegetables	Carrot	Grilled
Vegetables	Carrot	Fried
Vegetables	Carrot	Roasted
Vegetables	Cauliflower	Boiled
Vegetables	Cauliflower	Grilled
Vegetables	Cauliflower	Fried
Vegetables	Cauliflower	Roasted
Vegetables	Onion	Boiled
Vegetables	Onion	Grilled
Vegetables	Onion	Fried
Vegetables	Onion	Roasted
Vegetables	Tomatoe	Boiled
Vegetables	Tomatoe	Fried
Vegetables	Tomatoe	Roasted

Table 34. Correlations between heat damage markers (furosine, HMF and furfural), and the results of antioxidant capacity measured with Folin-Ciocalteu, FRAP and DPPH after digestion and *in vitro* fermentation processes, depending on the differents foods groups.

CEREALS	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	1935	1405	.41	.07	.09
			[-.29, .83]	[-.59, .67]	[-.57, .68]
Folin-Ciocalteu Fermentation	18638	5744	-.49	.49	.42
			[-.85, .21]	[-.20, .86]	[-.29, .83]
FRAP Digestion	4.12	4.29	.34	-.25	-.28
			[-.37, .80]	[-.76, .45]	[-.77, .43]
FRAP Fermentation	105	17.8	-.11	.52	.37
			[-.69, .56]	[-.16, .87]	[-.34, .81]
DPPH Digestion	4.98	5.16	-.64*	-.16	-.04
			[-.91, -.02]	[-.72, .52]	[-.65, .60]
DPPH Fermentation	104	23.4	.12	-.25	-.27
			[-.55, .70]	[-.76, .45]	[-.77, .43]
EGGS	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	2542	1664	.65	-.66	.83
			[-.83, .99]	[-.99, .82]	[-.65, 1.00]
Folin-Ciocalteu Fermentation	49849	4956	.66	-.51	.36
			[-.82, .99]	[-.99, .88]	[-.92, .98]
FRAP Digestion	5.90	0.83	.41	-.63	.47
			[-.91, .98]	[-.99, .84]	[-.90, .99]
FRAP Fermentation	282	12.0	.14	.13	-.34
			[-.95, .97]	[-.95, .97]	[-.98, .92]
DPPH Digestion	9.99	0.81	-.04	.02	.77
			[-.96, .96]	[-.96, .96]	[-.74, .99]
DPPH Fermentation	235	70.6	.36	-.41	.93
			[-.92, .98]	[-.98, .91]	[-.31, 1.00]
FISH	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	2626	825	-.02	.32	-.53
			[-.82, .80]	[-.67, .90]	[-.94, .49]
Folin-Ciocalteu Fermentation	59530	22284	-.09	.13	-.36
			[-.84, .78]	[-.76, .85]	[-.91, .64]
FRAP Digestion	2.51	0.64	.43	.44	.12
			[-.59, .92]	[-.58, .92]	[-.77, .85]
FRAP Fermentation	319	130	-.09	.13	-.37
			[-.84, .78]	[-.76, .85]	[-.91, .63]
DPPH Digestion	14.2	8.95	.21	.22	.07
			[-.73, .87]	[-.72, .88]	[-.79, .83]
DPPH Fermentation	214	36.7	-.26	-.32	-.12
			[-.88, .70]	[-.90, .67]	[-.85, .76]

FRUITS	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	598	512	-.57	-.64	-.28
			[-.99, .87]	[-.99, .84]	[-.98, .93]
Folin-Ciocalteu Fermentation	31752	8985	.64	.63	.87
			[-.83, .99]	[-.84, .99]	[-.56, 1.00]
FRAP Digestion	5.81	3.81	-.55	-.20	.24
			[-.99, .87]	[-.97, .94]	[-.94, .98]
FRAP Fermentation	126	39.2	-.09	-.52	-.96*
			[-.97, .95]	[-.99, .88]	[-1.00, .04]
DPPH Digestion	22.2	33.5	-.66	-.63	-.43
			[-.99, .82]	[-.99, .84]	[-.98, .90]
DPPH Fermentation	144	24.3	-.00	-.99**	-.53
			[-.96, .96]	[-1.00, -.69]	[-.99, .88]
LEGUMES	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	1803	1532	.46	.21	-.33
			[-.71, .95]	[-.83, .92]	[-.94, .78]
Folin-Ciocalteu Fermentation	43519	19159	.77	-.23	-.21
			[-.36, .98]	[-.92, .82]	[-.92, .83]
FRAP Digestion	6.95	4.52	-.39	.17	-.89*
			[-.95, .75]	[-.84, .91]	[-.99, -.05]
FRAP Fermentation	240	101	.72	-.16	-.33
			[-.44, .98]	[-.91, .84]	[-.94, .78]
DPPH Digestion	38.7	5.21	.06	-.17	-.78
			[-.87, .89]	[-.91, .84]	[-.98, .34]
DPPH Fermentation	187	56.3	-.84	-.01	-.70
			[-.99, .16]	[-.88, .88]	[-.98, .48]
MEAT	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	2616	998	.00	-.02	-.10
			[-.51, .52]	[-.53, .50]	[-.59, .43]
Folin-Ciocalteu Fermentation	49297	13682	-.05	-.12	-.12
			[-.55, .48]	[-.60, .42]	[-.60, .42]
FRAP Digestion	3.05	0.68	-.58*	.06	-.00
			[-.84, -.10]	[-.47, .56]	[-.51, .51]
FRAP Fermentation	278	73.7	-.17	-.23	-.18
			[-.63, .37]	[-.67, .32]	[-.64, .36]
DPPH Digestion	14.6	6.10	.48	.31	.22
			[-.04, .80]	[-.24, .71]	[-.33, .66]
DPPH Fermentation	481.7	288	-.42	-.38	-.18
			[-.77, .12]	[-.75, .16]	[-.63, .37]

VEGETABLES	M	SD	FUROSINE	HMF	FURFURAL
Folin-Ciocalteu Digestion	461	394	.80**	.80**	-.02
			[.52, .92]	[.53, .93]	[-.49, .47]
Folin-Ciocalteu Fermentation	27727	13643	-.08	-.15	-.22
			[-.54, .42]	[-.59, .36]	[-.64, .29]
FRAP Digestion	5.20	5.70	.80**	.74**	-.04
			[.52, .92]	[.40, .90]	[-.51, .45]
FRAP Fermentation	149	66.0	-.04	-.08	-.14
			[-.51, .45]	[-.54, .42]	[-.58, .36]
DPPH Digestion	17.4	20.3	.84**	.75**	-.03
			[.60, .94]	[.42, .90]	[-.50, .46]
DPPH Fermentation	136.3	51.4	-.22	-.16	-.08
			[-.63, .29]	[-.60, .34]	[-.54, .42]

Note. SD is used to represent standard deviation. Values in square brackets indicate the 95% confidence interval for each correlation. The confidence interval is a plausible range of population correlations that could have caused the sample correlation. * indicates $p < .05$, ** indicates $p < .01$.

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Conflicts of Interest

The authors declare no conflict of interest.

References

1. Delgado-Andrade, C.; Seiquer, I.; Haro, A.; Castellano, R.; Navarro, M.P. Development of the Maillard Reaction in Foods Cooked by Different Techniques. Intake of Maillard-Derived Compounds. Food Chem. 2010, 122, 145-153, doi:10.1016/j.foodchem.2010.02.031.

2. Ramírez-Anaya, J. del P.; Samaniego-Sánchez, C.; Castañeda-Saucedo, Ma. C.; Villalón-Mir, M.; de la Serrana, H.L.-G. Phe-nols and the Antioxidant Capacity of Mediterranean Vegetables Prepared with Extra Virgin Olive Oil Using Different Do-mestic Cooking Techniques. Food Chem. 2015, 188, 430-438, doi:10.1016/j.foodchem.2015.04.124.

3. Rufián-Henares, J.A.; Guerra-Hernández, E.; García-Villanova, B. Maillard reaction in enteral formula processing: furosine, loss of o-phthaldialdehyde reactivity, and fluorescence. Food Res. Int. 2002, 35, 527-533, doi: 10.1016/S0963-9969(01)00152-1.

4. Rizzi, G.P. Chemical Structure of Colored Maillard Reaction Products. Food Rev. Int. 1997, 13, 1-28, doi:10.1080/87559129709541096.

5. Rufián-Henares, J.A.; Guerra-Hernández, E.; García-Villanova, B. Colour measurement as indicator for controlling the man-ufacture and storage of enteral formulas. Food Control 2006, 17, 489-493, doi: 10.1016/j.foodcont.2005.02.011.

6. Baltes, W. Chemical Changes in Food by the Maillard Reaction. Food Chem. 1982, 9, 59-73, doi:10.1016/0308-8146(82)90069-3.

7. Baxter, J.H. Free Amino Acid Stability in Reducing Sugar Systems. J. Food Sci. 1995, 60, 405-408, doi:10.1111/j.1365-2621.1995.tb05682.x.

8. Finot, P.A.; Magnenat, E. Metabolic Transit of Early and Advanced Maillard Products. Prog. Food Nutr. Sci. 1981, 5, 193-207.

9. Pigman, W.; Johnson, K.C. Reactions of Carbohydrates with Nitrogenous Substances.1 III. The Tetraacetates of N-D-Glucosylaniline. J. Am. Chem. Soc. 1953, 75, 3464-3466, doi:10.1021/ja01110a047.

10. Rufián-Henares, J.A.; García-Villanova, B.; Guerra-Hernández, E. Generation of furosine and color in infant/enteral formu-la-resembling systems. J Agric Food Chem. 2004, 52, 5354-5358, doi: 10.1021/jf040088q.

11. Finot, P.A.; Bricout, J.; Viani, R.; Mauron, J. Identification of a New Lysine Derivative Obtained upon Acid Hydrolysis of Heated Milk. Experientia 1968, 24, 1097-1099, doi:10.1007/BF02147778.

12. Heyns, K.; Heukeshoven, J.; Brose, K.H. Degradation of Fructose Amino Acids to N-(2-Furoylmethyl)Amino Acids. Interme-diates in Browning Reactions. Angew. Chem. Int. Ed Engl. 1968, 7, 628-629, doi:10.1002/anie.196806281.

13. Lee, H. s.; Nagy, S. Quality Changes and Nonenzymic Browning Intermediates in Grapefruit Juice During Storage. J. Food Sci. 1988, 53, 168-172, doi:10.1111/j.1365-2621.1988.tb10201.x.

14. Rufián-Henares, J.A.; García-Villanova, B.; Guerra-Hernández, E. Occurrence of Furosine and Hydroxymethylfurfural as Markers of Thermal Damage in Dehydrated Vegetable. Eur. Food Res. Technol. 2008, 228, 249-256, doi:10.1007/s00217-008-0929-4.

15. Rufián Henares, J.; Guerra-Hernández, E.; Garcia-Villanova, B. Effect of Red Sweet Pepper Dehydration Conditions on Maillard Reaction, Ascorbic Acid and Antioxidant Activity. J. Food Eng. 2013, 118, 150-156, doi:10.1016/j.jfoodeng.2013.03.034.

16. Tressl, R.; Wondrak, G.T.; Garbe, L.A.; Krüger, R.P.; Rewicki, D. Pentoses and Hexoses as Sources of New Melanoidin-like Maillard Polymers. J. Agric. Food Chem. 1998, 46, 1765-1776, doi:10.1021/jf970973r.

17. Plaza, M.; Amigo-Benavent, M.; Castillo, M.D. del; Ibáñez, E.; Herrero, M. Facts about the Formation of New antioxidants in Natural Samples after Subcritical Water Extraction. Food Res. Int. 2010, 43, 2341-2348, doi:10.13039/100012818.

18. Yilmaz, Y.; Toledo, R. Antioxidant Activity of Water-Soluble Maillard Reaction Products. Food Chem. 2005, 93, 273-278, doi:10.1016/j.foodchem.2004.09.043.

19. Chen, X.-M.; Kitts, D.D. Correlating Changes That Occur in Chemical Properties with the Generation of Antioxidant Capacity in Different Sugar-Amino Acid Maillard Reaction Models. J. Food Sci. 2011, 76, C831-C837, doi:10.1111/j.1750-3841.2011.02215.x.

20. Morales, F.J.; Babbel, B.B. Antiradical efficiency of Maillard reaction mixtures in a hydrophilic media. J. Agric. Food Chem. 2002, 50, 2788-2792, doi: 10.1021/jf011449u.

21. Plengmuankhae, W.; Tantitadapitak, C. Low temperature and water dehydration increase the levels of asiaticoside and madecassoside in Centella asiatica (L.) Urban. South Afric. J. Bot. 2015, 97, 196-203, doi: 10.1016/j.sajb.2015.01.013.

22. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to In Vitro Digestion-Fermentation. Antioxidants 2020, 9, 1312, doi:10.3390/antiox9121312.

23. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Foods of Animal Origin Submitted to In Vitro Digestion-Fermentation. Antioxidants 2021, 10, 445, doi: 10.3390/antiox10030445.

24. Delgado-Andrade, C.; Rufián-Henares, J.A.; Morales, F.J. Lysine availability is diminished in commercial fibre-enriched breakfast cereals. Food Chem. 2007, 100, 725-731, doi: 10.1016/j.foodchem.2005.10.031.

25. Hinojosa-Nogueira D, Pérez-Burillo S, Navajas-Porras B, Ortiz-Viso B, de la Cueva SP, Lauria F, Fatouros A, Priftis KN, González-Vigil V, Rufián-Henares JÁ. Development of an Unified Food Composition Database for the European Project "Stance4Health". Nutrients. 2021, 13, 4206, doi: 10.3390/nu13124206.

26. Pérez-Burillo, S.; Rufián-Henares, J.A.; Pastoriza, S. Towards an Improved Global Antioxidant Response Method (GAR+): Physiological-Resembling in Vitro Digestion-Fermentation Method. Food Chem. 2018, 239, 1253-1262, doi:10.1016/j.foodchem.2017.07.024.

27. Pérez-Burillo, S.; Molino, S.; Navajas-Porras, B.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; López-Maldonado, A.; Pasto-riza, S.; Rufián-Henares, J.Á. An In Vitro Batch Fermentation Protocol for Studying the Contribution of Food to Gut Microbi-ota Composition and Functionality. Nat. Protoc. 2021, 16, 3186-3209, doi:10.1038/s4º596-021-00537-x.

28. Pastoriza, S.; Delgado-Andrade, C.; Haro, A.; Rufián-Henares, J.A. A Physiologic Approach to Test the Global Antioxidant Response of Foods. The GAR Method. Food Chem. 2011, 129, 1926-1932, doi:10.1016/j.foodchem.2011.06.009.

29. Bedinghaus, A.J.; Ockerman, H.W. Antioxidative Maillard Reaction Products from Reducing Sugars and Free Amino Acids in Cooked Ground Pork Patties. J. Food Sci. 1995, 60, 992-995, doi:10.1111/j.1365-2621.1995.tb06277.x.

30. Somoza, V. Five Years of Research on Health Risks and Benefits of Maillard Reaction Products: An Update. Mol. Nutr. Food Res. 2005, 49, 663-672, doi:10.1002/mnfr.200500034.

31. Friedman, M. Food Browning and Its Prevention: An Overview. J. Agric. Food Chem. 1996, 44, 631-653, doi:10.1021/jf950394r.

32. Förster, A.; Kühne, Y.; Henle, T. omas Studies on Absorption and Elimination of Dietary Maillard Reaction Products. Ann. N. Y. Acad. Sci. 2005, 1043, 474-481, doi:10.1196/annals.1333.054.

33. Yamagishi, S.; Nakamura, K.; Inoue, H. Possible Participation of Advanced Glycation End Products in the Pathogenesis of Osteoporosis in Diabetic Patients. Med. Hypotheses 2005, 65, 1013-1015, doi:10.1016/j.mehy.2005.07.017.

34. del Castillo, M.D.; Corzo, N.; Polo, M.C.; Pueyo, E.; Olano, A. Changes in the Amino Acid Composition of Dehydrated Orange Juice during Accelerated Nonenzymatic Browning. J. Agric. Food Chem. 1998, 46, 277-280, doi:10.1021/jf9705882.

35. Rufían-Henares, J.A.; de la Cueva, S.P. Assessment of Hydroxymethylfurfural Intake in the Spanish Diet. Food Addit. Contam. Part Chem. Anal. Control Expo. Risk Assess. 2008, 25, 1306-1312, doi:10.1080/02652030802163406.

36. Pérez-Burillo, S.; Pastoriza, S.; Jiménez-Hernández, N.; D'Auria, G.; Francino, M.P.; Rufián-Henares, J.A. Effect of Food Thermal Processing on the Composition of the Gut Microbiota. J. Agric. Food Chem. 2018, 66, 11500-11509, doi:10.1021/acs.jafc.8b04077.

37. Hidalgo, F.J.; Zamora, R. Characterization of the Products Formed during Microwave Irradiation of the Nonenzymatic Browning Lysine/CE)-4,5-Epoxy-(E)-2-Heptenal Model System. J. Agric. Food Chem. 1995, 43, 1023-1028, doi:10.1021/jf00052a033.

38. Chao, P.; Hsu, C.; Yin, M. Analysis of Glycative Products in Sauces and Sauce-Treated Foods. Food Chem. 2009, 113, 262-266, doi:10.1016/j.foodchem.2008.06.076.

39. Foerster, A.; Henle, T. Glycation in Food and Metabolic Transit of Dietary AGEs (Advanced Glycation End-Products): Stud-ies on the Urinary Excretion of Pyrraline. Biochem. Soc. Trans. 2003, 31, 1383-1385, doi:10.1042/bst0311383.

40. Carvalho, D.O.; Correia, E.; Lopes, L.; Guido, L.F. Further Insights into the Role of Melanoidins on the Antioxidant Potential of Barley Malt. Food Chem. 2014, 160, 127-133, doi:10.1016/j.foodchem.2014.03.074.

41. Martín, M.Á.; Ramos, S.; Mateos, R.; Rufián-Henares, J.A.; Morales, F.J.; Bravo, L.; Goya, L. Biscuit Melanoidins of Different Molecular Masses Protect Human HepG2 Cells against Oxidative Stress. J. Agric. Food Chem. 2009, 57, 7250-7258, doi:10.1021/jf9006032.

42. Seiquer, I.; Ruiz-Roca, B.; Mesías, M.; Muñoz-Hoyos, A.; Galdó, G.; Ochoa, J.J.; Navarro, M.P. The Antioxidant Effect of a Diet Rich in Maillard Reaction Products Is Attenuated after Consumption by Healthy Male Adolescents. In Vitro and in Vivo Comparative Study. J. Sci. Food Agric. 2008, 88, 1245-1252, doi:10.1002/jsfa.3213.

43. Manzocco, L.; Calligaris, S.; Mastrocola, D.; Nicoli, M.C.; Lerici, C.R. Review of Non-Enzymatic Browning and Antioxidant Capacity in Processed Foods. Trends Food Sci. Technol. 2000, 11, 340-346, doi:10.1016/S0924-2244(01)00014-0.

44. Samaras, T.S.; Camburn, P.A.; Chandra, S.X.; Gordon, M.H.; Ames, J.M. Antioxidant Properties of Kilned and Roasted Malts. J. Agric. Food Chem. 2005, 53, 8068-8074, doi:10.1021/jf051410f.

45. Papetti, A.; Daglia, M.; Aceti, C.; Quaglia, M.; Gregotti, C.; Gazzani, G. Isolation of an in Vitro and Ex Vivo Antiradical Mel-anoidin from Roasted Barley. J. Agric. Food Chem. 2006, 54, 1209-1216, doi:10.1021/jf058133x.

DISCUSIÓN INTEGRADORA

En la presente tesis doctoral se ha estudiado la funcionalidad de diferentes tipos de microbiota intestinal en relación a la capacidad antioxidante. Además, se ha examinado el daño térmico en alimentos cocinados con diferentes procesados térmicos. Llegados a este punto, es conveniente hacer una discusión que integre todos los resultados obtenidos de esta investigación.

1. Ensayos con microbiota intestinal de adultos y niños sanos: diferencias en los valores de capacidad antioxidante total

La microbiota intestinal se compone en un 90% de *Firmicutes* y *Bacteroidetes*. Con la introducción de la alimentación sólida en edad preescolar aumentan estos filos de bacterias, que son dominantes para toda la vida. Se puede afirmar que la microbiota intestinal de niños preescolares y escolares es semejante a la de los adultos en términos de composición global [1-4].

En esta tesis doctoral se ha estudiado la funcionalidad de diferentes tipos de microbiotas intestinales, tanto de personas de edad adulta sin patologías previas, como en escolares [5-8]. En este punto de la discusión integradora se van a estudiar las diferencias entre la capacidad antioxidante obtenida tras fermentar alimentos con microbiota de adulto sano y de niño escolar sano, ya que las diferencias entre los diferentes grupos de escolares se estudiaron previamente teniendo en cuenta el consumo de capacidad antioxidante diario en cada grupo [8].

En la **Tabla 35** se pueden ver las diferencias significativas que se produjeron teniendo en cuenta los distintos grupos de alimentos tras su

digestión y fermentación *in vitro* con microbiota de adulto sano y niño sano. Para ello se realizó un análisis de la varianza (ANOVA), seguido de la prueba de Duncan para comparar las medias que mostraban una variación significativa ($p < 0.05$).

Tabla 35. Diferencias significativas entre la capacidad antioxidante total obtenida de la digestión y fermentación *in vitro* de alimentos con microbiota intestinal de adultos y niños sanos en los distintos grupos de alimentos. Las diferencias significativas entre los grupos de alimentos ($p < 0.05$) están marcadas con un círculo negro.

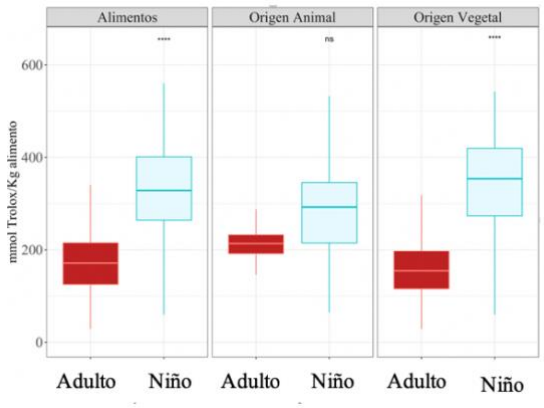
Grupos de alimentos	**Capacidad antioxidante total**	**DPPH**	**FRAP**	**Folin-Ciocalteu**
Cereales	Adulto vs niño	●		●
Frutas	Adulto vs niño	●		
Legumbres	Adulto vs niño			
Frutos secos	Adulto vs niño	●		
Aceites	Adulto vs niño			
Tubérculos	Adulto vs niño	●		
Hortalizas	Adulto vs niño	●		●
Lácteos	Adulto vs niño			
Huevos	Adulto vs niño		●	●
Carnes	Adulto vs niño	●	●	●
Pescados	Adulto vs niño	●		

En el método DPPH hubo diferencias significativas para casi todos los grupos de alimentos. Sin embargo, en el método FRAP, sólo se encontraron diferencias para los grupos de huevos y carnes. Por último, para el método de Folin-Ciocalteu se encontrarion diferencias para los grupos de cereales,

hortalizas, huevos y carnes. Por lo tanto, se entiende que ambas microbiotas intestinales se comportaron de forma diferente tras fermentar el mismo alimento una vez digerido.

A continuación, en la **Figura 51**, se pueden observar las diferencias de forma más gráfica, viendo así que tipo de microbiota fue capaz de liberar más compuestos con capacidad antioxidante dependiendo del origen de los alimentos. La **figura 51A** representa las diferencias para el método DPPH, **51B**, para FRAP y, **51C** para Folin-Ciocalteu.

A



(Figura 51 continuación)

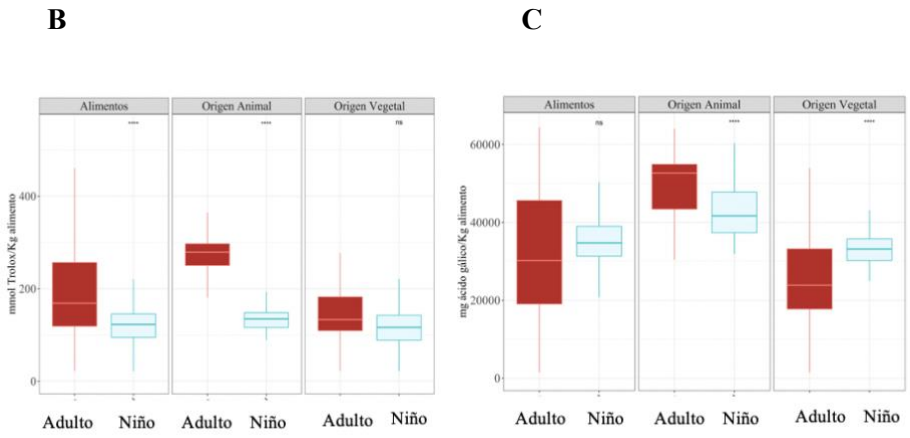


Figura 51. Capacidad antioxidante obtenida tras la digestión y fermentación de alimentos *in vitro*, con microbiota de adulto sano y niño sano. (**51A**) DPPH; (**51B**) FRAP; (**51C**) Folin-Ciocalteu. El análisis estadístico se realizó mediante la prueba de t de Student. Las comparaciones se realizaron utilizando "adulto" como grupo de referencia. Etiquetas estadísticas: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Para el método DPPH (**Figura 51A**) hubo diferencias significativas si se tienen en cuenta todos los grupos de alimentos, así como aquellos de origen vegetal. La microbiota intestinal de niños delgados fue capaz de generar más compuestos con capacidad antioxidante de forma estadísticamente significativa que la microbiota intestinal de adultos. Sin embargo, para el método FRAP (**Figura 51B**) no ocurrió lo mismo, dándose diferencias significativas para los alimentos de origen animal y todos los alimentos de forma general, y en este caso fue la microbiota intestinal de los adultos la que generó más compuestos antioxidantes de forma significativa. Por último, para Folin-Ciocalteu (**Figura 51C**) no hubo diferencias significativas si se consideraban todos los alimentos en general, pero sí las hubo cuando se consideraron por separado aquellos de origen vegetal y animal. La microbiota de los adultos fue capaz de generar más compuestos antioxidantes de aquellos

alimentos de origen animal, mientras que en el grupo de alimentos de origen vegetal ocurrió lo contrario.

Con los resultados expuestos se puede llegar a la conclusión de que ambas microbiotas tienen una funcionalidad diferente. Esto coincide con publicaciones anteriores [9,10] en las que se ha visto que la microbiota de niños sanos tienen diferencias funcionales con respecto a la de adultos sanos. Además, la microbiota intestinal en escolares es más maleable a los factores ambientales que la de los adultos. Hollister et al., [10] encontraron que escolares de entre 7 y 12 años poseen una microbiota similar a la adulta, taxones y genes funcionales similares, pero funcionalidad de la misma variaba significativamente. Es decir, desde la perspectiva funcional se encontraron diferencias significativas con respecto a la abundancia relativa de genes implicados en la síntesis de vitaminas, degradación de aminoácidos, etc.

Otro factor a tener en cuenta son las diferencias en el estilo de vida, que influyen en la composición de la microbiota intestinal [9]. Aquí es importante destacar que los adultos eran españoles, mientras que los niños que donaron las heces para los ensayos eran griegos, pudiendo ser esta una de las causas de las diferencias entre la funcionalidad de ambas microbiotas. Ringen-Kulka et al., [11] también estudiaron las diferencias entre las microbiotas intestinales entre adultos y niños de la misma zona geográfica y llegaron a la conclusión de que, incluso viviendo en el mismo lugar, había diferencias entre ambas.

2. Correlaciones entre marcadores de daño térmico y valores de capacidad antioxidante en ensayos con microbiota intestinal de niños

A lo largo de esta tesis doctoral se ha hablado en varias ocasiones de la reacción de Maillard, producida en los alimentos tras su procesado térmico. Los productos hidrosolubles del pardeamiento químico generados durante el tratamiento térmico llevan a cabo la recaptación de radicales [12]. Dicha capacidad antioxidante es proporcional al grado de pardeamiento [13] y tiene una estrecha correlación con los compuestos generados a partir de las etapas intermedias y tardías, así como con el tipo de azúcar implicado en la reacción [14]. A pesar de la pérdida parcial de compuestos naturales con actividad antioxidante que puede producirse durante el procesado de los alimentos, las propiedades antioxidantes podrían mantenerse e incluso aumentar debido a la formación de nuevos compuestos.

En el último capítulo de esta tesis doctoral se muestran las correlaciones de marcadores de daño térmico, como son furosina, HMF y furfural, con la capacidad antioxidante obtenida de digerir y fermentar, con microbiota de adulto sano, aquellos alimentos cocinados que se sometieron al análisis de estos marcadores de daño térmico. En esta ocasión, se realizarán las mismas correlaciones, pero esta vez con la capacidad antioxidante total obtenida de fermentar dichos alimentos con microbiota intestinal de niños sanos, obesos, celíacos y con alergia a las proteínas de la leche de vaca.

En la **Tabla 36** se pueden observar las correlaciones entre los marcadores de daño térmico y los resultados de tres métodos de capacidad antioxidante, DPPH, FRAP y Folin-Ciocalteu pertenecientes a las muestras con microbiota intestinal de los diferentes grupos de escolares.

Tabla 36. Correlaciones entre los marcadores de daño térmico (furosina, HMF y furfural) y la capacidad antioxidante medida en alimentos tras su digestión y fermentación *in vitro* mediante Folin-Ciocalteu (mg de ácido gálico equivalente/kg de alimento), FRAP (mmol de Trolox equivalente/kg de alimento) y DPPH (mmol de Trolox equivalente/kg de alimento) en función del tipo de microbiota usada para la fermentación *in vitro*.

Niño sano			
Ensayo	**Furosina**	**HMF**	**Furfural**
DPPH	.27*	.16	.15
	[.03, .49]	[-.09, .39]	[-.10, .38]
FRAP	.20	.20	-.00
	[-.05, .43]	[-.05, .43]	[-.25, .25]
Folin-Ciocalteu	-.02	-.01	-.27*
	[-.26, .23]	[-.26, .24]	[-.49, -.03]
Niño obeso			
Ensayo	**Furosina**	**HMF**	**Furfural**
DPPH	-.12	-.24	-.25*
	[-.35, .13]	[-.46, .01]	[-.47, -.00]
FRAP	.00	.16	.17
	[-.24, .25]	[-.09, .39]	[-.08, .40]
Folin-Ciocalteu	.04	-.13	-.26*
	[-.21, .28]	[-.37, .12]	[-.48, -.01]
Niño celíaco			
Ensayo	**Furosina**	**HMF**	**Furfural**
DPPH	.03	.01	-.24
	[-.22, .27]	[-.24, .26]	[-.46, .01]
FRAP	.08	.10	-.10
	[-.17, .32]	[-.16, .34]	[-.34, .15]
Folin-Ciocalteu	-.21	-.26*	-.35**
	[-.44, .04]	[-.47, -.01]	[-.55, -.11]
Niño con alergia a las proteínas de la leche de vaca			
Ensayo	**Furosina**	**HMF**	**Furfural**
DPPH	.22	.03	-.13
	[-.03, .44]	[-.22, .28]	[-.36, .12]
FRAP	.09	.33**	.15
	[-.16, .33]	[.09, .53]	[-.10, .39]
Folin-Ciocalteu	-.08	-.20	-.10
	[-.32, .17]	[-.43, .05]	[-.34, .15]

Nota. Las correlaciones se obtuvieron mediante el método de Pearson Los valores entre corchetes indican el intervalo de confianza del 95% para cada correlación [15]. Etiquetas estadísticas: *: $p < 0,05$, **: $p < 0,01$.

Entre los diferentes grupos de niños se pueden ver algunas similitudes y diferencias en los resultados. Los grupos de niños delgados, obesos y celíacos tuvieron correlación negativa significativa para furfural y los resultados de capacidad antioxidante de Folin-Ciocalteu. En general, las correlaciones con el método de Folin-Ciocalteu fueron todas negativas, al contrario que ocurrió con los métodos DPPH y FRAP, que generalmente tuvieron correlaciones positivas. El grupo de los niños con alergia, en concreto, tuvo una correlación positiva y moderadamente significativa entre el HMF y el método FRAP. Por su parte, una de las diferencias más llamativas fue que para el grupo de los niños obesos, las correlaciones con DPPH fueron todas negativas, en concreto destaca la del furfural, que además fue significativa.

Para estudiar más a fondo las distintas correlaciones de marcadores de daño térmico con la capacidad antioxidante total obtenida en cada grupo de niños, se tuvo en cuenta el cocinado empleado, puesto que de esta variable depende la producción de furosina, HMF y furfural. En la **Figura 52** se pueden observar dichas correlaciones.

Para los alimentos hervidos las correlaciones eran en su mayoría negativas, destacando aquellas entre el HMF y el furfural con los datos de capacidad antioxidante total de Folin-Ciocalteu para el grupo de niños sanos, siendo significativas. Para los alimentos fritos se encontraron correlaciones significativas y negativas entre la furosina y el furfural con el método Folin-Ciocalteu para los resultados obtenidos de los ensayos con microbiota de niños celiacos. En aquellos alimentos cocinados a la parrilla la única correlación estadísticamente significativa fue aquella entre el contenido en furosina y la capacidad antioxidante obtenida del método Folin-Ciocalteu para el grupo de niños sanos. En los alimentos que fueron horneados hubo

correlación negativa y estadísticamente significativa entre el contenido en furfural y la capacidad antioxidante obtenida del método Folin-Ciocalteu para niño celiaco. Por último, en los alimentos tostados, se encontraron correlaciones negativas y estadísticamente significativas entre el contenido en HMF y furfural y la capacidad antioxidante obtenida del método Folin-Ciocalteu para niños sanos.

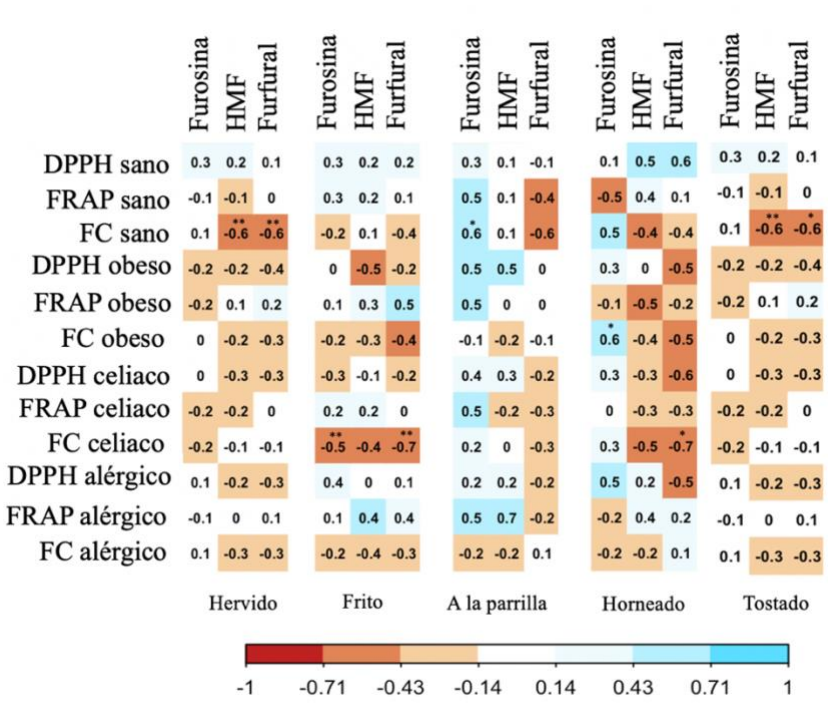


Figura 52. Correlaciones entre los marcadores de daño térmico (furosina, HMF y furfural) y la capacidad antioxidante medida en alimentos tras su digestión y fermentación *in vitro* mediante Folin-Ciocalteu (FC) (mg de ácido gálico equivalente/kg de alimento), FRAP (mmol de Trolox equivalente/kg de alimento) y DPPH (mmol de Trolox equivalente/kg de alimento) en función del tipo de microbiota usada para la fermentación *in vitro*, así como dependiendo de la técnica de cocción empleada. Etiquetas estadísticas: *: $p < 0.05$.

Destacan las correlaciones entre el método Folin-Ciocalteu para los grupos de niños sanos, celíacos y obesos por sus correlaciones significativas con el contenido en marcadores de daño térmico. Estas son en su mayoría negativas, excepto en dos ocasiones en las que se produjo correlación positiva y significativa con el contenido en furosina, concretamente con los resultados de Folin-Ciocalteu para niños delgados y obesos. En los alimentos hervidos las correlaciones negativas y significativas tienen sentido, ya que el medio de cocción es agua y los compuestos fenólicos podrían quedarse en el medio acuoso, impidiendo su digestión y posterior fermentación. Sin embargo, en alimentos tostados no hay medio de cocción acuoso, pero el daño térmico es mayor, teniendo en cuenta, además, que el único alimento sometido al tostado fue el pan (el daño térmico es mayor por su composición). En este caso aquellos compuestos fenólicos que pudieran estar presentes podrían ser destruidos por el tratamiento térmico. Lo mismo ocurre con alimentos fritos. En cuanto a las correlaciones positivas con el contenido en furosina, es importante destacar que este compuesto aparece en etapas tempranas de la reacción de Maillard, por lo que el daño térmico aún no es muy alto y sí se producen compuestos con capacidad antioxidante.

Por lo tanto, la microbiota intestinal de los diferentes grupos de niños no se comportan de la misma forma, puesto que las correlaciones no siguen un patrón común para los 4 tipos de microbiota usadas en la fermentación. Esto es normal dado que cada grupo de niños posee una patología, excepto el de los niños sanos, lo que puede llevar a un desequilibrio en la microbiota intestinal y hacer que se comporten de forma diferente ante un mismo agente [2].

Además, se encontraron diferencias con respecto a los adultos, ya que este grupo las correlaciones significativas fueron positivas. Con respecto a

esto, cabe destacar que, como se dijo en el punto anterior, aunque la microbiota intestinal a partir de los tres años ya es muy parecida a la de los adultos [2,16], hay diferencias funcionales [1,3,4,9], es decir, son parecidas a nivel global pero no están aún del todo desarrolladas.

Referencias bibliográficas

1. Reigstad, C.S.; Salmonson, C.E.; Rainey, J.F., III; Szurszewski, J.H.; Linden, D.R.; Sonnenburg, J.L.; Farrugia, G.; Kashyap, P.C. Gut Microbes Promote Colonic Serotonin Production through an Effect of Short-Chain Fatty Acids on Enterochromaffin Cells. FASEB J. 2015, 29, 1395-1403, doi:10.1096/fj.14-259598.

2. Álvarez, J.; Fernández Real, J.M.; Guarner, F.; Gueimonde, M.; Rodríguez, J.M.; Saenz de Pipaon, M.; Sanz, Y. Microbiota intestinal y salud. Gastroenterol. Hepatol. 2021, 44, 519-535, doi:10.1016/j.gastrohep.2021.01.009.

3. Henao-Mejia, J.; Strowig, T.; Flavell, R.A. Microbiota Keep the Intestinal Clock Ticking. Cell 2013, 153, 741-743, doi:10.1016/j.cell.2013.04.043.

4. Hansen, C.H.F.; Nielsen, D.S.; Kverka, M.; Zakostelska, Z.; Klimesova, K.; Hudcovic, T.; Tlaskalova-Hogenova, H.; Hansen, A.K. Patterns of Early Gut Colonization Shape Future Immune Responses of the Host. PLoS ONE 2012, 7, doi:10.1371/journal.pone.0034043.

5. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Foods of Animal Origin Submitted to In Vitro Digestion-Fermentation. Antioxidants 2021, 10, 445, doi:10.3390/antiox10030445.

6. Navajas-Porras, B.; Pérez-Burillo, S.; Valverde-Moya, Á.J.; Hinojosa-Nogueira, D.; Pastoriza, S.; Rufián-Henares, J.Á. Effect of Cooking Methods on the Antioxidant Capacity of Plant Foods Submitted to in Vitro Digestion-Fermentation. Antioxidants 2020, 9, 1312.

7. Navajas-Porras, B.; Pérez-Burillo, S.; Hinojosa-Nogueira, D.; Douros, K.; Pastoriza, S.; Rufián-Henares, J.Á. The Gut Microbiota of Obese Children Releases Lower Antioxidant Capacity from Food than That of Lean Children. Nutrients 2022, 14, 2829, doi:10.3390/nu14142829.

8. Navajas-Porras, B.; Pérez-Burillo, S.; Hinojosa-Nogueira, D.; Douros, K.; Pastoriza, S.; Rufián-Henares, J.Á. The Intake of Antioxidant Capacity of Children Depends on Their Health Status. Nutrients 2022, 14, 3965, doi:10.3390/nu14193965.

9. Derrien, M.; Alvarez, A.-S.; de Vos, W.M. The Gut Microbiota in the First Decade of Life. Trends Microbiol. 2019, 27, 997-1010, doi:10.1016/j.tim.2019.08.001.

10. Hollister, E.B.; Riehle, K.; Luna, R.A.; Weidler, E.M.; Rubio-Gonzales, M.; Mistretta, T.-A.; Raza, S.; Doddapaneni, H.V.; Metcalf, G.A.; Muzny, D.M.; et al., Structure and Function of the Healthy Pre-Adolescent Pediatric Gut Microbiome. Microbiome 2015, 3, 36, doi:10.1186/s40168-015-0101-x.

11. Ringel-Kulka, T.; Cheng, J.; Ringel, Y.; Salojärvi, J.; Carroll, I.; Palva, A.; Vos, W.M. de; Satokari, R. Intestinal Microbiota in Healthy U.S. Young Children and Adults—A High Throughput Microarray Analysis. PLOS ONE 2013, 8, e64315, doi:10.1371/journal.pone.0064315.

12. Lee, H. s.; Nagy, S. Quality Changes and Nonenzymic Browning Intermediates in Grapefruit Juice During Storage. J. Food Sci. 1988, 53, 168-172, doi:10.1111/j.1365-2621.1988.tb10201.x.

13. Rufián-Henares, J.A.; García-Villanova, B.; Guerra-Hernández, E. Occurrence of Furosine and Hydroxymethylfurfural as Markers of Thermal Damage in Dehydrated Vegetables. Eur. Food Res. Technol. 2008, 228, 249-256, doi:10.1007/s00217-008-0929-4.

14. Rufián-Henares, J.Á.; Guerra-Hernández, E.; García-Villanova, B. Effect of Red Sweet Pepper Dehydration Conditions on Maillard Reaction, Ascorbic Acid and Antioxidant Activity. J. Food Eng. 2013, 118, 150-156, doi:10.1016/j.jfoodeng.2013.03.034.

15. Cumming, G. The New Statistics: Why and How. Psychol. Sci. 2014, 25, 7-29, doi:10.1177/0956797613504966.

16. Zhong, H.; Penders, J.; Shi, Z.; Ren, H.; Cai, K.; Fang, C.; Ding, Q.; Thijs, C.; Blaak, E.E.; Stehouwer, C.D.A.; et al., Impact of Early Events and Lifestyle on the Gut Microbiota and Metabolic Phenotypes in Young School-Age Children. Microbiome 2019, 7, 2, doi:10.1186/s40168-018-0608-z.

CONCLUSIONES

Conclusiones generales

Los resultados de la presente tesis doctoral muestran cómo la capacidad antioxidante de los alimentos aumenta considerablemente tras el proceso de fermentación *in vitro* con microbiota intestinal. Esta capacidad antioxidante dependerá del tipo de alimento, así como de las técnicas culinarias empleadas. Estos dos factores influyen sobre la capacidad antioxidante total del alimento, así como sobre la microbiota intestinal, haciendo que esta sea capaz de extraer compuestos con actividad antioxidante tras la fermentación.

Conclusiones específicas

1. La capacidad antioxidante de los alimentos tras la fermentación *in vitro* de los mismos es de tres a cuatro veces mayor que tras la digestión *in vitro*. La microbiota intestinal es capaz de liberar compuestos con capacidad antioxidante tras su acción fermentativa.

2. En los ensayos realizados con microbiota intestinal de adulto sano, la capacidad antioxidante fue mayor en alimentos cocinados. Destacaron el cacao y las legumbres por la capacidad antioxidante que poseían con respecto a los demás grupos de alimentos de origen vegetal. Sin embargo, el café y el grupo de cereales fueron los que más capacidad antioxidante aportaron a la dieta teniendo en cuenta la ingesta diaria.

3. En el grupo de alimentos de origen animal, para los ensayos realizados con microbiota intestinal de adulto sano, destacaron por su capacidad antioxidante carnes y pescados con respecto a los demás grupos de alimentos. Estos dos grupos, junto con los productos lácteos, fueron los

grupos que más capacidad antioxidante aportaron a la dieta teniendo en cuenta la ingesta diaria.

4. En general, la capacidad antioxidante de los alimentos fue mayor que la descrita por otros autores que no realizaban digestión y/o fermentación previamente. Esto es debido a que estos alimentos poseen gran cantidad de compuestos bioactivos que se liberan tras la digestión y fermentación *in vitro*.

5. En los ensayos realizados con microbiota intestinal de diferentes grupos de niños se vio que la capacidad antioxidante total de los alimentos está influenciada por la microbiota intestinal, obteniendo resultados más bajos en el grupo de niños obesos que los obtenidos en el grupo de niños sanos.

6. Tras evaluar las capacidades antioxidantes procedentes de los ensayos con microbiota de niños sanos, celíacos, obesos y con alergia a las proteínas de la leche de vaca, se vio que, en general, los cereales, las frutas y las hortalizas mostraron una mayor contribución a la ingesta diaria de antioxidantes, mientras que los tubérculos, el pescado y la carne mostraron una mayor capacidad antioxidante por tamaño de ración. Los alimentos fermentados con la materia fecal de niños sanos y alérgicos a las proteínas de la leche de vaca mostraron una mayor capacidad antioxidante, lo que podría implicar que existe un mayor papel de la microbiota intestinal en este ámbito.

7. El tostado y la fritura produjeron las mayores concentraciones de compuestos de Maillard, mientras que el hervido fue el cocinado que menos produjo. Para los resultados obtenidos de los ensayos con heces de adulto, el contenido de marcadores de daño térmico mostró una correlación positiva significativa con los datos de digestión *in vitro* en los alimentos fritos

y asados, y con la fermentación *in vitro* en los alimentos asados. Para los resultados obtenidos de los ensayos con heces de los diferentes grupos de niños, muchas de las correlaciones entre capacidad antioxidante total y marcadores de daño térmico fueron negativas, lo que muestra que hay diferencias funcionales entre las microbiotas intestinales de adultos y niños, siendo la de estos últimos menos capaz de extraer compuestos con capacidad antioxidante cuando el alimento está más procesado térmicamente.

General conclusions

The results of this doctoral thesis show that the antioxidant capacity of foods increases considerably after the *in vitro* fermentation process by the gut microbiota. This antioxidant capacity will depend on the type of food as well as on the culinary techniques used. These two factors influence the total antioxidant capacity of the food, as well as the gut microbiota, making it capable of extracting compounds with antioxidant activity after fermentation.

Specific conclusions

1. The antioxidant capacity of foods after *in vitro* fermentation is three to four times higher than after *in vitro* digestion. The gut microbiota is able to release compounds with antioxidant capacity following fermentation.

2. In the tests carried out with healthy adult gut microbiota, the antioxidant capacity was higher in cooked foods. Cocoa and legumes stood out for their antioxidant capacity compared to the other plant food groups. However, coffee and the cereal group were those that contributed most to the overall dietary antioxidant capacity, taking into account daily intake.

3. In the group of foods of animal origin, for the tests carried out with the gut microbiota of healthy adults, meat and fish stood out for their antioxidant capacity compared to the other food groups. These two groups, together with dairy products, were the groups with the highest antioxidant capacity in the diet, taking into account daily intake.

4. In general, the antioxidant capacity of the foods was higher than that reported by other authors without prior digestion and/or fermentation. This is related with the large number of bioactive compounds that are released after digestion and *in vitro* fermentation of foods.

5. In the tests carried out with the gut microbiota of different groups of children, it was observed that the total antioxidant capacity of foods is influenced by the gut microbiota, obtaining lower results from the group of obese children than those obtained by the group of healthy children.

6. After evaluating the antioxidant capacities from microbiota assays of healthy, celiac, obese and cow's milk proteins allergic children, it was found that, in general, cereals, fruits and vegetables showed a higher contribution to their daily antioxidant intake, while tubers, fish and meat showed a higher antioxidant capacity per serving size. Foods fermented with faecal matter from healthy children and those allergic to cow's milk proteins showed a higher antioxidant capacity, which could imply that there is a deeper role of the gut microbiota in this area.

7. Roasting and frying produced the highest concentrations of Maillard compounds, while boiling produced the least. For the results obtained from the adult faeces tests, the content of heat damage markers showed a significant positive correlation with *in vitro* digestion data for fried and roasted foods, and with *in vitro* fermentation for roasted foods. For the results obtained from the stool assays of the different groups of children, many of the correlations between total antioxidant capacity and heat damage markers were negative, showing that there are functional differences between the gut microbiota of adults and children, with the latter being less able to extract compounds with antioxidant capacity when food suffered a more intense heat processing.