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Departamento de Nutrición y Bromatología

Programa de doctorado en nutrición y ciencias de los alimentos

PERFIL LIPÍDICO Y PÉPTIDOS CON ACTIVIDAD BIOLÓGICA EN LA LECHE MATERNA

Tesis doctoral

SILVIA SÁNCHEZ HERNÁNDEZ

CSIC Consejo Superior de Investigaciones Científicas

CIAL Instituto de Investigación en Ciencias de la Alimentación

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Memoria presentada por:

SILVIA SÁNCHEZ HERNÁNDEZ

Para optar al Grado de

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"La vida no va de encontrarse a uno mismo, sino de crearse a uno mismo"

Silvia


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"In all mammalian species the reproductive cycle comprises both pregnancy and breastfeeding: in the absence of latter, none of these species, man included, could have survived" **Bo Vahlquist, 1981**


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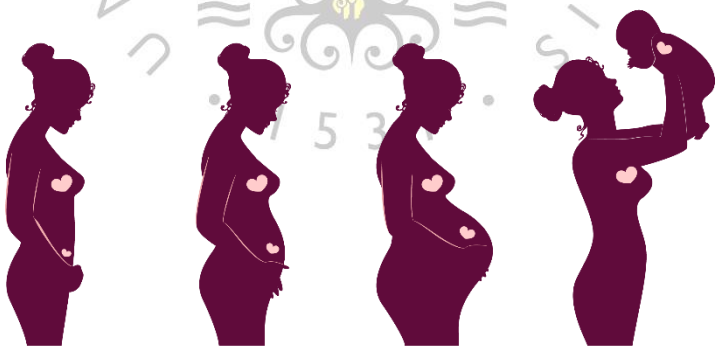


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En esta Tesis Doctoral se han realizado estudios encaminados a evaluar el estado nutricional durante el embarazo y la posible influencia tanto del índice de masa corporal de la madre como la fase de la lactancia en la composición de la leche materna. En primer lugar, se ha llevado a cabo un estudio de la composición corporal de mujeres embarazadas residentes en la provincia de Granada durante el primer trimestre de embarazo mediante el uso de medidas antropométricas. Además, se ha determinado la adherencia a la dieta mediterránea y se ha estimado la ingesta de nutrientes mediante un cuestionario de frecuencia de consumo de alimentos durante ese mismo periodo. Este estudio pone de manifiesto que las mujeres embarazadas no han alcanzado algunas recomendaciones establecidas para esta etapa de la vida. La composición de la dieta en cuanto a macronutrientes no era adecuada según las recomendaciones y también se han encontrado ingestas inadecuadas de varios micronutrientes que son relevantes durante el embarazo.

En segundo lugar, se han estudiado los principales cambios en el perfil de ácidos grasos de muestras de leche materna recogidas en diferentes momentos de la lactancia y se han comparado con distintas fórmulas infantiles. Para ello, se han determinado los ácidos grasos mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS/MS), encontrándose diferencias significativas entre las muestras de leche materna y las fórmulas infantiles, principalmente en ácidos grasos esenciales (α-linolénico) y ácidos grasos poliinsaturados de cadena larga (DHA y EPA). Además, se pone de manifiesto que la leche materna se trata de un fluido dinámico y cambiante con el tiempo.

En tercer lugar, se han llevado a cabo un estudio del perfil proteico y peptídico de la leche materna, simulando la digestión gastrointestinal en condiciones del lactante. se ha caracterizado el perfil de proteínas mediante espectrometría de masas MALDI-TOF, demostrando ser una herramienta con una alta eficacia para asociar el perfil proteico de la leche con la composición corporal de la madre. Seguidamente, se ha empleado un modelo *in vitro* para evaluar la degradación de las proteínas y la liberación de péptidos. Mediante la aplicación de técnicas como la electroforesis en gel de poliacrilamida, SDS-PAGE y la

cromatografía líquida de alta eficacia (UPLC), se ha observado que durante la fase gástrica existe una alta resistencia de las proteínas más abundantes del suero humano, α-lactolbúmina y lactoferrina al contrario de lo que ocurre con las caseínas, especialmente la β-caseína que se hidrolizan en gran medida debido a su estructura flexible. En cambio, la digestión intestinal da lugar a una rápida hidrólisis de las proteínas intactas que quedaban tras la fase gástrica. Estos datos son similares a los observados *in vivo*. Los péptidos identificados en los digeridos de la leche materna mediante cromatografía líquida acoplada a espectrometría de masas (HPLC–MS/MS) pertenecieron a veinte proteínas diferentes, encontrándose noventa secuencias habían sido identificadas previamente en digestiones de recién nacidos tras el consumo de leche materna. Las principales bioactividades asociadas a estos péptidos fueron antioxidantes, opioides, inmunomoduladoras, antimicrobianas y antibacterianas; y se identificaron con predominio de las tres proteínas más abundantes, como son la β-caseína, lactoferrina y α-lactoalbúmina, coincidiendo total o parcialmente con péptidos bioactivos descritos en la bibliografía, incluso encontrados en digeridos in vivo.

El último objetivo ha sido estudiar el perfil de compuestos fenólicos individuales en muestras de leche materna de diferentes etapas de la lactancia y fórmulas infantiles mediante un método de cromatografía líquida acoplada a espectrometría de masas (UPLC-MS/MS). Se han conseguido cuantificar 26 compuestos fenólicos (diez ácidos hidroxibenzoicos, siete ácidos hidroxicinámicos, cuatro flavonoides, tres hidroxibenzaldehídos y otros dos compuestos fenólicos), encontrándose que la leche materna contiene el doble de compuestos fenólicos individuales que las fórmulas infantiles, con una mayor proporción de ácidos hidroxibenzoicos. Algunos de estos compuestos fenólicos no habían sido descritos previamente en leche materna. Por el contrario, las fórmulas infantiles mostraron una mayor proporción de ácidos hidroxicinámicos. Por último, se ha estudiado el contenido total de compuestos fenólicos mediante el ensayo de Folin-Ciocalteu y la actividad antioxidantecon los ensayos DPPH, ABTS y FRAP. La leche materna ha demostrado tener mayor contenido en compuestos fenólicos totales, encontrando sólo pequeñas variaciones entre las

diferentes etapas de la lactancia. Sin embargo, las fórmulas infantiles han mostrado una mayor capacidad antioxidante para el ensayo DPPH y ABTS.


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LISTA DE ABREVIATURAS

AAP	Colegio americano de pediatras	*American Academy of Pediatrics*
ALA	Ácido α-linolénico	*α-linolenic acid*
ARA	Ácido araquidónico	*Arachidonic acid*
BAL	Lipasa activada por sales biliares	*Bile activated lipase*
BIOPEP	Base de datos de péptidos bioactivos	*Database of bioactive peptides*
BMI	Índice de masa corporal	*Body mass index*
DHA	Ácido docosahexaenocio	*Docosahexaenoic acid*
EPA	Ácido eicosapentanoico	*Eicosapentaenoic acid*
ESPGHAN	Sociedad Europea de Gastroenterología, Hepatología y Nutrición Pediátrica	*European Society for Pediatric Gastroenterology Hepatology and Nutrition*
FA	Ácidos grasos	*Fatty acids*
FI	Fórmulas infantiles	*Infant formulas*
GC-MS/MS	Cromatografía de gases acoplada a espectrometría de masas	*Gas Chromatography–Mass Spectrometry*
HM	Leche materna	*Human milk, breast milk*
HPLC–MS/MS	Cromatografía líquida de alta resolución acoplada a espectrometría de masas	*High-Performance Liquid Chromatography- Mass Spectrometry*
ISAK	Sociedad Internacional para el Avance de la Cineantropometría	*International Society for the Advancement of the Kinanthropometry*
LA	Ácido linoleico	*Linoleic acid*
LC-FA	Ácidos grasos de cadena larga	*Long chain FA*
MALDI-TOF	Ionización mediante láser asistida por matriz con detección de masas por tiempo de vuelo	*Matrix Assisted Laser Desorption/ Ionization- Time-Of-Flight*

MBPDB	Base de datos de péptidos bioactivos de la leche	*Milk bioactive peptide database*
MC-FA	Ácidos grasos de cadena media	*Medium chain FA*
MFGM	Membrana de glóbulo de grasa de leche	*Milk fat globule membrane*
MUFA	Ácidos grasos mono insaturados	*Monounsaturated FA*
NPN	Nitrógeno no proteico	*Non-protein nitrogen*
PUFA	Ácidos grasos poliinsaturados	*Polyunsaturated fatty acids*
RDA	Ingesta diaria recomendada	*Recommended Dietary Allowances*
SC-FA	Ácidos grasos de cadena corta	*Short chain FA*
sIgA	Inmunoglobulina A secretora	*Secretory Immunoglobulin A*
SDS-PAGE	Electroforesis en gel de poliacrilamida	*Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis*
SFA	Ácidos grasos saturados	*Saturated FA*
TAG	Triglicéridos	*Triacylglycerols, triglycerides*
UNICEF	Fondo de las Naciones Unidas para la Infancia	*United Nations Children's Fund*
UPLC	Cromatografía líquida de alta eficacia	*Ultra Performance Liquid Chromatography*
WHO	Organización Mundial de la Salud	*World Health Organization*

1. INTRODUCCIÓN


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1.1 Valoración antropométrica nutricional de la mujer embarazada. Requerimientos nutricionales

El embarazo o gestación es el período que transcurre entre la implantación en el útero del óvulo fecundado y el momento del parto. Durante la gestación tienen lugar los procesos fisiológicos de crecimiento y desarrollo del feto en el interior del útero materno. El embarazo humano dura unas 40 semanas desde el primer día de la última menstruación o 38 semanas desde la fecundación (Inatal, 2021).

La situación ideal sería aquella en la que la mujer inicia la gestación con un peso óptimo y un buen estado nutricional. En este caso las necesidades energéticas serán aquellas que le permitan satisfacer el incremento de las demandas metabólicas de la gestación, manteniendo una ganancia de peso adecuada para asegurar el crecimiento del feto, la placenta y los tejidos maternos adicionales. Por ello es fundamental mantener una composición corporal adecuada, así como preservar una reserva de energía suficiente para iniciar la lactancia materna tras el parto (FAO, 2001).

Se han propuesto varias herramientas para evaluar la composición corporal materna y los cambios que ocurren durante el embarazo. El índice de masa corporal (*body mass index*, BMI) es un indicador de utilidad en los estudios de la morbilidad y mortalidad asociados a elevados niveles de adiposidad, sin embargo, no refleja los cambios que se producen durante el embarazo, ni discrimina las proporciones del peso corporal correspondientes a músculos, huesos y tejido adiposo, y menos aún el predominio regional o topográfico de este último (Orozco-Muñoz et al., 2009). De manera similar, el uso de la bioimpedancia, aunque discrimina entre masa de tejido magro y adiposo, no proporciona una medida de la composición corporal materna que sea independiente del feto y de los tejidos de soporte (Widen & Gallagher, 2014). El método más utilizado para medir los cambios en la composición corporal materna durante el embarazo, y en particular, los cambios en la grasa subcutánea, ya que son reproducibles con un entrenamiento específico y el cumplimiento de protocolos definidos es la antropometría, como las medidas del grosor de los pliegues cutáneos y las circunferencias (Kannieappan et al.,

2013). La Sociedad Internacional para el Avance de la Cineantropometría (*International Society for the advancement of the Kinanthropometry*, ISAK), creada el 20 de Julio de 1986 en Glasgow (Escocia), propone procedimientos de medición antropométrica mejorados que son reconocidos mundialmente (Schuindt da Silva & Soares-Vieira, 2020).

La circunferencia media del brazo es un indicador sencillo, económico y con bajo error en su medición. Ésta refleja la masa corporal total, es decir tejido graso y muscular, siendo un buen indicador del estado nutricional Así mismo, se han propuesto otras mediciones como indicadores del estado nutricional materno como es la circunferencia de la pierna o pantorrilla. Ambos, también son indicadores fáciles y económicos de medir, y las variaciones durante la gestación se deben fundamentalmente a modificaciones de la grasa y del contenido de agua específico de la gestación. Los pliegues subcutáneos reflejan la distribución y la reserva de la grasa subcutánea, observándose que las medidas de ciertos pliegues cutáneos aumentan durante el embarazo (Vida-Candel et al., 2016).

Los requerimientos nutricionales maternos están aumentados en comparación con las de la mujer sana en etapa no reproductiva, para mantener el metabolismo materno y la acumulación de tejido mientras se apoya el crecimiento y desarrollo fetal (Jardí et al., 2019). Es por esto que una alimentación inadecuada durante el embarazo, tanto por una ingesta dietética deficiente en macronutrientes y micronutrientes clave, como por los excesos nutricionales pueden tener un impacto sustancial en los resultados del embarazo y la salud neonatal (Mousa et al., 2019). El sobrepeso y la obesidad en las mujeres antes y durante el embarazo son uno de los factores de riesgo más importantes para las complicaciones maternas y fetales. Valores elevados de BMI pregestacional y una elevada ganancia de peso durante el embarazo aumentan el riesgo de hipertensión inducida por el embarazo, diabetes gestacional, preeclampsia, aborto espontáneo, cesáreas y complicaciones perinatales (parto prematuro, muerte fetal o malformaciones fetales). Por el contrario, el bajo aumento de peso durante el embarazo aumenta el riesgo de bajo crecimiento fetal, aborto espontáneo o parto prematuro, así como

una reducción en el transporte placentario de nutrientes fetales (Mazurek & Bronkowska, 2020). Además, estos factores también se relacionan con un mayor riesgo de desarrollar diferentes patologías como diabetes tipo II u obesidad en la etapa adulta del descendiente (Martínez-García et al., 2020).

Existen recomendaciones específicas para diferentes tipos de nutrientes durante el embarazo. Tanto la cantidad como la composición de las proteínas son importantes en el contexto de la calidad de la dieta. La proteína está involucrada en roles biológicos tanto estructurales (queratina, colágeno) como funcionales (enzimas, transporte de proteínas, hormonas); y su calidad está determinada por su digestibilidad y capacidad para satisfacer los requerimientos de aminoácidos indispensables para el crecimiento, reparación y mantenimiento (Mousa et al., 2019). La grasa en la dieta de la mujer embarazada es importante principalmente en el contexto de la composición de ácidos grasos (*fatty acids*, FA*)*, ya que los FA omega-3 son beneficiosos para el desarrollo del cerebro y el buen funcionamiento de la retina (Danielewicz et al., 2017). Diferentes organizaciones nacionales e internacionales abogan por la mejora de la dieta, como la adhesión al patrón de dieta mediterránea, que se caracteriza por un alto contenido de frutas, verduras, aceite de oliva, legumbres, productos lácteos y frutos secos, y recomiendan un consumo mínimo de carne roja, grasas animales, azúcares y sal (Dapcich et al., 2004).

1.2 Lactancia materna. Los beneficios de la leche materna

Durante el período inicial de la vida de cada mamífero, la primera y única fuente de alimentación que satisface todas las necesidades nutricionales es la leche (Garwolińska et al., 2018). La lactancia materna es una forma inigualable de proporcionar el alimento ideal para el crecimiento y desarrollo saludable de los recién nacidos; siendo también una parte integral del proceso reproductivo con importantes implicaciones para la salud de las madres (WHO, 2002). De hecho, la leche materna (*human milk,* HM) está reconocida universalmente como el

"estándar de oro" para la nutrición de los recién nacidos durante los primeros meses de vida (Walker, 2010).

Distintos comités de nutrición encargados de proporcionar las directrices sobre la manera de alimentar a los recién nacidos y a los niños, como la Asociación Española de Pediatría, la Sociedad Europea de Gastroenterología, Hepatología y Nutrición Pediátrica (*European Society for Pediatric Gastroenterology Hepatology and Nutrition*, ESPGHAN), la Sociedad Española de Gastroenterología, Hepatología y Nutrición Pediátrica y el colegio americano de pediatras (*American Academy of Pediatrics*, AAP) (Martín-Martínez, 2005), así como organismos internacionales como la Organización Mundial de la Salud (*World Health Organization*, WHO) y el Fondo de las Naciones Unidas para la Infancia (*United Nations Children's Fund,* UNICEF), elaboraron conjuntamente el libro llamado "La Estrategia Global para la Alimentación del Lactante y el Niño Pequeño", en el que recomiendan la lactancia materna exclusiva durante los primeros seis meses de vida del bebé, seguida de una lactancia materna continuada con alimentos complementarios adecuados hasta los 2 años o más (WHO, 2003, 2017).

La lactancia materna exclusiva significa que el bebé sólo recibe HM. No se le da ningún otro líquido o sólido -ni siquiera agua-, a excepción de la solución de rehidratación oral o las gotas o jarabes de vitaminas, minerales o medicamentos (Motee & Jeewon, 2014). A pesar de estas recomendaciones, muchos países no alcanzan estos objetivos de lactancia materna, habiendo una gran variación entre países. La Figura 1 muestra la prevalencia mundial de niños que reciben HM a los 12 meses de edad.

Si valoramos las tasas de lactancia en relación con la renta económica del país, observamos notables diferencias. En los países de renta alta la duración de la lactancia materna es menor que en los de renta baja y media. La lactancia materna es uno de los pocos comportamientos positivos para la salud que está más extendido en los países de ingresos bajos y medios que en los de ingresos altos. Sin embargo, incluso en los países de ingresos bajos y medios, sólo el 37% de los

bebés menores de 6 meses son alimentados exclusivamente con HM (AEP, 2016; Victora et al., 2016).

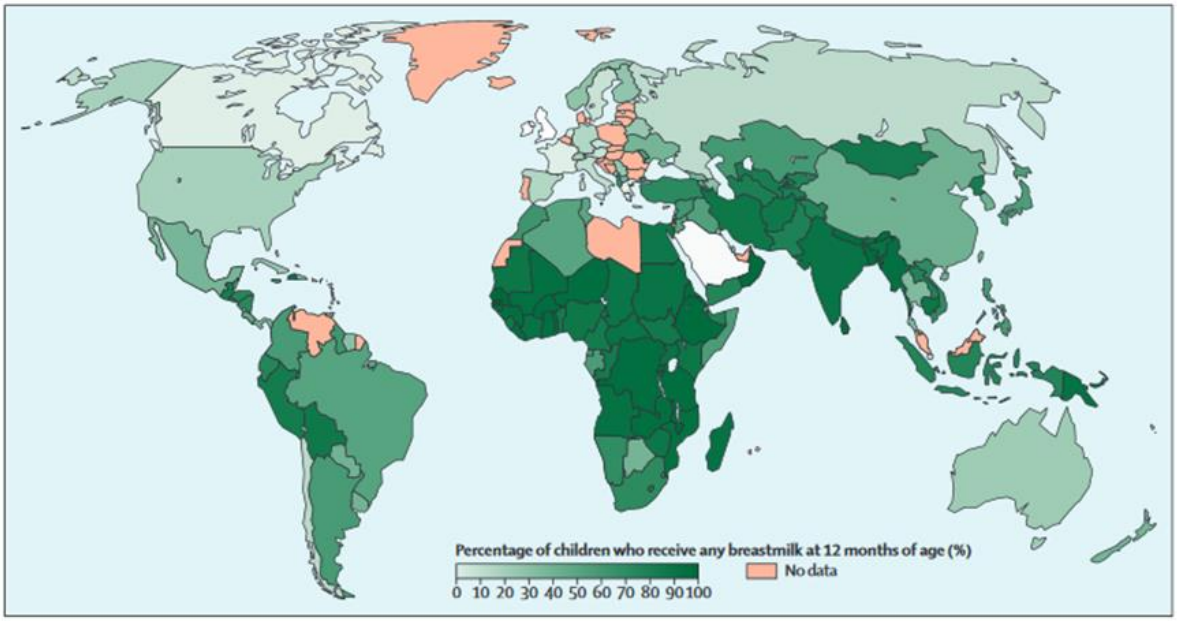


Figura 1. Distribución mundial del porcentaje de niños que reciben lactancia materna a los 12 meses de edad (Victora et al., 2016).

Como se muestra en la Figura 2, según el Instituto Nacional de Estadística, España, considerada un país de renta alta, a las 6 semanas de edad tiene una tasa de lactancia materna del 68,40%, reduciéndose en un 52,48% a los 3 meses y en un 24,72% a los 6 meses de lactancia. Si miramos por comunidades autónomas, Andalucía tiene una tasa de lactancia ligeramente inferior a la media nacional, siendo del 66,83% a las 6 semanas, del 46,88% a los 3 meses y del 20,20% a los 6 meses de lactancia (INE, 2021). Estos valores son similares a los globales europeos, pero están muy lejos de las recomendaciones de la WHO-UNICEF. Mejorar las tasas de lactancia materna en el mundo podría salvar la vida de más de 820.000 niños menores de 5 años cada año, la mayoría (87%) menores de 6 meses (UNICEF, 2017).

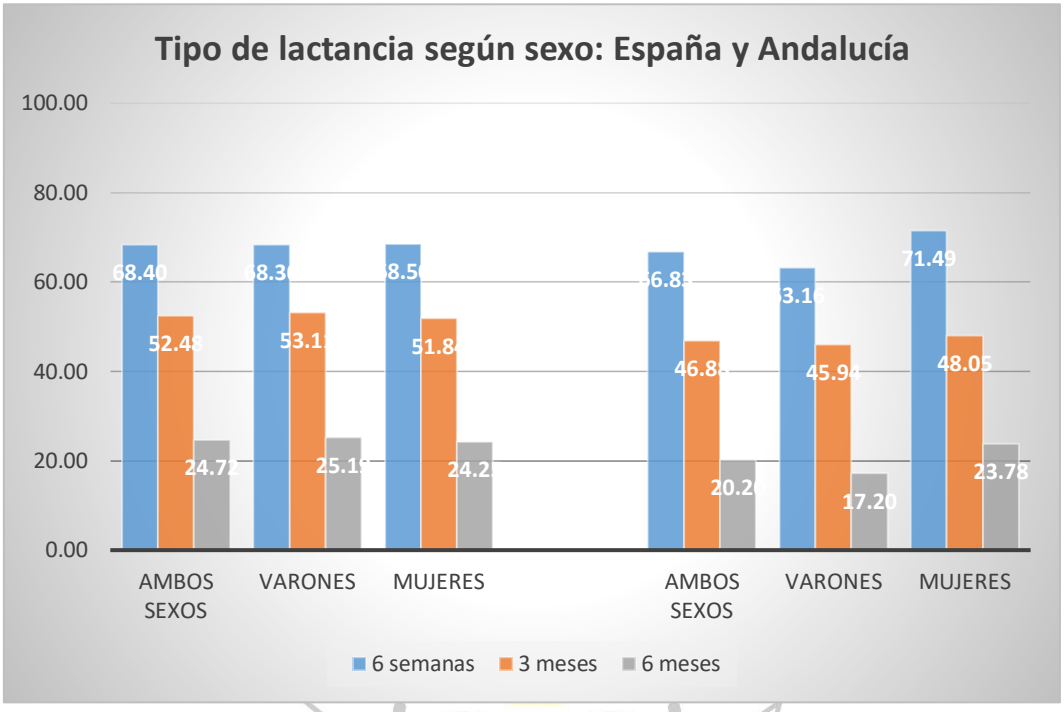


Figura 2. Tasa de lactancia (%) según sexo del bebé en España y en Andalucía a las 6 semanas, 3 meses y 6 meses de edad (INE, 2021).

Beneficios para el recién nacido y la madre

La lactancia materna ofrece una serie de beneficios a corto y largo plazo para la salud y desarrollo tanto del recién nacido como para la madre (Grote et al., 2016; Ip et al., 2007). La lactancia materna exclusiva posee un fuerte efecto protector durante los primeros meses de vida, reduciendo especialmente la morbilidad y la mortalidad por enfermedades infecciosas en la infancia, con una reducción del 88% de la mortalidad en comparación con los bebés que nunca han sido amamantados (Victora et al., 2016).

Durante los primeros 6 meses de vida, los niños alimentados exclusivamente con HM tienen un menor riesgo de desarrollar diarrea (Boone et al., 2016), infecciones de las vías respiratorias superiores (Horta & Victora, 2013b), enterocolitis necrotizante (Altobelli et al., 2020; Nolan et al., 2020), infecciones gastrointestinales (Jakaitis & Denning, 2014), infecciones de las vías

urinarias (Chamova et al., 2018; Mårild et al., 2007), otitis media aguda (Kørvel-Hanquist et al., 2017; Lodge et al., 2016) y síndrome de muerte súbita del lactante (Goldberg et al., 2018; Thompson et al., 2017), en comparación con los niños alimentados con leche artificial. También se reducen los eventos de enfermedad en general y las hospitalizaciones (Mosca & Giannì, 2017).

Por otro lado, los beneficios futuros de la lactancia materna son los que persisten, o incluso se manifiestan, tras el cese de la misma. Los principales beneficios a largo plazo de la lactancia materna son un efecto protector contra la presión arterial sistólica elevada, la leucemia infantil, la dermatitis atópica, el asma infantil, el colesterol total y un mayor rendimiento en las pruebas de inteligencia y desarrollo cognitivo que los que son amamantados durante periodos más cortos, o no son amamantados (Binns et al., 2016; Brock & Long, 2019; Peres et al., 2018). Además, cada vez hay más estudios que demuestran que la lactancia materna protege contra el sobrepeso/obesidad, con una reducción del 13%, y la diabetes de tipo 2 en la infancia y en la edad adulta (Horta et al., 2015; Horta & Victora, 2013a).

La lactancia materna también beneficia a las madres, favoreciendo la disminución de la pérdida de sangre en el postparto y la reducción de la amenorrea durante la lactancia, el cáncer de mama y de ovarios, la diabetes de tipo 2, la obesidad, la osteoporosis, las enfermedades cardiovasculares, la retención de peso y la depresión postnatal (Chowdhury et al., 2015; Hoddinott et al., 2008). Además, en el caso de tratarse de madres con un BMI elevado, la lactancia materna puede ayudar a un retorno más rápido hacia un BMI normal.

1.3 Composición de la leche materna

La HM, a diferencia de las fórmulas infantiles (*infant formulas*, FI), es un biofluido dinámico y específico del individuo, caracterizado por una gran variabilidad en su composición, tanto en lo que respecta a los componentes nutricionales como a las moléculas bioactivas, los anticuerpos, las enzimas y las

hormonas, los cuales presentan beneficios para la salud, ya que protegen contra la infección y la inflamación, contribuyendo a la maduración inmunológica y a la colonización microbiana saludable (Ballard & Morrow, 2013; Hoddinott et al., 2008).

La HM se reconoce como el único alimento capaz de satisfacer todas las necesidades del recién nacido al proporcionarle todos los macronutrientes (lípidos, proteínas e hidratos de carbono), micronutrientes (vitaminas y minerales), así como parte de las enzimas digestivas y hormonas. Además de estos nutrientes, la HM es rica en células inmunitarias y otras numerosas moléculas bioactivas, algunas de las cuales son derivadas de las proteínas y los lípidos (Martin et al., 2016).

La composición de la HM ha sido ampliamente estudiada, y se ha encontrado una marcada variación tanto interindividual como intraindividual. Es cambiante a lo largo de la lactancia, durante diferentes momentos del día, e incluso dentro de una misma tetada. Por ejemplo, la leche anterior (leche del principio de la toma) es más fina con un mayor contenido en lactosa, lo que satisface la sed del bebé, y la leche posterior (leche del final de la toma), es más cremosa y tiene un contenido mayor en energía y en grasa. De esta forma se va "adaptando" a las necesidades del niño a medida que va siendo alimentado (Saarela et al., 2007).

La HM pasa por varias etapas diferenciadas: el pre-calostro, el calostro, la leche de transición y la leche madura. El pre-calostro es el fluido biológico segregado por algunas mujeres durante el último trimestre de embarazo. El calostro es el primer líquido amarillento y cremoso producido por las madres después del parto, el cual difiere en volumen, aspecto y composición. Éste es producido aproximadamente durante los primeros 5 días postparto y en bajas cantidades (de 2 a 20 ml por toma), siendo esto suficiente para satisfacer las necesidades del recién nacido. Es rico en proteínas como la lactoferrina y especialmente en inmunoglobulinas, como la inmunoglobulina A secretora (*secretory immunoglobulin A*, sIgA), vitaminas liposolubles (E, A, K), carotenos y algunos minerales (sodio, magnesio, zinc). Sin embargo, el calostro contiene

concentraciones relativamente más bajas de lactosa, grasa, vitaminas hidrosolubles y minerales como el potasio y el calcio que la leche madura (Ballard & Morrow, 2013).

Inmediatamente después llega la leche de transición, que comparte algunas de las características del calostro, pero que representa un período de "aumento" en la producción de leche para satisfacer las necesidades nutricionales y de desarrollo del lactante en rápido crecimiento. Suele producirse entre el 5º y el 14º día postparto, pudiendo llegar a segregar hasta 660 ml/día. Se trata de una leche más calórica, conteniendo mayores niveles de grasa, lactosa y vitaminas hidrosolubles (Ballard & Morrow, 2013).

A partir de los 15 días, y entre cuatro y seis semanas después del parto, la HM se considera totalmente madura y su composición permanece relativamente constante, aunque se producen sutiles cambios en la composición de la leche a lo largo de la lactancia (Ballard & Morrow, 2013). El volumen promedio de leche madura es de 700 a 900 ml/día durante los primeros 6 meses postparto, y de 500 ml/día durante los siguientes 6 meses postparto. La Tabla 1 representa una visión general de la composición de la HM en los distintos estadíos de la lactancia.

Los componentes nutricionales de la HM provienen de tres fuentes: por síntesis en la glándula mamaria, por ingesta alimentaria y por las reservas maternas. La atención a la dieta materna es importante para ciertos nutrientes, pero en general, la calidad nutricional de la HM se conserva en gran medida (Ballard & Morrow, 2013).

Tabla 1. Composición nutricional de la leche materna en las diferentes fases de la lactancia materna.

Nutriente	Calostro	Leche de transición	Leche madura
Energía, kcal/100mL	67.1	73.5	69.0
Proteínas, g/100mL	2.3	1.6	1.0
Lactosa, g/100mL	5.3	6.4	7.2
Grasas, g/100mL	2.9	3.6	4.2
Colesterol, g/100mL	2.7	2.4	1.6

1.3.1 Lípidos

Los lípidos de la leche están presentes en forma de glóbulos de grasa láctea distribuidos homogéneamente en la fase acuosa de la leche. El tamaño medio de los glóbulos de grasa de la HM, dependiendo de la fase de lactancia, varía entre 0,1 y 15 μm. Estos glóbulos se componen de un núcleo de triglicéridos (*triacylglycerols*, TAG) rodeados de una fina membrana de grasa (*milk fat globule membrane*, MFGM). Los TAG representan entre el 98-99% de la grasa de la leche total, y las propiedades de éstos en la leche están muy influidas por su composición en FA. En menor porcentaje se encuentra la contribución de los fosfolípidos (0,5-1%), esteroles como el colesterol (0,2%) y el contenido restante de lípidos está compuesto por FA libres, diacilglicéridos y monoacilglicéridos, los cuales deben estar presentes en cantidades traza, puesto que son subproductos producto de la lipólisis (Demmelmair & Koletzko, 2018).

El contenido medio de grasa en el calostro es de 2,2 g/100 ml, aumentando a 3,0 g/100 ml en la leche de transición y 3,4 g/100 ml en la leche madura (Gidrewicz & Fenton, 2014). Los lípidos son uno de los componentes más importantes de la HM, siendo una fuente rica en nutrientes esenciales como los ácidos grasos poliinsaturados (*polyunsaturated fatty acids*, PUFA), vitaminas liposolubles y compuestos bioactivos como fosfolípidos, plasmalógenos y esfingolípidos (Keikha et al., 2017). Proporcionan una parte importante, entre el

40-55%, de la ingesta total de energía de los lactantes. Además, la grasa de la leche es portadora de sabor y aroma. Se trata del macronutriente de la leche que experimenta una mayor variación, ya que el contenido de grasa aumenta con el tiempo o la "maduración de la HM, sigue un patrón diurno, tendiendo a ser más alto durante el día e inferior durante la noche, y además varía desde el inicio hasta el final de la toma, siendo de dos a tres veces superior en la leche posterior que en la leche anterior, como se muestra en la Figura 3A/B (Khan et al., 2013). Cabe destacar que aunque la cantidad de grasa por volumen es notablemente diferente entre la leche anterior y posterior, la composición en FA de la grasa láctea total no es diferente (Hall, 1979).

Son numerosos estudios han demostrado los importantes efectos biológicos de los lípidos de la HM sobre la salud neonatal, por ejemplo, sobre la función gastrointestinal, el crecimiento adecuado del lactante, el desarrollo y la función neurocognitiva, la regulación de la inflamación y el riesgo de infección, la función inmunitaria y la reducción del riesgo de enfermedades metabólicas y cardiovasculares posteriores en la edad adulta (Koletzko, 2017).

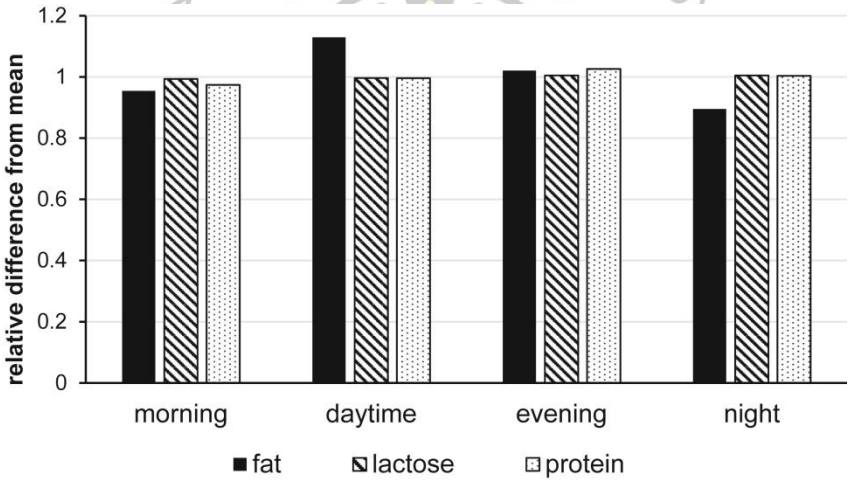


Figura 3A. Cambios que se producen en el contenido de macronutrientes (proteínas, hidratos de carbono y lípidos) en la leche materna según el momento del día (Demmelmair & Koletzko, 2018).

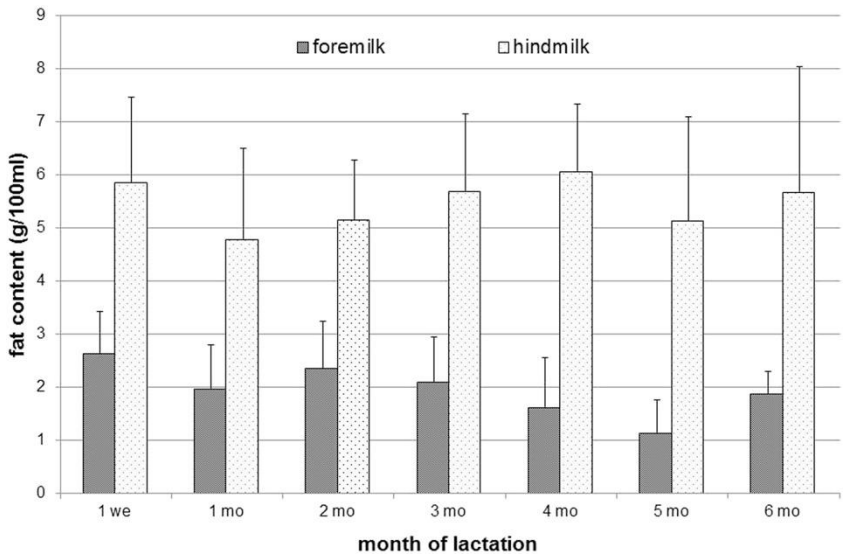


Figura 3B. Contenido en grasa de la leche anterior y posterior de la toma a lo largo de los primeros 6 meses de lactancia materna (Demmelmair & Koletzko, 2018).

1.3.1.1 Ácidos grasos

Los FA de la HM proceden de la movilización de las reservas endógenas de la madre, de la síntesis en el hígado o el tejido mamario, y de la dieta de la madre, aunque la composición de los FA no es alterada por cambios en la dieta de ésta (Siziba et al., 2019). Los FA pueden clasificarse de acuerdo con los siguientes criterios:

i. Número de átomos de carbonos:
   - FA de cadena corta (*short chain FA*, SC-FA): 2 a 10 carbonos.
   - FA de cadena media (*medium chain FA*, MC-FA*)*: 12 a 16 carbonos.
   - FA de cadena larga (*long chain FA*, LC-FA): más de 16 carbonos.

ii. Presencia y número de dobles enlaces:
   - FA saturados (*saturated FA*, SFA): no posee dobles enlaces
   - FA monoinsaturados (*monounsaturated FA*, MUFA): posee un doble enlace

- FA poliinsaturados (*poliunsaturated FA*, PUFA): posee más de un doble enlace

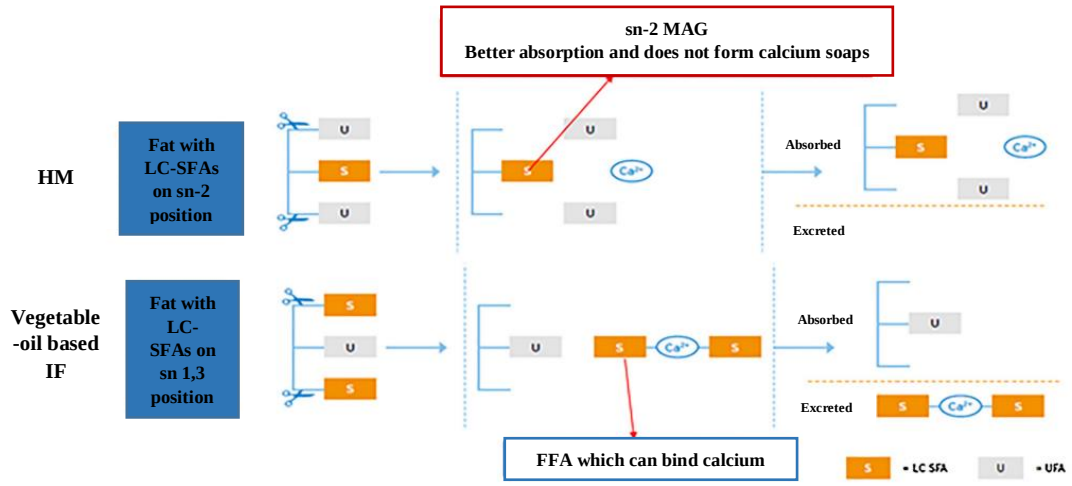
iii. Posición relativa del doble enlace respecto al átomo de carbono terminal (omega):
- ω-3/n-3 FA
- ω-6/n-6 FA

iv. Configuración geométrica:
- cis-FA
- trans-FA.

v. Fuente:
- Esencial: no sintetizado por el organismo, por lo que debe obtenerse de la dieta
- No esencial: sintetizado por el organismo.

Al contrario de lo que ocurre con la leche de vaca, los SC-FA contribuyen relativamente poco al contenido total de grasa de la HM, conteniendo principalmente FA que tienen entre 10 y 20 átomos de carbono. Casi la mitad de éstos son SFA, representando aproximadamente el 35-40% (Ramiro-Cortijo et al., 2020). El ácido palmítico (C16:0), uno de los principales SFA, proporciona aproximadamente el 25% de todos los FA de la leche, y, por lo tanto, la mayor parte del contenido total de SFA. La distribución de los FA entre la posición externa y la posición sn-2 en la molécula de glicerol modifica la tasa de captación intestinal de FA. Mientras que en la HM alrededor del 70% del ácido palmítico está esterificado en la posición β (posición sn-2) de los TAG, en los aceites vegetales y en la leche de vaca el ácido palmítico suele estar esterificado en las posiciones sn-1,3 de los TAG, como se muestra en la Figura 4 (Mehrotra et al., 2019). Estas diferencias en la estereoespecificidad del ácido palmítico dentro de la molécula de los TAG tienen influencia en la absorción de nutrientes, ya que la digestión de los TAG mediante la lipasa pancreática con el ácido palmítico en la posición sn-2 maximiza la absorción de grasa en general, ácido palmítico en particular y calcio. Por el contrario, la escisión del ácido palmítico en las posiciones sn-1 y sn-3 produce ácidos palmíticos libres que se unen al calcio en la

luz intestinal, dando lugar a la formación de jabones de calcio insolubles que posteriormente son excretados (Miles & Calder, 2017).



HM: leche materna; IF: fórmulas infantiles; LC-SFAs: ácidos grasos saturados de cadena larga; UFA: ácidos grasos insaturados; MAG: monoacilglicéridos; FFA: ácidos grasos libres; Ca: calcio.

Figura 4. Impacto en la absorción de grasa y calcio según la estereoespecificidad del ácido palmítico en los triglicéridos de la leche materna y de las fórmulas infantiles elaboradas a base de grasas vegetales. Adaptado de Mehrotra y col. (2019).

La proporción de MUFA es más estable que la de los SFA y representan alrededor del 45%-50% de la HM durante la lactancia. Aproximadamente, el 36% de estos MUFA lo constituye el ácido oleico (C18:1 n-9) y cumple una importante función en la reducción del punto de fusión de los TAG, proporcionando así la liquidez necesaria para la formación, el transporte y el metabolismo del glóbulo graso de la leche (Koletzko, 2017).

Los PUFA representan aproximadamente el 15% del total de lípidos de la HM y se han estudiado ampliamente por sus funciones biológicas de desarrollo, cardioprotectoras, anticancerígenas, antiinflamatorias y antioxidantes. Los LC-

PUFA principales cuantitativamente son el ácido dihomo-γ-linolénico (C20:3) y el ácido araquidónico (*arachidonic acid*, ARA C20:4) pertenecientes a la serie w-6 y el ácido eicosapentaenoico (*eicosapentaenoic acid*, EPA C20:5) y el ácido docosahexaenoico (*docosahexaenoic acid*, DHA C22: 6) pertenecientes a la serie w-3. La HM también contiene dos FA esenciales, el ácido linoleico (*linoleic acid*, LA C18: 2 w-6) al 15% y el ácido α-linolénico (*α-linolenic acid*, ALA C18: 3 w-3) al 0,35%. Estos dos FA esenciales se convierten, respectivamente, en AA y EPA, dando lugar este último al DHA, tal y como se muestra en la Figura 5.

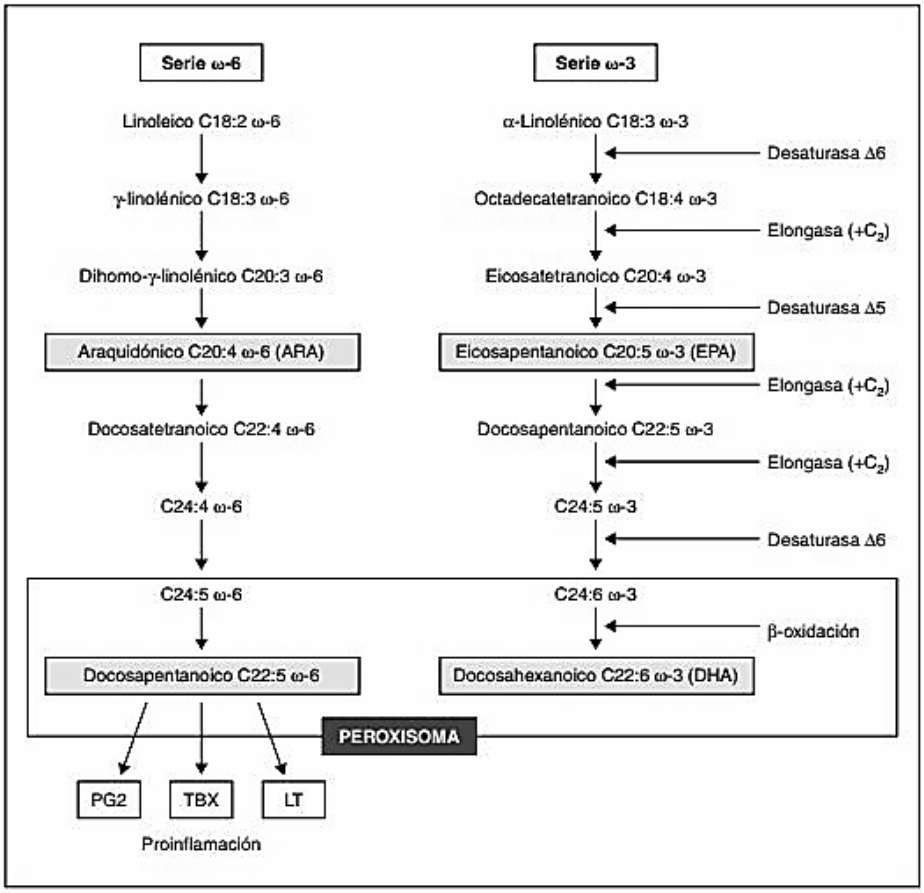


Figura 5. Biosíntesis de los ácidos grasos poliinsaturados de cadena larga (LC-PUFA) (w-6/w-3) a través de sus precursores ácido linoleico y ácido α-linolénico (Martín-Martínez, 2005).

Un aporte adecuado de FA esenciales, en particular durante los dos primeros años de vida, es vital para asegurar el desarrollo, regular el crecimiento, las respuestas inflamatorias, la función inmunológica y juegan un papel clave en la estructura y función del tejido neural, la estructura de la membrana celular, el desarrollo cognitivo y los sistemas motores en los recién nacidos (Barreiro et al., 2018; Siziba et al., 2020). Sin embargo, la síntesis de AA y DHA está limitada en el recién nacido debido a la actividad enzimática inmadura. Por este motivo, una correcta alimentación por parte de la madre es importante para el aporte de estos FA esenciales, aunque cabe destacar que se ha observado que la HM casi siempre es adecuada en nutrientes esenciales, incluso cuando su propia nutrición es inadecuada (Martin et al., 2016). Además, varios estudios no encontraron relación significativa entre el BMI y el contenido en grasa de la HM. Este hallazgo parece ser razonable con las necesidades nutricionales del lactante, pues sería totalmente contraproducente para el desarrollo del bebé que si la madre presenta acumulación de grasa corporal ésta pasara en gran cantidad en la HM afectando a su contenido, y por tanto a los requerimientos del lactante y su estado nutricional (Llorente-Romero et al., 2021).

A pesar de la gran cantidad de estudios llevados a cabo sobre la composición de FA en la HM, la mayoría de éstos han investigado las variaciones en la composición entre países y grupos demográficos. La repetición de estudios de cohortes de nacimiento realizados con varios años de diferencia, en poblaciones con algunos cambios en los componentes sociodemográficos, dietéticos y de estilo de vida, podría ser más informativa a este respecto (Siziba et al., 2020). Además, cabe destacar que estos estudios también son útiles para evaluar el avance de métodos analíticos cada vez más sensibles y precisos, como pueden ser los basados principalmente en la espectrometría de masas combinada con métodos de separación de alta resolución, que no están aún extendidos en estudios de HM.

1.3.2 Proteínas

Las proteínas de la HM proporcionan una fuente importante de aminoácidos para el crecimiento del lactante, y juegan un papel fundamental en la digestión y absorción de muchos otros componentes de la HM, como el hierro, el calcio y la vitamina B12. Otras de las funciones que se les atribuyen a las proteínas son el desarrollo de la microflora intestinal y la mejora de la función inmunológica, proporcionando una defensa contra bacterias patógenas, virus y levaduras (Hendricks & Guo, 2014; Lönnerdal, 2003). El contenido medio de proteínas disminuye rápidamente durante el primer mes de lactancia, gradualmente del segundo al sexto-séptimo mes de lactancia y se estabiliza a partir de entonces. El contenido en proteína varía de 1.4-2.0 g/100ml durante la lactancia temprana, 0.8-1.3 g/100ml durante los 3-6 meses de lactancia, a 0.7- 0.8 g/100ml después de seis meses (Guo, 2014), ya que lo requerimientos en proteínas según las ingestas diarias recomendadas (*Recommended Dietary Allowances*, RDA) desde el nacimiento hasta el quinto mes de vida son de 2.2 g/kg/día y entre el quinto mes y un año de 1.6 g/kg/día (Elango & Ball, 2016).

Las proteínas de la HM se pueden clasificar en tres grupos: mucinas, caseínas y proteínas del suero. Son las proteínas del suero las más abundantes, mientras que las caseínas se presentan en una menor proporción. Las mucinas, también conocidas como MFGM, contribuyen solo con un pequeño porcentaje del contenido total de proteínas de la HM (≈1%) (Lönnerdal, 2003). Sin embargo, son importantes desde el punto de vista biológico e incluyen la osteopontina, la lipasa activada por sales biliares (*bile activated lipase*, BAL), o la lactoperoxidasa, entre otras (Zhu & Dingess, 2019). La proporción de suero/caseína en la HM fluctúa entre el 70:30 y el 80:20 durante el inicio de la lactancia, ya que la concentración de proteína del suero es muy alta y la de caseína es prácticamente indetectable. A medida que avanza la lactancia, la síntesis de caseína en la glándula mamaria y la producción en la leche aumenta, mientras que la concentración de proteínas del suero disminuye, obteniendo una proporción del 50:50 al final de la lactancia (Guo, 2014). Esta proporción es significativamente mayor en comparación con la leche

de otros mamíferos. En la leche de vaca, las proteínas del suero representan solo el 18% de las proteínas de la leche, siendo de un 82% el contenido en caseínas (ratio 20:80) (Liao et al., 2017).

Las proteínas que componen la fracción del suero de la HM incluyen predominantemente: α-lactoalbúmina, lactoferrina, sIgA, albúmina sérica y lisozima. Más del 25% del contenido de proteína del suero de la HM está compuesta por α-lactoalbúmina. Ésta es esencial en el proceso de biosíntesis de lactosa, y, además, la generación de péptidos se cree que puede estar implicada en favorecer la absorción de cationes divalentes, aumentando así la absorción de minerales como el calcio como el zinc. Sin embargo, sólo una pequeña parte del calcio total que se encuentra en la HM se une a la α-lactoalbúmina (Hendricks & Guo, 2014).

La lactoferrina es otro de los componentes más importantes de las proteínas de la HM, constituyendo alrededor de un 10-20% de las mismas. Esta proteína se encuentra en cantidades muy elevadas en el calostro, descendiendo y manteniéndose a lo largo de la lactancia. Posee actividad antimicrobiana, antiviral y antiinflamatoria, ya que previene la propagación de bacterias potencialmente patógenas al unirse fuertemente a una proporción importante del hierro de la HM (a dos átomos de hierro por cada molécula de lactoferrina), facilitando así su absorción. Además, posee actividad inmunomodulante y anticarcinogénica (Hendricks & Guo, 2014).

Hay cinco tipos básicos de anticuerpos: inmunoglobulinas A, M, D, G y E, todos ellos presentes en la HM. De todas ellas la más abundante es la inmunoglobulina A (>90%), que se encuentra generalmente en forma de sIgA, llamada así porque está formada por dos moléculas de inmunoglobulina A unidas con un componente secretor. Estas colecciones de anticuerpos son transmitidas al lactante debido a que la madre produce anticuerpos contra los patógenos de su entorno, de manera que éste recibe la protección que más necesita contra los agentes infecciosos que es más probable encuentre. Por lo tanto, la lactancia materna proporciona inmunidad dirigida. Además, las sIgA evitan que el lactante

sufra daños ya que previenen enfermedades sin causar inflamación, a diferencia de la mayoría de los otros anticuerpos, de manera que protegen las distintas superficies mucosas del intestino (Lawrence, 2005).

La fracción de caseína está compuesta por: β-caseína, κ-caseína y α-s1-caseína, que contribuyen al patrón característico de aminoácidos de la HM, siendo altamente digeribles. Funcionalmente, su propiedad más importante es la capacidad para formar agregados estables que incluyen calcio y fósforo. Las micelas de caseína de la HM varían de 20 a 55 nm de tamaño en comparación con las de la leche de vaca, que varían de 100 a 150 nm de diámetro. La β-caseína es la caseína predominante de la HM. Se trata de una proteína altamente fosforilada cuyo contenido decrece a lo largo de la lactancia. Durante la digestión, se forman fosfopéptidos, que pueden aumentar la absorción de calcio al aumentar su solubilidad, lo que se suma a la alta biodisponibilidad del calcio en la HM. Los fosfopéptidos de caseína también pueden contribuir a la absorción de zinc y otros cationes divalentes. Por otra parte, la κ-caseína es una proteína altamente glicosilada, que proporciona defensa contra las infecciones, ya que se ha demostrado que inhibe la adherencia de *Helicobacter pylori* a la mucosa gástrica humana y de *Streptococcus pneumoniae* y *Hemophilus influenzae* a las células epiteliales del tracto respiratorio humano. También promueve el crecimiento de *Bifidobacterium bifidum*, reduciendo el crecimiento de microorganismos patógenos intestinales en lactantes.

La fracción de nitrógeno no proteico (*non-protein nitrogen*, NPN) representa una fracción importante en la HM, alrededor del 20-25% del nitrógeno total. Los componentes principales de esta fracción son la urea, ácido úrico, creatinina, creatina, pequeños péptidos, nucleótidos, nucleósidos y aminoácidos libres (Lönnerdal, 2003).

Las tecnologías proteómicas han avanzado enormemente para tener un profundo conocimiento sobre las proteínas de la HM, como es el caso del trabajo realizado para caracterizar las MFGM. Además, la cantidad de proteínas caracterizadas en el suero humano ha aumentado constantemente a lo largo de los

últimos años. Gracias al desarrollo de la proteómica, y al mejorar la espectometría de masas en tándem de cromatografía líquida 2D (LC-MS / MS), más de 300 proteínas han podido ser identificadas en la HM, muchas de las cuales son activas y desempeñan diferentes funciones en la protección del lactante (Gao et al., 2012; Liao et al., 2011). Sin embargo, existe información limitada sobre la posible influencia del estado nutricional o de salud de la madre sobre la distribución de las proteínas en la HM.

1.3.2.1 Péptidos con actividad biológica

La concentración media total de péptidos en la HM es de 11.0 μg/ml. Teniendo en cuenta que la concentración total de proteínas de la HM es de 8-10 mg/ml (10.000 μg/ml), es obvio que sólo una pequeña fracción (≈0,1%) de todas las proteínas de la HM está en forma de péptidos (Lönnerdal, 2016).

Es interesante saber que la proteólisis de la HM comienza en la glándula mamaria, de manera que parte de estos péptidos derivados de las proteínas de la HM son liberados antes de que el lactante la consuma, aunque es posible que estos péptidos se liberen sólo en cantidades mínimas (Wada & Lönnerdal, 2020). Sin embargo, y a lo largo del tracto gastrointestinal, estas proteínas se pueden fragmentar totalmente por la acción de las proteasas lácteas, gástricas, intestinales y bacterianas, y ser usadas como fuente de aminoácidos que el bebé puede absorber y utilizar para la síntesis y el crecimiento de proteínas. Algunas proteínas pueden sufrir una proteólisis parcial dando lugar a fragmentos biológicamente activos (fosfopéptidos de caseína), experimentar una proteólisis reducida como ocurre con la lactoferrina o incluso una proteólisis nula, como pasa con la sIgA, llegando a resistir a la digestión y encontrándose totalmente intactas en las heces. Los péptidos biológicamente activos se definen como fragmentos que permanecen inactivos en las secuencias de proteínas precursoras, pero cuando se liberan por la acción de enzimas proteolíticas, pueden interactuar con receptores seleccionados y regular las funciones fisiológicas del cuerpo (Beverly et al., 2020). El tamaño de

las secuencias bioactivas varía generalmente de 2 a alrededor de 20 residuos de aminoácidos, y se sabe que algunos de los péptidos tienen múltiples funciones.

Se han identificado muchos péptidos de la HM con bioactividades que pueden ser útiles para el bebé, además de la importancia nutricional que tienen, contribuyendo al alto valor biológico atribuido a la HM (Gan et al., 2019; Wada & Lönnerdal, 2020). Los efectos biológicos que se han demostrado en sistemas experimentales para estos péptidos incluyen actividades opioides, anticrobianas, antioxidantes, inmunomoduladoras, antihipertensivas, antitrombóticas y anticolesterolémicas, entre otras (Hernández-Ledesma et al., 2007; Mohanty et al., 2016). En muchos casos, los péptidos liberados tienen una bioactividad distinta y más potente que sus proteínas originales intactas. Sin embargo, la mayoría de los péptidos bioactivos se han identificado a partir de leche y productos lácteos digeridos *in vitro* (Dallas et al., 2014; Deglaire et al., 2019; Elwakiel et al., 2020; Wada & Lönnerdal, 2015), mientras que muy pocos lo han hecho en HM no digerida o *in vivo* (Beverly et al., 2019; Dallas et al., 2013; Wada et al., 2017).

Las caseínas, y en particular la β-caseína, son las proteínas más susceptibles a la proteólisis, y, por tanto, la mayor fuente de péptidos bioactivos, dando lugar a fosfopéptidos de caseína durante la digestión. La alta sensibilidad de las caseínas a las enzimas digestivas puede atribuirse a la estructura abierta y suelta de la micela de caseína (Wada & Lönnerdal, 2020). La estructura de la κ-caseína contiene un enlace peptídico muy sensible, que se escinde rápidamente durante la digestión, lo que da lugar a la formación de un glicomacropéptido. La α-lactoalbúmina es otra proteína que se digiere con relativa facilidad y no deja restos en las heces de los lactantes. Durante el paso por el estómago y la parte superior del intestino delgado, la digestión de la α-lactoalbúmina da lugar a la formación de varios péptidos pequeños, probablemente de naturaleza transitoria. Se ha demostrado *in vitro* que estos péptidos tienen actividad prebiótica, participan en la estimulación inmunitaria y mejoran la absorción de minerales (Lönnerdal, 2016).

No obstante, el número de péptidos biológicamente activos que se han descrito en las bases de datos de péptidos bioactivos, tales como la *database of*

bioactive peptides, (BIOPEP) y en la base de datos de péptidos bioactivos de la leche (*milk bioactive peptide database*, MBPDB) es limitado, ya que los péptidos bioactivos derivados de la proteína de la HM se han investigado menos que los de la leche de vaca, presumiblemente debido a la escasez de muestras disponibles (Minkiewicz et al., 2019; Nielsen, Beverly, Qu, et al., 2017).

1.3.3 Hidratos de Carbono

Los hidratos de carbono de la HM están compuestos por monosacáridos, como glucosa y galactosa; disacáridos, como lactosa y lactulosa; oligosacáridos; y algunos sacáridos más complejos, como las glicoproteínas. El contenido en hidratos de carbono es de 7 g/100ml, de los cuales el 90% es en forma de lactosa, representando aproximadamente el 7%. Ésta ayuda a la absorción de minerales, sobre todo de calcio, aunque también es la que más probabilidades tiene de contribuir a síndromes de malabsorción e intolerancia debido a alteraciones metabólicas, como la intolerancia a la lactosa, malabsorción de lactosa o galactosemia. A diferencia de las proteínas y las grasas, la cantidad de lactosa es relativamente estable, ya que su concentración es baja en el calostro, pero aumenta rápidamente a una concentración promedio de 6 g/L. El contenido de lactosa es especialmente elevado entre el cuarto y el séptimo mes de lactancia y disminuye durante los meses posteriores. Se trata del nutriente con menos probabilidades de verse afectado por la nutrición materna (Eriksen et al., 2018).

1.3.4 Micronutrientes: Vitaminas y minerales

La HM contiene cantidades adecuadas de la mayoría de las vitaminas para apoyar el crecimiento infantil normal, excepto el de vitamina D y K. Para evitar una deficiencia de vitamina D, una mineralización ósea inadecuada y afecciones como el raquitismo. se recomienda la exposición a la luz solar y los suplementos de vitamina D para los lactantes alimentados exclusivamente con HM. La vitamina K es esencial para la proteína involucrada en la coagulación sanguínea. Por esto,

después del nacimiento, se recomienda su suplementación. En la HM, los principales minerales son el calcio, el fósforo, el magnesio, el potasio, el sodio y el cloruro, que contribuyen en una variedad de funciones fisiológicas, forman parte esencial de muchas enzimas y son biológicamente importantes para diversas estructuras moleculares (Pietrzak-Fiećko & Kamelska-Sadowska, 2020).

1.3.5 Propiedades antioxidantes de la leche materna. Compuestos fenólicos

La HM es una fuente de antioxidantes exógenos que permite a los recién nacidos enfrentarse al estrés oxidativo que se produce de manera fisiológica en el momento del parto, y, además, ayudan a prevenir y a proteger contra las enfermedades en la infancia (Živković et al., 2015). La HM es fuente de múltiples componentes antioxidantes, tanto enzimáticos como no enzimáticos que previenen el enranciamiento oxidativo. Sin embargo, en los últimos años han crecido las evidencias sobre las altas propiedades antioxidantes y bioactivas de los denominados compuestos fenólicos. Son metabolitos secundarios exclusivos de las plantas, cuya estructura molecular incluye al menos un grupo fenólico: un anillo aromático unido al menos a un grupo funcional hidroxilo. Su función está relacionada principalmente con la pigmentación y la protección contra patógenos y depredadores. Existen más de 10.000 compuestos fenólicos diferentes, desde los más simples hasta los más complejos, y su análisis y características son indicativos de su gran diversidad en la naturaleza (Tsopmo, 2018).

El interés por los compuestos fenólicos ha aumentado debido a sus importantes efectos biológicos. Sus beneficios incluyen la protección contra el estrés oxidativo y la inflamación, inhiben la agregación plaquetaria, poseen funciones inmunológicas, participan en la protección directa del ADN y en la promoción de la apoptosis, entre otras (Vázquez et al., 2015). Los compuestos fenólicos pueden clasificarse en los siguientes grupos: ácidos fenólicos, flavonoides, estilbenos, cumarinas, lignanos y taninos (Figura 6).

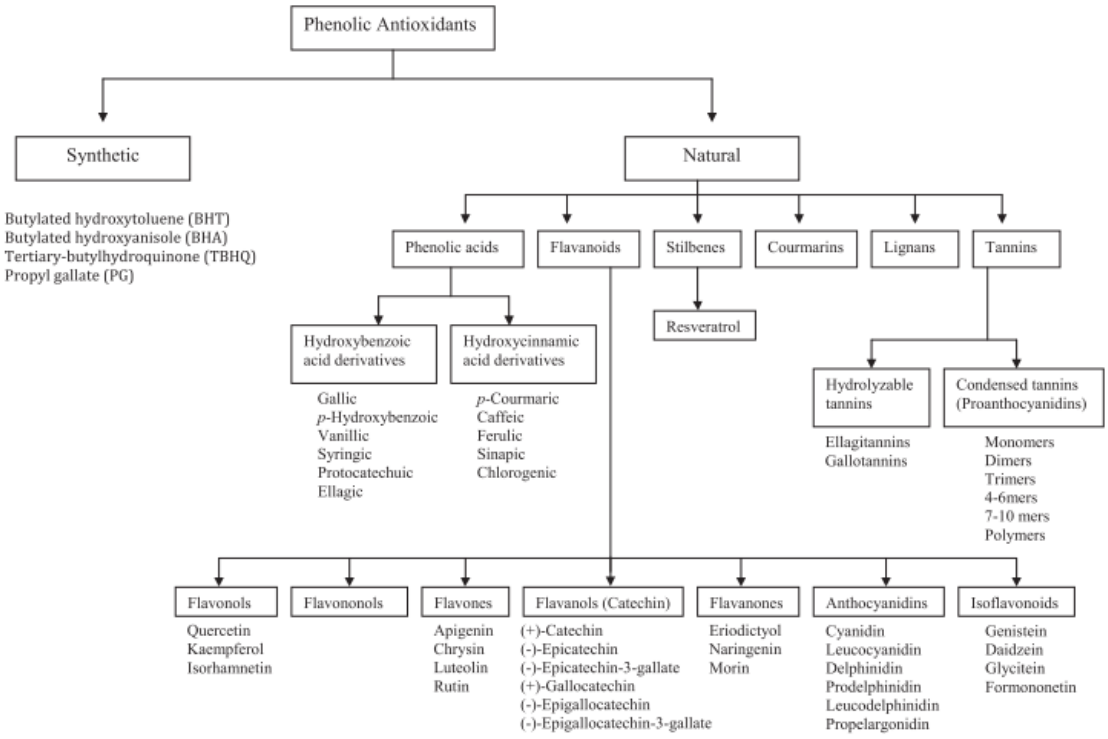


Figura 6. Clasificación de las principales familias de compuestos fenólicos (Shahidi & Ambigaipalan, 2015).

Durante las últimas décadas, algunos estudios se han centrado en el estudio de los flavonoides en la HM, al constituir el principal subgrupo. Entre ellos, han sido descritos en bibliografía la quercetina, luteína, naringerina, daidzeina, genisteína, equol, epicatequina, etc (Song et al., 2013).

Sin embargo, la presencia de ácidos fenólicos en la HM ha sido escasamente investigada (Li et al., 2009). Éstos se clasifican en derivados del ácido hidroxicinámico e hidroxibenzoico. El primer grupo incluye compuestos como los derivados del ácido gálico, que han sido ampliamente estudiados por sus propiedades antimicrobianas, antiinflamatorias, cardiovasculares o gastrointestinales, entre otros (Kahkeshani et al., 2019), o el ácido protocatechuico, al que se le ha reportado una actividad antiinflamatoria y una capacidad de mejorar las defensas antioxidantes (Juurlink et al., 2014). El segundo grupo incluye por ejemplo el ácido clorogénico, al que se le ha atribuido efectos anti obesidad,

antioxidantes, antiinflamatorios, antihipertensivos y antimicrobianos (Naveed et al., 2018). La mayoría de ellos forman parte de la dieta humana y también se consumen como preparados medicinales, y muchos de los efectos beneficiosos para la salud se han atribuido por sus propiedades biológicas.

1.3.6 Condiciones digestivas del recién nacido. Simulación gastrointestinal

La digestión es un proceso complejo esencial para la salud del ser humano, ya que se encarga de la transformación de los alimentos ingeridos en moléculas más simples que pueden ser absorbidas. Los ensayos *in vivo*, y especialmente en humanos, implican limitaciones técnicas, éticas y financieras. Por tanto, es necesario desarrollar alternativas *in vitro* para una mejor comprensión de la cinética digestiva, ya que tienen la ventaja de que ser más rápidos y menos laboriosos. Además, los ensayos *in-vitro* tienen una mejor reproducibilidad y menor variabilidad interindividual y, por lo tanto, son óptimos para los experimentos de screening (o cribado). Pero necesitan ser validados con *datos in vivo.*

Existen varias diferencias entre los modelos de digestión *in vitro* entre bebés y adultos, principalmente en algunas enzimas digestivas y en el pH gástrico. Generalmente éstos últimos consideran tres fases principales: (i) fase oral en la boca durante el cual los alimentos sólidos se mastican y se transforman en un bolo blando que se puede tragar, (ii) fase gástrica y (iii) fase duodenal. Sin embargo, una particularidad de los recién nacidos (de 0 a 6 meses) es que se alimenten exclusivamente con comidas líquidas a base de leche, lo que reduce considerablemente la fase oral del proceso digestivo, ya que el contacto de la comida líquida con la cavidad bucal y con los componentes activos de la saliva se limita al tiempo de deglución (5 a 10 segundos) (Bourlieu et al., 2014).

La fase gástrica tiene lugar en el estómago bajo la acción del jugo gástrico. Los dos tipos principales de enzimas gástricas contenidas en estas secreciones son la lipasa gástrica humana y la pepsina las cuales catalizan la hidrólisis de los lípidos

y las proteínas, respectivamente. Sin embargo, mientras que existe un rápido desarrollo de lipasa a partir de la undécima semana de gestación, llegando a alcanzar niveles de actividad adulta al nacer; la pepsina presenta un desarrollo lento, que, a pesar de ser segregadas a partir de la décimo cuarta semana de gestación, tan sólo alcanza el 18% de la actividad adulta cuatro semanas después del nacimiento, aumentándose con la edad posnatal. Esto quiere decir que mientras existe una extensa digestión de las grasas en el estómago, las proteínas presentan una digestión mínima (Lindquist & Hernell, 2010; Mlenard et al., 1995). Además, el pH gástrico en ayunas del lactante es menos ácido que el de un adulto (respectivamente 4 a 5 frente a 2 en ayunas), lo que reduce también significativamente la digestión de las proteínas, ya que la actividad óptima de la pepsina se encuentra en un pH entre 1.5-2.2 (Figura 7).

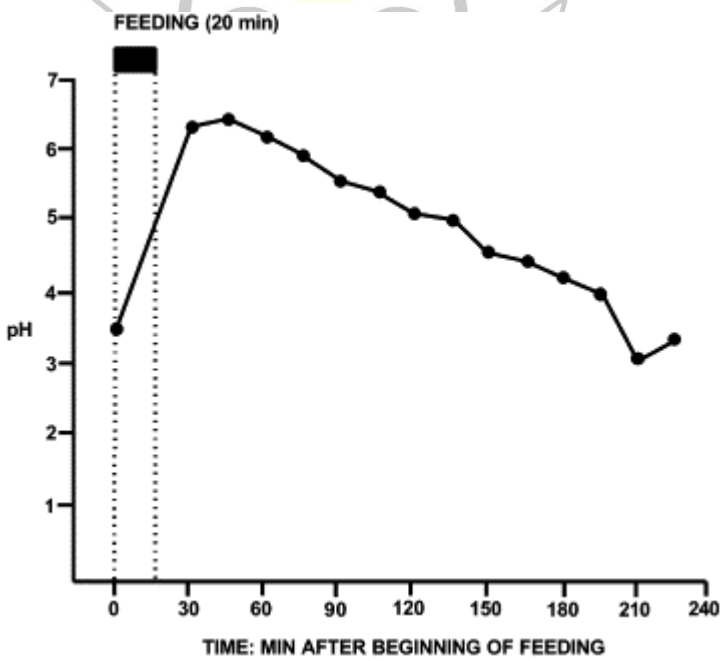


Figura 7. Evolución del pH gástrico durante la digestión del lactante (Chatterton et al., 2004).

Después de la fase gástrica, las secreciones gástricas se vacían al duodeno, donde el proceso digestivo se lleva a cabo por la acción de las enzimas pancreáticas y las secreciones biliares. Las sales biliares juegan un papel esencial en la lipólisis,

absorción y transporte de lípidos. Sin embargo, en los recién nacidos el metabolismo y el recambio de las sales biliares es activo pero inmaduro. La proteólisis en los bebés tiene un pH y concentraciones de tripsina similares a las del intestino de los adultos, mientras que las quimotripsinas y las carboxipeptidasas-B solo representan alrededor del 10% al 60% de la actividad encontrada en los adultos. Con respecto a la digestión de lípidos, la actividad y la producción de la lipasa gástrica son similares en bebés y en adultos (Shani-Levi et al., 2017). Los parámetros digestivos del lactante a término y del adulto se resumen en la Figura 8.

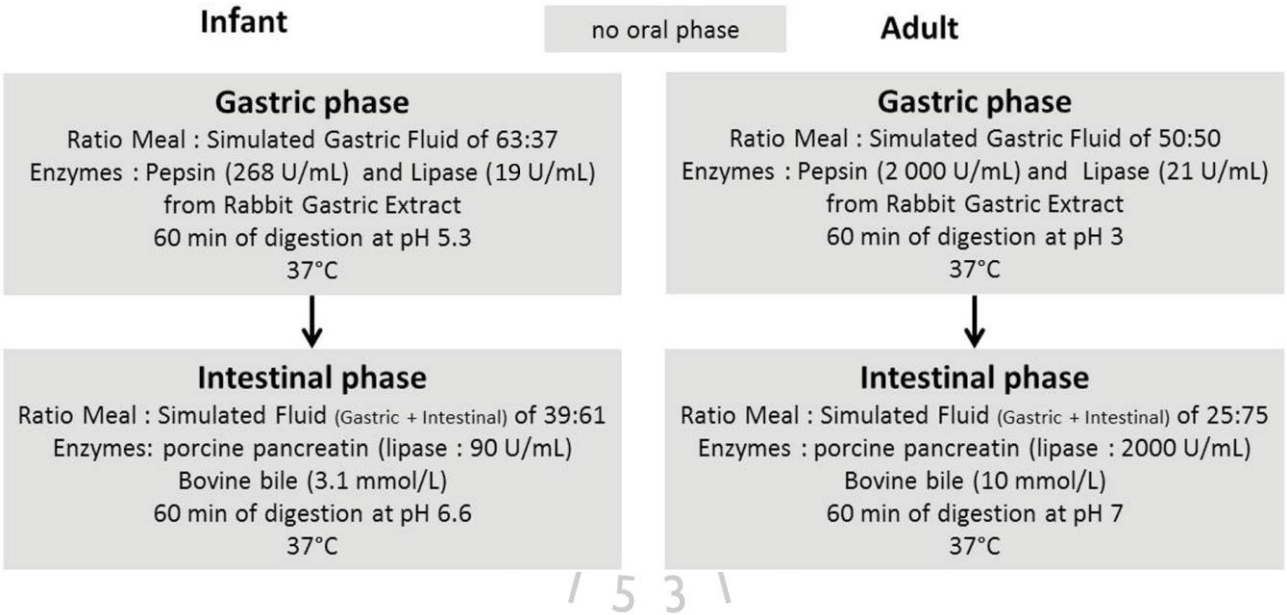


Figura 8. Condiciones digestivas del lactante y del adulto utilizadas en los modelos de digestión estática *in vitro* (Ménard et al., 2018).

Al igual que se ha encontrado un consenso internacional para simular la digestión en la etapa adulta con un modelo estático *in vitro* mediante la red de científicos INFOGEST (Minekus et al., 2014), recientemente se ha propuesto un modelo fisiológicamente relevante de digestión estática *in vitro* en la etapa infantil (Ménard et al., 2018). Para ello, se recopilaron todos los datos fisiológicos sobre las condiciones digestivas en el recién nacido, teniendo en cuenta que la

funcionalidad del tracto gastrointestinal humano se desarrolla en el primer año de vida; los recién nacidos (<28 días de vida) y los lactantes de hasta seis meses poseen un sistema digestivo inmaduro en comparación con los lactantes mayores (>6 meses) o el tracto gastrointestinal completamente maduro de un adulto. Esta inmadurez afecta tanto a parámetros enzimáticos (tipos de enzimas y nivel de actividad) como no enzimáticos (dieta a base de leche, frecuencia de alimentación, concentraciones de sales biliares) (Shani-Levi et al., 2017).

Recientemente, ha existido un creciente interés por intentar correlacionar datos de digestión *in-vitro* e *in-vivo* (Bohn et al., 2018). Con este objetivo, existen estudios que han comparado la degradación de las proteínas, el perfil de péptidos resistentes a la digestión, las secuencias bioactivas y los aminoácidos libres, empleando datos de modelos animales porcinos (Egger et al., 2017; Miralles et al., 2021) o datos humanos (Sanchón et al., 2018). En cuanto a las condiciones de digestión infantil, un estudio llevado a cabo por Ménard *et al.* desarrolló un sistema de digestión gastrointestinal dinámica *in-vitro* bicompartimental simple que se validó comparando la cinética de la proteólisis obtenida en este sistema *in-vitro* con datos obtenidos *in-vivo* recopilados en lechones, obteniendo también una buena correlación entre los resultados de ambos ensayos (Ménard et al., 2014). Sin embargo, la mayoría de estudios se han llevado a cabo en condiciones gastrointestinales del adulto, por lo que sería interesante obtener datos de correlación utilizando resultados disponibles de digestión infantil.

1.3.7 Alimentación artificial. Fórmulas infantiles

Como sustituto de la HM destinado a satisfacer los requisitos nutricionales normales de los bebés, las IF son alimentos manufacturados para la alimentación de recién nacidos y bebés que intentan igualar, en la medida de lo posible, la composición de la HM, especialmente el perfil lipídico (Lopes et al., 2018). Las IF están altamente reguladas por diferentes autoridades en todo el mundo, como la Comisión del Codex Alimentarius de la Organización de las Naciones Unidas para la Agricultura y la Alimentación, la Autoridad Europea de Seguridad Alimentaria,

la ESPGHAN o la OMS, para garantizar la ingesta adecuada de nutrientes durante la infancia (Zou et al., 2016). Dado que la HM es un fluido complejo y dinámico muy difícil de imitar, la mayoría de las mejoras de las IF han consistido en la adición de componentes identificados individualmente como bioactivos en HM. En particular, algunos de estos componentes que pueden agregarse son los LC-PUFA, lactoferrina, nucleótidos, aminoácidos esenciales, oligosacáridos, prebióticos o probióticos, entre otros (Bourlieu et al., 2017).

La mayoría de las fórmulas comerciales para lactantes se formulan utilizando leche de vaca como sustituto de la fuente de proteína de la HM (por ejemplo, leche desnatada, caseínas, combinación de caseínas y proteínas de suero, proteína de suero parcialmente hidrolizada, etc.). El contenido en proteínas de las IF difiere tanto en cantidad como en contenido con respecto al de la HM. Contienen cantidades considerablemente más altas de proteínas, y aunque se ha reducido durante las últimas décadas, esto sigue siendo un hecho (Lönnerdal, 2014). A diferencia de la HM, aproximadamente el 80% de estas proteínas están presentes en forma de caseínas. Sus proteínas predominantes son la β-lactoglobulina, perteneciente a la fracción del suero, y la α-s2-caseína, perteneciente a las caseínas, ausentes ambas en la HM. Sin embargo, éstas pueden desencadenar en respuestas inmunogénicas a las proteínas de la leche de vaca y propiciar otras alergias alimentarias posteriores en la vida cuando son consumidas por bebés susceptibles (Zhu & Dingess, 2019). Poseen cantidades muy inferiores de lactoferrina y la inmunoglobulina G es predominante en éstas. Las principales diferentes pueden observarse en la Tabla 2 / Figura 9. Aparte de las diferencias en la composición de las proteínas, el procesado térmico de las IF también afecta a la digestibilidad de las proteínas, pudiendo generar diferencias en los patrones de péptidos durante la digestión (Deglaire et al., 2016).

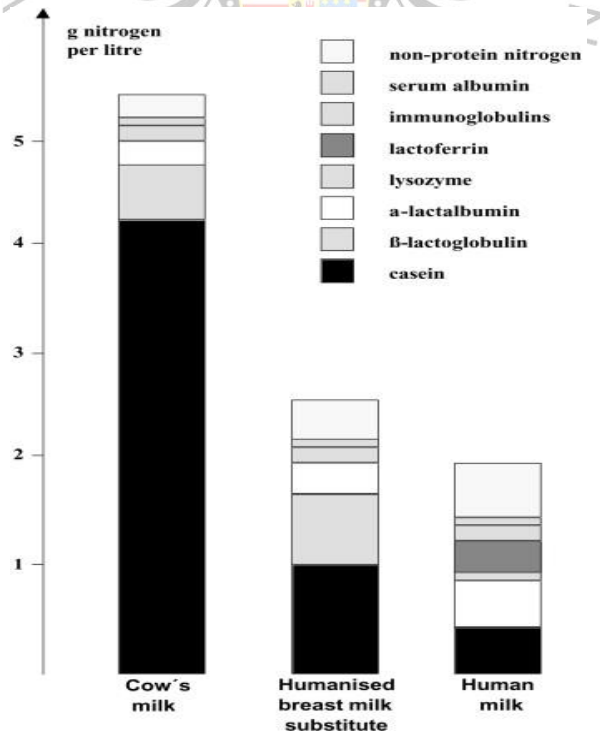
Actualmente se emplea una combinación de aceites vegetales (aceite de coco, soja, canola, cártamo alto oleico, girasol alto oleico u oleína de palma) en la formulación de IF para imitar, al menos parcialmente, el perfil lipídico de la HM. Pero mientras que en la HM la composición de FA es dinámica y modulada por la

dieta, en las IF el contenido en lípidos es estática. Al igual que la HM, la grasa proporciona aproximadamente el 40-50% de la energía, pero, aunque los fabricantes de las IF han conseguido igualar algunos de los FA predominantes en la HM, las mezclas de aceites para lactantes no se acercan a la presencia o concentración de los más de 150 FA y otros componentes identificados en la HM, ni a la estructura de los TAG. No obstante, en las IF, los LC-PUFA se obtienen añadiendo aceite de pescado, aceite de algas rico en DHA de *Crypthecodinium cohnii*, aceite de hongos rico en ARA de *Mortierella alpina* o fosfolípidos de yema de huevo (lecitina / fosfatidilcolina) (Mazzocchi et al., 2018; Su et al., 2017). Los ácidos láurico y mirístico, combinados entre sí, no deben superar el 20% del total de FA, así como el contenido de FA trans no debe superar el 3% del total de FA, ya que se trata de componentes endógenos de la grasa láctea (FAO, 2016).

Sin embargo, y aunque la composición de las IF ha cambiado con frecuencia a lo largo de los años con un conocimiento cada vez mayor de la nutrición infantil (como por ejemplo el ajuste de la proporción de proteínas del suero:caseína y la adición de lactoferrina), siguen existiendo diferencias que aún persisten, como es la digestibilidad de las proteínas, la absorción de aminoácidos y la presencia o abundancia de péptidos bioactivos específicos entre la HM y las IF.

Tabla 2 / Figura 9. Diferencias en la composición de proteínas entre la leche materna y la leche de vaca. Adaptada de Guo y col. (2014).

	Leche materna	**Leche de vaca**
Caseínas totales	0,3 g/100g	2,6 g/100g
α_{s1} caseína	Trazas	40
α_{s2} caseína	-	8
β-caseína	85	38
κ-caseína	15	12
Tamaño de micela, nm	50	150
Proteínas del suero	0,7 g/100g	0,8 g/100g
α-lactoalbúmina	26	17
β-lactoglobulina	-	43
Lactoferrina	26	Trazas
Albúmina sérica	10	5
Lisozima	10	Trazas
Inmunoglobulinas	16 (IgA)	10 (IgG)
Otras	12	24

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2. OBJETIVOS


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Entre los factores que pueden influir en la composición y actividad biológica de la HM se encuentran factores maternos, que incluyen tanto factores nutricionales (hábitos alimentarios, estado nutricional y antropométrico de la madre y estilos de vida), como no nutricionales (nivel socioeconómico y/o educativo, clase social, hábito tabáquico y/o consumo de medicamentos o drogas o la ganancia de peso de la madre durante el embarazo); factores genéticos como la edad materna, raza/etnia o antecedentes familiares de enfermedad, como la obesidad, hipertensión o diabetes de la madre previo y durante el embarazo; y factores ambientales, como la contaminación ambiental, los pesticidas o la exposición a contaminantes químicos. Además, la HM también varía su composición a lo largo de la toma (siendo la parte inicial más rica en agua y la parte final más rica en grasa), a lo largo del día (variación diurna, especialmente en el contenido de grasa) y a lo largo de la lactancia.

Se ha demostrado que la adiposidad materna puede desempeñar un papel fundamental en la regulación de las concentraciones de hormonas como la leptina y macronutrientes presentes en la HM, así como en la cantidad y calidad de leche producida a lo largo de la lactancia. Esto plantea la posibilidad de que los cambios metabólicos asociados con la obesidad de la madre puedan afectar a la composición de la HM. Además, se recomienda el mantenimiento de un estado nutricional saludable durante el embarazo y la lactancia para garantizar un suministro adecuado de todos los nutrientes. Sin embargo, actualmente no hay evidencia científica suficiente para asociar las características maternas con un perfil de composición de la leche.

Objetivo general

Durante el año 2018 y 2019, se llevó a cabo un estudio de investigación sobre el análisis de marcadores biológico/nutricionales durante el embarazo y su influencia en el parto y en la lactancia materna. La población de estudio fueron mujeres embarazadas reclutadas en el Servicio de Ginecología y Obstetricia del Hospital Universitario Virgen de las Nieves de Granada, España, a las cuales se les hizo un seguimiento desde la semana 10-12 de gestación, coincidiendo con su

primera visita prenatal, hasta el post parto. El principal objetivo de esta Tesis Doctoral fue determinar el impacto que pueden tener las condiciones antropométricas y nutricionales de la madre en la composición de la HM, así como los cambios producidos en dicha composición a lo largo de la lactancia materna. Para cumplir este objetivo se plantearon los siguientes objetivos parciales:

Objetivo 1

Evaluar el estado nutricional y antropométrico de un grupo representativo de embarazadas de la provincia de Granada. Estudiar la ingesta dietética de dicha población y determinar las deficiencias en la ingesta de macro y micronutrientes respecto a las recomendaciones diarias recomendadas.

Objetivo 2

Determinar los principales cambios en el perfil de ácidos grasos de la HM de mujeres lactantes de Granada durante el primer mes de lactancia. Comparar con diferentes IF comercializadas en España.

Objetivo 3

Caracterizar el perfil de proteínas y péptidos de la HM durante el primer mes de lactancia. Evaluar el efecto de la composición corporal en este perfil.

Objetivo 4

Evaluar el efecto de la simulación de la digestión gastrointestinal en condiciones del lactante en la degradación de las proteínas y la liberación de péptidos, identificando aquellos con actividad biológica. Contrastar los resultados con datos disponibles *in-vivo* tras la ingesta de HM.

Objetivo 5

Estudiar el perfil de compuestos fenólicos individual, el contenido total de compuestos fenólicos y la actividad antioxidante de la HM durante el primer mes de lactancia. Comparar con diferentes IF comercializadas en España.

3. RESULTADOS


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Publicación I

Anthropometric and nutritional assessment of pregnant women from Southern Spain during early pregnancy

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Abstract

Specific maternal anthropometric and nutritional characteristics have a direct impact on the short and long term health status of offspring, so an evaluation of the nutritional status during pregnancy is essential for the optimization of maternal, fetal and neonatal health. The objective of this study was to describe a population of Granada pregnant women assessing their body composition using anthropometric measurements and determining the adherence to the Mediterranean diet and estimated nutrients intake using validated questionnaires. Anthropometric measurements showed that almost 40% of the studied cohort had overweight or obesity and that, as expected, there was a positive correlation between increased body mass index and increased skinfolds and circumferences. We found no association between quality of the diet and obesity, even though the resulting score was consistent with high adherence to the Mediterranean diet. However, the observed population did not reach some recommendations established for this life stage, because the composition of the diet regarding macronutrients was not fully in line with the proposed range, and inadequate intakes of several micronutrients which are relevant during pregnancy were also found. Thus, the observed results support the development of nutritional education programs in the future, particularly to improve nutrition before and during pregnancy.

Keywords: pregnancy, body mass index, anthropometry, nutritional intake, Mediterranean diet

1. Introduction

The evaluation of nutritional status during pregnancy is essential for the optimization of maternal, fetal and neonatal health [1]. Specific maternal anthropometric characteristics, namely excessive maternal pre-pregnancy body mass index (BMI) and inadequate gestational weight gain (GWG), have a negative impact on the immediate and long-term health status of offspring. In particular, pre-pregnancy overweight/obesity or excessive gestational weight gain is associated with higher odds of large for gestational age, macrosomia and childhood

overweight/obesity [2–4], and it has been suggested to contribute to impaired childhood neurodevelopment [5]. On the other hand, underweight pre-pregnancy status and insufficient GWG might be accompanied with prematurity, intrauterine growth retardation and low birth weight [6,7], as well as an increased risk of maternal and perinatal death in large cohorts of study [8,9].

Overall, maternal body composition changes markedly across the course of pregnancy to support the growing fetus and prepare the mother for lactation [10]. A number of tools have been proposed to assess these changes, being BMI one of the most widely used parameters for assessing nutritional status and correlates with body composition measurements [11]. However, it does not provide information on the proportions of organ components, nor the regional distribution of fat tissue in the body [12]. Similarly, bioimpedance analysis, while differentiating between lean and adipose tissue mass, does not differentiate between maternal and fetal contributions [13]. Other techniques such as magnetic resonance imaging and computed tomography might expose the mother to ionizing radiation and lack portability while being expensive [14]. Anthropometric measurements, particularly skinfold thickness and mid-upper arm circumferences (MUAC), have been used extensively to estimate changes in body composition in pregnancy, as they indirectly assess the pregnant woman's fat reserve. When combined with BMI measurement, it has been shown that it can determine maternal nutritional state during the first weeks of pregnancy and provide useful information in body adiposity studies [15].

Pregnant women should also receive adequate and balanced nutrition throughout pregnancy to provide energy and nutrients for milk production and lactation [16,17]. The Mediterranean diet is characterized by an abundant consumption of plant-origin foods, mainly cereals, vegetables and fruit along with moderate consumption of dairy, fish and poultry products, and a low consumption of red meat with olive oil being the main source of fats [18]. The benefits of this diet during pregnancy for both the mother and the fetus on development have been previously explored [19]. However, evidence regarding the beneficial effects of

the adherence to Mediterranean diet on pregnancy outcomes and children's health is not consistent. In this sense, different populations account for different foods consumption even with the same adherence to the Mediterranean diet [20]. Complementing the assessment of the Mediterranean diet adherence with a validated evaluation of estimated nutrients intake might provide an insight into the benefits of this diet.

Following these observations and in order to provide a broader overview of the situation, we aimed to describe a population of pregnant women living in Southern Spain, a Mediterranean area, assessing their body composition using anthropometric measurements during the first trimester of gestation. Additionally, we determined the adherence to the Mediterranean diet using the PREDIMED (Prevention with Mediterranean Diet) validated questionnaire and we assessed the estimated nutrients intake.

2. Materials and methods

2.1. Participant characteristics

The present research is a secondary analysis of 56 pregnant women that participated in a larger prospective cohort study that aimed to evaluate the association between vitamin D deficiency during early pregnancy and the odds of preterm birth (Ref. doi:10.3390/nu12113279). Women were recruited during 2018-2019 at the obstetrics and gynecology services of the University Hospital "Virgen de las Nieves" of Granada, Spain, during their first routine prenatal control (weeks 10-12 of gestation). Out of the 380 women participating in the cohort study, 80 women were offered to additionally participate in the present descriptive study and 56 firmed the informed consent thus being included in the research. This study was approved by the Ethics Committee of the University of Granada (72-2015), and it was conducted in full accordance with the principals stipulated in the Declaration of Helsinki, reviewed in Fortaleza, Brazil, in 2003.

2.2 Participants Data

Inclusion criteria for the present descriptive study consisted of pregnant women older than 16 years old, capable of signing the informed consent and speak Spanish, between weeks 10-12 of gestation with singleton pregnancy determined by ultrasonography. Specific exclusion criteria that did not applied to the original cohort of study consisted of women with physical disabilities that might compromise study participation. Sociodemographic variables were collected by personal interview and consisted of maternal age, pre-gestational body mass index, smoking habit, and parity. Clinical variables were collected from medical records. In this sense, data regarding cases of miscarriage or stillbirth, weeks of gestation at delivery, birth weight, and mode of delivery (vaginal or cesarean section) were registered.

2.3 Anthropometric measurements

Height and weight were measured using a SECA mechanical scale with a measuring rod, with a capacity to support 220 kg, with minimal clothing and no shoes. Each measurement was repeated two times, and the mean value was calculated. BMI was calculated using the equation "weight in kg/height in m^2" and it was classified according to the standards established by the World Health Organization (WHO) (<18.5 kg/m^2, underweight; 18.5-24.9 kg/m^2, normal weight; 25.0-29.9 kg/m^2, overweight and >30.0 kg/m^2, obesity). Women's pre-pregnancy weight was self-reported by participants at the first prenatal visit. GWG was calculated based on the difference between self-reported pre-pregnancy weight and the weight at delivery obtained from medical records.

Anthropometric measurements were performed as per the International Society for the Advancement of Kinanthropometry (ISAK) by a level 1 anthropometrist accredited [21]. Assessed anthropometric variables consisted of those presented in **Table 1.** Measurements were taken using calibrated equipment. Slim Guide caliper, Cescorf tape measure and Slim Guide pachymeter were used to measure skinfold thicknesses, circumferences and diameters, respectively. For

data collection, template proposed by ISAK was used. Two measurements were taken each time and if the observed difference was greater than 7.5%, a third measurement was performed. The final measurement as calculated as the mean value of two measurements or the median of three (if three measurements were taken).

Table 1. Anthropometric measurements used in this study

Characteristics	Measures	Characteristics	Measure
Variables	Age (years)	Circumferences (cm)	Chest
	Weight (kg)		Minimum waist
	Height (cm)		Medium waist
	Body mass (kg)		Hip
			Relaxed arm
Skinfolds (mm)	Triceps		Flexed arm
	Biceps		Forearm
	Subscapular		Thigh at 1cm
	Supraspinale		Medium thigh
	Illiac crest		Calf
	Abdominal		Wrist
	Front thigh		Ankle
	Medial calf		
		Indicators	Fat mass (%)
Diameters (cm)	Acromiale-radiale		Muscle mass (%)
	Elbow		Bone mass (%)
	Knee		Body density
	Wrist		Somatotype
	Ankle		Waist-hit ratio

2.4 Adherence to the Mediterranean Diet and estimate nutrients intake

To estimate the mother's adherence to Mediterranean diet, a survey validated by the PREDIMED study was used [22]. The total score ranged from 0 (minimum adherence) to 14 (maximum adherence). The score for wine consumption was eliminated given that alcoholic beverages should not be consumed during this period. The adherence was defined as low when the total of these 13 items did not reach a score of 7 points. Each question was scored as 0 or 1. The index consists of twelve questions on frequency of food consumption and two questions on eating habits considered to be characteristic of the Spanish Mediterranean diet. Based on previous studies on MD score, women were

classified into high-MD adherence (score >8; range 0-14) and low-MD adherence group (score ≤ 8).

A semi-quantitative food frequency questionnaire (FFQ) including a detailed food frequency section, comprising 101 items that assessed maternal dietary habits during pregnancy (first trimester of gestation) was used. This FFQ was previously validated by Vioque *et al.*, (2013) in pregnant women from a Mediterranean area [23] and includes data on milk consumption and dairy (8 items); eggs, meat and fish (22 items); vegetables and pulses (18 items); fresh and dried fruits (14 items); bread, cereals and first courses (6 items); oils and fats (3 items); sweets and other foods (7 items); alcoholic and non-alcoholic beverages (10 items); and others (13 items). The response categories used to indicate the usual frequency of food consumption over the course of time were: never or less than once per month, 1–3 times per month, once per week, 2–4 times per week, 5–6 times per week, once a day, 2–3 times per day, 4–5 times per day and more than 6 times per day. The response for each food item was converted to an average daily intake for each participant using the software Dietowin n® 8.0 [24]. These data were compared with nutritional reference values for pregnant women [25].

2.5 Statistical analyses

Normality of variables was analyzed using the Kolmogorov-Smirnov test. Categorical variables were reported as relative frequency (percentage) and numerical variables were reported as mean ±standard difference or median and interquartile range based on the results of the normality test. To assess differences between variables across BMI groups, the Spearman rho test was used. P-values <0.05 were considered statistically significant. All analyses were performed using the software SPPS version 25 (IBM Crop®, Armonk, NY, USA). Graph Pad Prism version 8.0.1 for Windows (La Jolla, CA, USA) was used for graphics.

3. Results and discussion

3.1 Anthropometric measurements

A total number of 56 pregnant women were classified according to their pre-gestational BMI, as shown in **Table 2**. The mean pre-gestational BMI was 24.46 ± 5.06 kg/m^2, 8.9% were underweight (< 18.5 kg/m^2), 53.6% were in the normal weight category (18.5-24.9 kg/m^2), 30.4% in the overweight category (25.0-29.9 kg/m^2) and 7.1% of the women were obese (> 30.0 kg/m^2). These values are in line with those obtained by Fatta *et al.*, who observed that out of 1,000 Caucasian women recruited with a singleton pregnancy during the first trimester of pregnancy 3.1%, 50.2%, 27.7%, and 19.0% were underweight, normal weight, overweight and obese respectively [26]. Similarly, Hernández-Díaz *et al.*, who evaluated a cohort of study consisting of 582 Cuban women, also observed a prevalence of overweight/obesity of 30% and a prevalence of underweight around 8% [12].

Table 2. Participants' demographic characteristics based on pre-gestational BMI (n=56)

Pre-gestational BMI, kg/m^2	Underweight	Normal-weight	Overweight	Obesity	Total
Age, years	30.6 ± 1.14	31.70 ± 5.88	30.50 ± 6.01	34.20 ± 4.38	31.52 ± 5.51
Pre-gestational weight, kg	54.06 ± 6.60	59.86 ± 6.08	74.28 ± 6.54	100.4 ± 11.04	67.10 ± 14.34
Height, m	1.69 ± 0.08	1.65 ± 0.07	1.65 ± 0.04	1.65 ± 0.07	1.66 ± 0.06
Pre-gestational BMI, kg/m^2	18.33 ± 0.68	21.94 ± 1.64	27.10 ± 1.53	36.79 ± 1.81	24.46 ± 5.01
Parity					
Nulliparity, %	40	45.5	72.2	37.5	48.1
Multiparity, %	60	54.5	27.8	62.5	51.9
Smoking status					
Smoker, %	20	20.4	23.8	30	21.7
Non-smoker, %	80	79.6	76.2	70	78.3
GWG, kg	14.40 ± 6.19	12.72 ± 4.52	13.00 ± 4.65	7.83 ± 7.25	12.45 ± 5.17
Length of pregnancy, days	277 ± 10	274 ± 14	278 ± 9	274 ± 10	275 ± 12
Final gestational weight, kg	64.66 ± 6.39	72.37 ± 7.55	87.43 ± 5.48	104.21 ± 18.98	79.53 ± 15.26
Final pregnancy BMI, kg/m^2	23.75 ± 1.85	26.96 ± 2.32	31.64 ± 2.08	38.39 ± 5.25	29.31 ± 5.12
Mode of delivery					
Vaginal, %	80	69.8	100	70	78.0
Cesarean section, %	20	30.2	0	30	22.0
Birth weight, g	3.378 ± 0.638	3.218 ± 0.488	3.342 ± 0.432	3.359 ± 0.561	3.279 ± 0.491

Underweight: BMI ≤ 18.5; Normal weight: BMI 18.6 - 24.9; Overweight: BMI 25.0 - 29.9; Obesity: BMI ≥ 30.0; GWG: gestational weight gain. Data presented as mean (SD).

No statistically significant differences between groups were found for any of the variables with the exemptions of pre-gestational weight, final gestational weight, pre-gestational and final pregnancy BMI. Although mean gestational weight gain was not significantly different between BMI groups, there was a trend towards decreased GWG along with increased BMI was observed. This trend could be explained since overweight and obese women already have large fat reserves for energy during labor and lactation, which could ultimately decrease weight gain compared to women with smaller fat reserves [27]. According to the 2009 guidelines from The Institute of Medicine (IOM), recommended GWG should be as follows: between 12.5-18 kg for underweight women, 11.5-16 kg for normal weight women, 7-11.5 kg for overweight women; and 5-9 kg for obese women [28]. All observed values in the present research were in line with these guidelines with the exemption of GWG among overweight women which was notably higher (13 ± 4.65 kg). The observed higher GWG in relation to the guidelines may be a consequence of several factors, such as lifestyle changes (lower level of physical activity during pregnancy or inadequate diet), low knowledge about the importance of an adequate weight gain or increased consumption of processed food and prepared meals [29].

Martínez-Hortelano *et al.*, evaluated the average GWG of the global population based on the aforementioned recommended guidelines, obtaining an average GWG in Europe of 13.60 kg, which correlates with our results (12,45 ± 5.17 kg) [29]. Mean GWG in the present study was lower than the values observed by Ramón-Arbués *et al.* and Ma *et al*. in Spanish and Chinese women (14,02 kg and 15,9 kg respectively) [30,31], but they were in line with those observed by Kac *et al.*, in Brazilian women (12.9 kg) [32]. The differences in GWG and pre-pregnancy BMI across regions could be influenced by countries' income, as overweight and obesity tend to be more prevalence among high or middle-income countries. On the other hand, differences between populations within the same countries can be consequence of socioeconomic status disparities. In this sense, in low/middle income countries the prevalence of overweight/obesity is higher among the wealthier populations, while the opposite happens in high income

countries where the prevalence of overweight / obesity tends to be higher among the poorest individuals [33]. Interestingly, in Spain, as a consequence of the economic crisis of 2012, the prevalence of obesity increased along with decreased quality of the diet [34].

Anthropometric characteristics of pregnant women are shown in **Table 3** and the average values of the skinfold thicknesses, circumferences and diameters are represented in **Figures 1A, B** and **C**.

There were significant differences between almost all the variables studied in the group of skinfold thicknesses, circumferences and diameters (p value <0.05). As expected, a positive correlation was observed between BMI, skinfold and circumferences, as a higher BMI correlated with higher skinfold and circumferences values. These results can be explained since higher BMI is indicative of body fat accumulation [35]. Changes in skinfold thickness and circumferences have been widely used to estimate subcutaneous fat changes in pregnant women, as it is suggested that more maternal fat is accumulated centrally than peripherally [10].

Skin thickness does not vary across trimesters of gestation which enables nutritional assessment during pregnancy thus being of great importance in primary health care [36]. Pérez A *et al.*, carried out a cross-sectional study in pregnant women with normal weight during the second and third trimesters of pregnancy measuring the mid-arm circumference, the mid-thigh circumference, the calf circumference and triceps, biceps, subscapular, mid-thigh and calf skinfolds, finding significant differences between trimesters of gestation only for lower extremities measurements. This author suggested that such variability is primarily caused by changes in fat and water content related to gestation. Edema becomes increasingly common as pregnancy progresses [37]. WHO corroborated that measurements of the upper extremities, both circumferences and skin thickness, do not vary across trimesters of gestation, are largely independent of gestational age and are considered a proxy indicator for maternal pre-pregnancy weight or weight during early pregnancy [36].

Table 3. Anthropometrical measurements based on maternal BMI in the first trimester of gestation (n=56).

Measure	Underweight	Normalweight	Overweight	Obesity	ρ-test	p-value
Skinfold (mm)						
Triceps	12.84 ± 1.86	17.95 ± 4.02	28.65 ± 5.74	34.30 ± 2.33	0.862	**<0.0001**
Biceps	5.10 ± 1.24	6.93 ± 2.71	12.74 ± 3.77	19.80 ± 4.60	0.754	**<0.0001**
Subscapular	7.90 ± 1.02	11.83 ± 3.17	22.44 ± 8.26	30.50 ± 6.89	0.833	**<0.0001**
Supraspinale	14.80 ± 4.67	20.22 ± 6.53	30.97 ± 5.87	36.40 ± 8.08	0.673	**<0.0001**
Illiaccrest	9.70 ± 3.99	12.60 ± 4.87	22.85 ± 5.87	32.80 ± 5.76	0.814	**<0.0001**
Abdominal	21.13 ± 7.60	26.87 ± 7.17	30.35 ± 7.74	35.08 ± 1.55	0.457	**<0.05**
Front thigh	22.00 ± 5.69	30.46 ± 7.04	41.11 ± 6.73	52.50 ± 8.62	0.753	**<0.0001**
Medial calf	12.10 ± 4.80	19.33 ± 6.04	30.36 ± 5.36	37.20 ± 6.34	0.737	**<0.0001**
Circumferences (cm)						
Chest	83.09 ± 0.51	84.39 ± 8.25	93.57 ± 5.01	108.70 ± 8.38	0.799	**<0.0001**
Minimum waist	72.12 ± 2.43	75.26 ± 4.31	84.41 ± 5.50	102.75 ± 5.10	0.936	**<0.0001**
Medium waist	81.30 ± 1.89	86.05 ± 5.08	96.16 ± 5.87	114.98 ± 10.48	0.915	**<0.0001**
Hip	90.08 ± 3.88	96.04 ± 6.20	106.78 ± 6.00	121.75 ± 6.64	0.932	**<0.0001**
Relaxed arm	24.72 ± 1.08	27.22 ± 1.63	31.60 ± 1.94	38.48 ± 2.05	0.962	**<0.0001**
Flexed arm	25.10 ± 0.87	27.47 ± 1.73	31.69 ± 2.14	37.62 ± 2.45	0.943	**<0.0001**
Forearm	23.25 ± 0.51	23.50 ± 1.15	25.09 ± 1.59	27.48 ± 1.21	0.737	**<0.0001**
Thigh at 1cm	50.28 ± 1.19	56.27 ± 3.52	61.87 ± 3.88	68.90 ± 2.61	0.899	**<0.0001**
Medium thigh	44.34 ± 2.24	49.12 ± 3.89	55.22 ± 4.10	62.75 ± 3.07	0.887	**<0.0001**
Calf	32.62 ± 1.49	35.82 ± 2.92	40.67 ± 4.93	44.92 ± 1.76	0.836	**<0.0001**
Wrist	14.53 ± 0.10	15.57 ± 2.31	15.35 ± 0.62	16.5 0± 0.89	-0.002	NS
Ankle	21.25 ± 0.17	21.48 ± 1.29	22.42 ± 1.35	23.93 ± 1.26	0.542	**<0.01**
Diameters (cm)						
Acromiale-radiale	34.27 ± 0.21	31.69 ± 1.40	31.55 ± 1.73	31.55 ± 1.50	-0.192	**<0.05**
Elbow	6.02 ± 0.33	6.17 ± 0.35	6.45 ± 0.31	6.66 ± 0.51	0.505	**<0.01**
Knee	8.82 ± 0.24	9.17 ± 0.51	9.71 ± 0.51	10.60 ± 0.78	0.727	**<0.0001**
Wrist	4.80 ± 0.60	4.83 ± 0.24	4.98 ± 0.37	5.68 ± 0.43	0.408	**<0.001**
Ankle	6.27 ± 0.25	6.61 ± 0.36	6.68 ± 0.34	6.77 ± 0.15	0.285	NS
Indicators						
Fat mass (%)	23.12 ± 2.04	29.05 ± 2.99	33.12 ± 0.69	37.68 ± 1.58		**<0.0001**
Muscle mass (%)	35.35 ± 1.50	32.38 ± 0.06	28.75 ± 2.51	26.65 ± 2.45		**<0.0001**
Bone mass (%)	17.01 ± 0.78	15.07 ± 1.23	13.03 ± 1.23	11.14 ± 0.59		**<0.0001**
Body density, m^3	1.045 ± 0.005	1.032 ± 0.007	1.022 ± 0.002	1.012 ± 0.004		**<0.0001**
Somatotype	3-2-4	5-4-2	7-6-1	9-8-1		
Waist-hip ratio	0.80 ± 0.02	0.78 ± 0.04	0.79 ± 0.04	0.84 ± 0.03		**<0.05**

Data presented as mean (SD). Significant differences p-value < 0.05

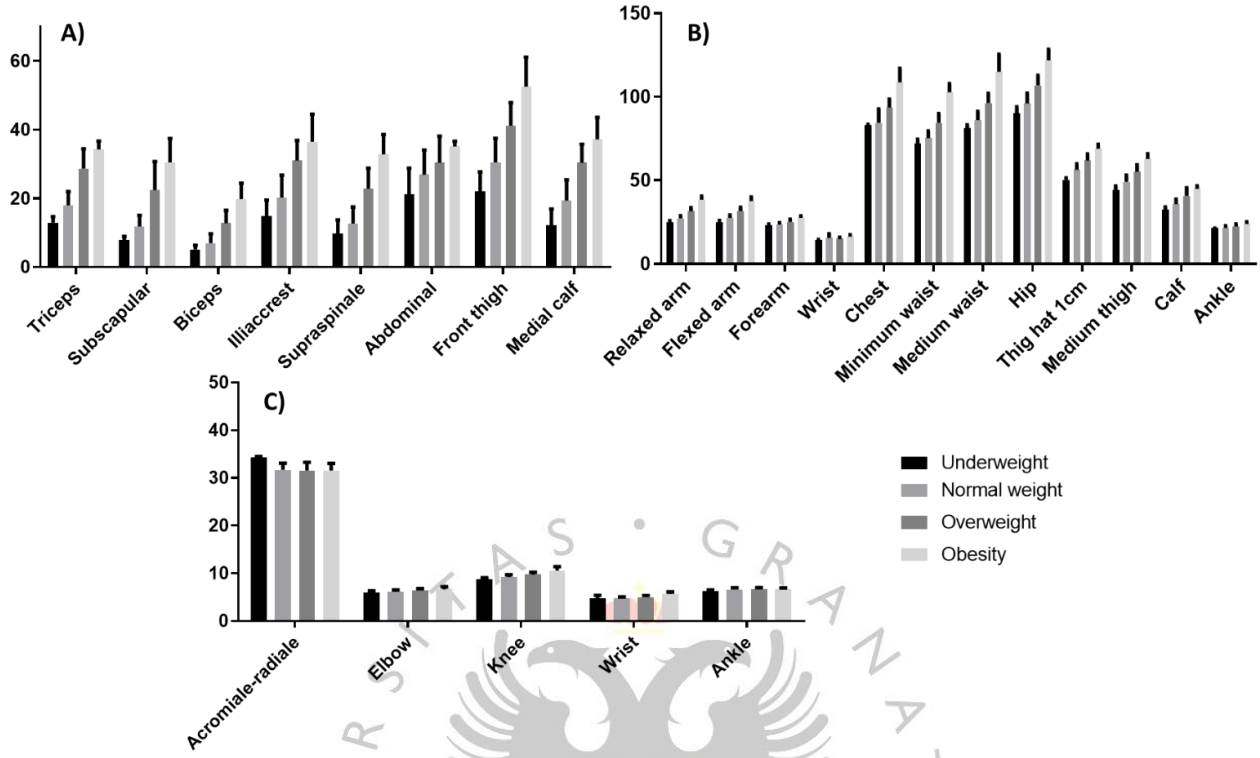

Figure 1. (A) Average values obtained of skinfold thicknesses, **(B)** circumferences and **(C)** diameters during the first trimester of pregnancy in our pregnant women.

Relaxed arm circumference, more commonly known as MUAC, and BMI presented the highest correlation among all the measures (r=0.962). MUAC is a widely accepted indicator of maternal nutritional status in pregnant women. Moreover, data on maternal MUAC have showed that this indicator is a significant predictor of birth weight and it is closely related to maternal weight but is independent of gestational age. However, the evidence regarding variations during the course of pregnancy is still limited [38].

Standard regression equations were used to convert skinfold thickness measurements to body fat estimates [39]. As expected, underweight and normal weight participants were within recommended values, with a total body fat mass mean percentage of 23.12% and 29.05 %, respectively. Groups with BMI > 25.00

exceed 30% fat mass, even further exceeding the limit value of 31-33% [15]. The percentage of total body fat mass is directly associated to BMI and it enables the determination, with a certain degree of accuracy, of the risk of suffering from certain pregnancy adverse outcomes pathologies related to overweight and obesity, such as gestational diabetes, cardiovascular diseases or preeclampsia [40].

Waist-hip ratio (WHR) is a specific and complementary anthropometric measure for assessing abdominal adiposity as part of the pregnant woman's fat body composition from early pregnancy. WHO establishes as normal levels for WHR values of 0.71-0.8 in women [41]. In our study, the values for this parameter were 0.80, 0.78, 0.79 and 0.84 for the underweight, normal weight, overweight and obese group, respectively. The latter was the only group that exceeded the WHO recommendations. Higher values indicate abdominovisceral obesity, which is associated with an increased cardiovascular risk and increased odds of developing hypertensive disorders and diabetes [42].

Anthropometric assessment has many advantages for nutritional evaluation: it is relatively simple, non-invasive and causes minimal discomfort to the patient. In developing countries, pregnant women usually initiate their prenatal care after the first months of pregnancy and thus pre pregnancy weight can be unknown; in such cases, total weight gain is difficult to determine. The measurement of MUAC and skinfolds, such as the bicipital, tricipital and the subscapular, has been proposed for its use in pregnant women, as it make it possible to evaluate changes in the adipose tissue of the body and, being an alternative or complementary measurement during prenatal care [38]. However, in late pregnancy abnormal body weight gain can be related to clinical edema. These findings indicate that changes in maternal weight or body composition in pregnancy usually occur after the first trimester. Therefore, accurate measurement of weight or body composition at any time in the first trimester may be used as a baseline for subsequent comparison [26].

3.2 Adherence to the Mediterranean Diet and estimate nutrients intake

The adherence of Mediterranean diet and distribution of macronutrient and micronutrient intakes are shown in **Table 3.** Regarding differences by maternal BMI, no associations were found between BMI and adherence of Mediterranean diet, energy intake or nutrient intakes (p-value >0.05).

We found no association between obesity and quality of the diet and the mean score obtained from the PREDIMED questionnaire was 9.35 ± 1.80 out of 13 points, indicative of an overall high adherence to Mediterranean diet. This score was higher than those found in other studies of pregnant women. Tomaino *et al*. evaluated adherence to DM in a sample of pregnant women followed in a hospital in the Canary Islands, obtaining low adherence with a mean score of 6.61 [43]; and Grillone *et al*. enrolled >18 years' pregnant women in North East of Italy (2nd-3rd trimester) and it had a mean value of 8.04 (SD 1.95), with low MD adherence in 63% of sample [44]. In addition, low adherence to DM may be related to future problems in the child. Fernández-Barrés *et al*. recruited, between 2003 and 2008, 1,827 Spanish pregnant women from the "Infancia y Medio Ambiente" cohort study, to evaluate associations between adherence to the MD during pregnancy and childhood overweight and abdominal obesity risk. Their data suggested that MD during pregnancy is not associated with measures of over- weight in 4 years old offspring, but is inversely associated with offspring waist circumference, a marker of abdominal obesity [45].

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Table 3. Daily intake of macronutrients and micronutrients during the first trimester of pregnancy

	All women	BMI kg/m^2		Spearman ρ-test	
		< 25 (n = 25)	≥ 25 (n = 14)	ρ - value	p-value
Mediterranean diet adherence	9.35 ± 1.80	9.34 ± 1.78	9.37 ±1.89		0.965
Nutrients (units/day)					
Energy, kcal	2,145.2 [1726.6-2449.3]	2,147.5 [1,719.2-2,426.5]	2,339.5 [1,689.6–2,941.6]	0.142	0.387
Proteins, g	106.4 ± 35.8	108.± 39.3	103,8 ± 32.2	0.024	0.886
Carbohydrates, g	190.8 ± 73.3	191.1 ± 66.5	196.4 ± 92.7	-0.009	0.954
Total fat, g	110.2 ± 37.3	103.9 ± 35.3	124.9 ± 40.3	0.271	0.096
SFA, g	30.6 [20.0-35.1]	29.6 [16.7-35.2]	32.0 [24.4–43.5]	0.190	0.247
MUFA, g	41.0 [33.7-57.7]	39.2 [33.8-50.6]	55.7 [33.6–73.6]	0.275	0.090
PUFA, g	13.0 [10.0-15.6]	12.7 [9.8-15.3]	14.4 [10.9–16.6]	0.195	0.235
Cholesterol, mg	406.6 [317.9-484.7]	406.6 [313.2-477.4]	422.5 [335.5–481.5]	0.014	0.931
Fibre, g	22.2 [18.1-28.8]	24.2 [18.3-32.3]	22.3 [17.0–34.3]	-0.014	0.931
Minerals					
Sodium, mg	2,744.8 [2,252.3-3,688.6]	2,714.6 [2,084.1-3,435.5]	3,121.4 [2,299.7–4,066.2]	0.185	0.259
Potasium, mg	3,596.9 [3,033.5-4864.3]	3,825.0 [3,160.6-4,846.4]	3,626.0 [2,914.1–5,298.0]	0.009	0.954
Magnesium, mg	343.2 [270.5-403.1]	357.0 [270.0-397.5]	341.1 [282.1–470.6]	-0.005	0.977
Calcium, mg	1,092.0 ± 378.7	1,120.0 ± 404.3	1,068.3 ± 370.0	-0.047	0.774
Phosphorus, mg	1,734.5 ± 656.9	1,569.3 ± 774.7	1,709.1 ± 498.3	0.095	0.565
Iron, mg	14.9 ± 5.2	15.3 ± 5.9	14.7 ± 4.9	-0.033	0.841
Iodine, µg	96.3 [72.9-120.7]	89.5 [67.2-119.6]	104.8 [90.2–121.8]	0.114	0.490
Vitamins					
Vitamin A, µg	840.0 ± 385.9	858.6 ± 430.3	859.1 ± 370.0	0.028	0.863
Tiamin,mg	1.2 [1.0-1.4]	1.3 [1.0-1.4]	1.2 [0.9-1.6]	-0.009	0.954
Riboflavin, mg	2.1 ± 0.7	2.1 ± 0.8	2.0 ± 0.6	-0.057	0.730
Niacin, mg	23.4 [21.1-32.1]	24.1 [21.4-31.9]	24.3 [21.3-34.2]	0.019	0.909
Pantotenic acid, mg	5.6 [5.2-6.9]	5.8 [5.3-7.2]	5.6 [4.8-6.6]	-0.109	0.508
Vitamin B6, mg	1.9 [1.6-2.4]	1.9 [1.6-2.4]	1.9 [1.6-2.9]	0.005	0.977
Biotin, mg	40.3 ± 14.3	40.8 ± 12.4	35.7 ± 16.1	-0.104	0.527
Folic acid, µg	287.3 ± 102.5	295.3 ± 108.7	269.4 ± 108.4	-0.057	0.730
Vitamin B12, µg	6.4 [4.5-7.5]	5.9 [4.4-7.6]	6.4 [4.6-7.0]	-0.047	0.774
Vitamin C, mg	181.8 [136.5-248.1]	212.8 [132.8-269.1]	163.9 [141.7-218.5]	-0.138	0.403
Vitamin D, µg	7.2 [5.6-10.2]	7.1 [5.8-9.6]	9.2 [7.8-11.2]	0.237	0.145
Vitamin E, mg	10.1 [7.5-13.5]	9.5 [6.5-13.3]	11.7 [10.5-18.8]	0.309	0.056
Vitamin K, µg	363.8 [291.4-427.5]	376.5 [283.4-537.0]	358.2 [292.6-380.9]	-0.052	0.752

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Data presented as mean (SD) or median [interquartile range]. Significant differences p-value < 0.05

Mean energy intake was 2,145.2 kcal/day. The WHO establishes a generic calculation between 1600 and 2000 kcal/day for women. It is estimated that an average intake of 300 extra calories is required during pregnancy which implies an approximate daily intake of 2,150-2,200 kcal/day [46]. Extra energy is required for the synthesis of new tissue (fetus, placenta and amniotic fluid) and the growth of existing the existing ones (uterus, breast and maternal adipose tissue) [47]. It should be noted that the mean energy intakes reported during pregnancy differed by geographic regions, with Europe having the second highest energy intake after the USA/Canada [48]. Although the energy intake was within the recommendations, an inadequate distribution of macronutrients was detected with high proteins intake (19.8% of energy) high fat content (46.2% of energy), and low carbohydrate amounts (35.6% of energy) **(Figure 2)**. Another study conducted in Spanish pregnant women also observed an excessive total fat content along with deficient carbohydrate intake in the cohort of study. Excessive consumption of saturated fat and low n-3 fatty acid intake, as well as a low-carbohydrate diet, have been related to adverse maternal and infant health outcomes, and might impair fetal growth [49].

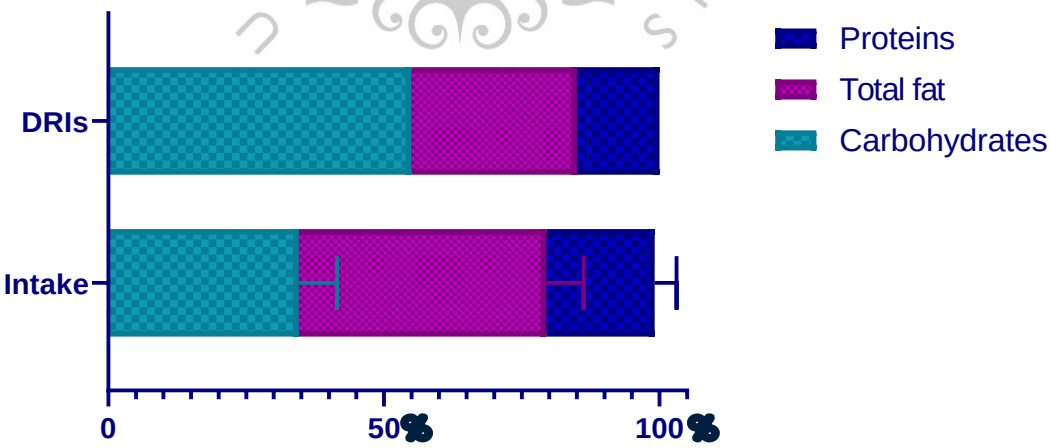

Figure 2. Percentage of distribution of the macronutrients (carbohydrates, total fat and proteins) content of participant's pregnant women and comparison with the recommendations [50]

Increased protein requirements are caused by the development of maternal, fetal, and placental tissue, as proteins are involved in both structural (keratin, collagen) and functional (enzymes, protein transport, hormones) biological roles. Approximately, 925g of protein accumulates throughout pregnancy, so the DRI for pregnant women is 1.1 g/kg/day of body weight instead of 0.8 g/kg or an additional 25 g/day to meet the needs of pregnancy [51]. Nevertheless, high maternal dietary protein intake is linked to intrauterine growth restriction and can lead to fetal or neonatal death due to ammonia toxicity. In the same fashion, both low and high maternal dietary protein intakes are associated to low birth weight [52].

Lipids are essential for the formation of cell membranes and hormones and are necessary for proper eye and brain development, especially during the prenatal period and into the first few years of the child's life [53]. Fat intake mainly comes from monounsaturated fatty acids (MUFAs) (41.0 g/day), due to the high consumption of olive oil which is rich in oleic acid, characteristic of the Mediterranean diet. Nevertheless, saturated fatty acids (SFAs) intake was also high (30.6 g/day). These are attributed to the consumption of animal origin products such as meat or dairy products and their derivatives. Finally, polyunsaturated fatty acids (PUFAs) such as omega-3 and omega-6 are found in smaller quantities, maybe due to the low consumption of fish, especially oily fish. The essential fatty acids, namely linoleic acid (omega-6) and linolenic acid (omega-3), are necessary for optimal formation of the brain and eyes. Vioque *et al*. (2013) found a fat intake distribution similar to that of our study in pregnant women at 10-13 weeks of gestation (46, 31 and 15 g/day of MUFAs, SFAs and PUFAs, respectively) [23]. Dietary intake of fatty acids, particularly PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is important during pregnancy in order to meet the requirements of the mother and the fetus on development. DHA can influence the development of the fetus brain and retina while EPA has been associated to decreased risk of preeclampsia [47].

Cholesterol content was above 300 mg/day in line with the SENC nutritional target, with a mean intake of 406.6 mg/day. When a high cholesterol

intake is combined with increased levels of progesterone and estrogen, cholesterol blood levels increase around 30-50% compared to pre-pregnancy levels. Continuate high cholesterol intakes throughout pregnancy might lead to adverse maternal and perinatal outcomes such as preterm birth, placental disfunction, fetus blood vessel lesions, gestational diabetes mellitus, and preeclampsia [54,55].

On the other hand, the fiber content was found to be slightly lower (22.2 g/day) than the values recommended for pregnant women (≥ 25 g/day). Together with the carbohydrate content, it can be attributed to low consumption of vegetables, fruits, whole grains, nuts and legumes. Consumption of plant-based foods helps to prevent digestive disorders such as constipation, a common side effect of pregnancy. They also promote satiety and might prevent an increase in cholesterol blood levels [53,56].

According to the micronutrient intake recommendations for pregnant women established by the Spanish Federation of Nutrition, Food and Dietetics (FESNAD), almost all vitamins and minerals evaluated reached the recommended values as shown in **Figure 3A** and **B**. Most insufficient intakes for the study population consisted of vitamin D, vitamin E, folic acid, iron and iodine.

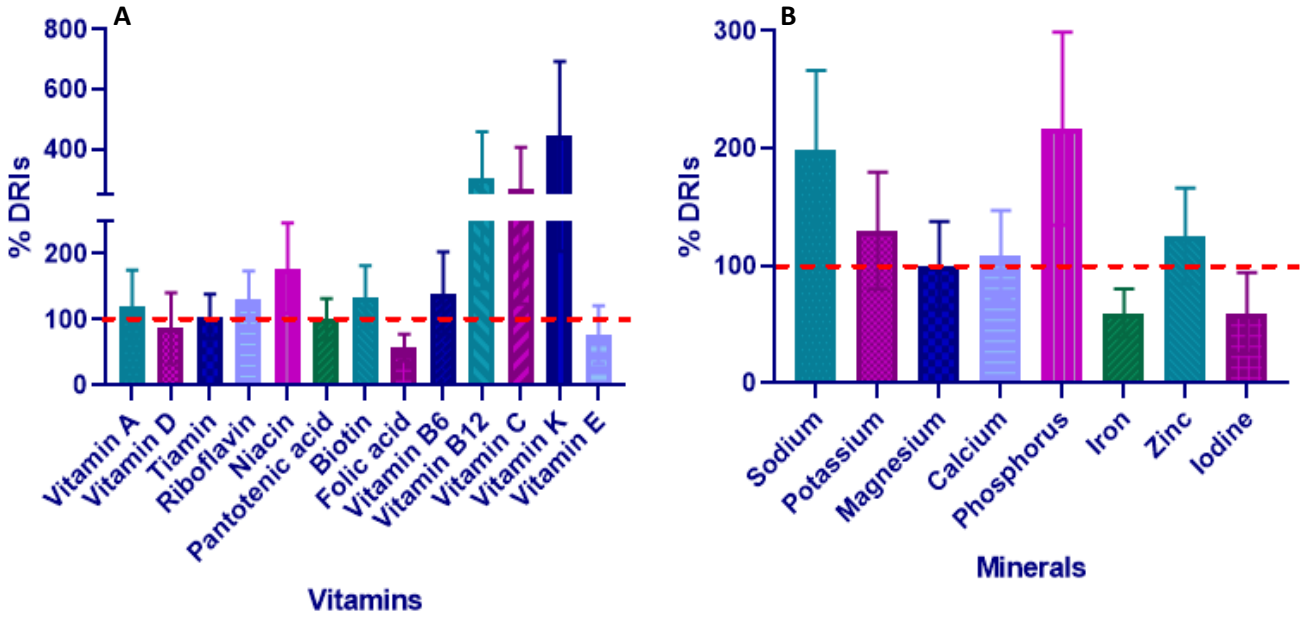

Figure 3 A/B. Percentage of micronutrient intakes (vitamins and minerals) of participant's pregnant women and comparison with the recommendations [25].

The average intake of folic acid, vitamin D and E were lower than the DRIs. The average vitamin D intake in the cohort of study was 86.6%. This secosteroid is essential for calcium absorption and bone maintenance and low levels of vitamin D are associated with congenital rickets and fractures, neurodevelopment, immune function and increased susceptibility to chronic disease. Furthermore, vitamin D deficiency has been linked to a number of pregnancy adverse outcomes such as preterm birth [57,58]. Vitamin D can be obtained through diet and supplements or via endogenous synthesis through ultraviolet light exposure. Vitamin D deficiency is common in pregnancy, especially in high-risk groups such as vegetarians, women who live in cold climates, and ethnic minority women with darker skin. However, in Granada, sun exposure is relatively high throughout the year, so it is likely that inadequacy will be lower than estimated in other studies.

Vitamin E is a fat-soluble nutrient of utmost importance important during the early stages of life, from the time of conception to the postnatal development of the infant. Low maternal vitamin E intakes during pregnancy, as well as vitamin D, are associated with increased risk of asthma during the first 10 years of life [59,60]. Moreover, second trimester vitamin E insufficiency is associated with hyperglycemia and insulin resistance [61].

Two B vitamins, folic acid and B12, should be given special attention. Insufficient folic acid intake is closely related to a diet poor in vegetables and fresh fruit. Early supplementation with folic acid is recommended before pregnancy and during the first 12 weeks of gestation to prevent fetal neural tube defects. Although the women in our study did not reach 100% of the recommendations for this vitamin through diet, Spain is the country with the highest consumption of food supplements, including folic acid supplements (97.8% of Spanish pregnant women) [62].

Iron deficiency continues to be one of the most prevalent nutritional deficiency diseases in the world and has a particularly high prevalence in pregnancy. There are significant negative outcomes to iron deficiency in pregnancy; these include maternal and infant mortality in severe cases, but also shortened gestation, prematurity, and poorer infant development in less severe

cases. In this case, iron supplements have routinely been recommended in pregnancy because iron needs nearly double during pregnancy [46].

The Spanish diet has changed substantially in recent years, moving away from the more traditional Mediterranean diet and towards the Western diet. Intake of cereals vegetables, fruits and legumes has decreased, and at the same time the intake of fat, red meat, eggs and dairy products has increased, providing an unbalanced energy profile [63]. Further, unhealthy practices associated with lower diet quality described in other studies have included sedentary lifestyle [63], smoking [64], low socioeconomic class and educational level [65].

Several limitations of this study must be considered when interpreting our findings. First, its observational nature, with dietary information recorded retrospectively (diet during the year prior to pregnancy self-reported at 12 weeks' gestation). Besides, this would entail a certain recall bias, which could particularly affect women with poor dietary habits, which could decrease the likelihood of obtaining significant differences between groups. Second, we have not taken into account certain factors, such as socio-economic or educational status, or unhealthy practices, such as smoking or sedentary lifestyles that may influence women's dietary habits. The strengths of this study include that we used a previously validated food frequency questionnaire in pregnant women in Spanish Mediterranean area, and that we compared with the FESNAD nutritional recommendations, as it distinguishes nutritional recommendations between adults and pregnant women. Lastly, anthropometric measurements were measured by an accredited anthropometrist and not self-reported.

In conclusion, our data suggest that a high proportion of pregnant women population sample did not have an adequate body composition at the beginning of pregnancy. In addition, they did not reach some recommendations established for this life stage. Composition of the diet regarding macronutrients was not fully in line according to recommendations and inadequate intakes of several micronutrients which are relevant during pregnancy were also found. Adequate maternal body composition and good diet quality in general, and the adherence to the MD in particular, can be associated with a reduced occurrence of some negative

outcomes in babies. In Spain, very limited dietary advice is provided to expectant mothers during the first prenatal visit and Andalusia is no exception to the trend affecting most developed countries. The observed results support the development of nutritional education programs in the future, particularly to improve nutrition before and during pregnancy.

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Conflict of Interests

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

References

1. Vida-Candel, R.; Sanchis-Valero, S.; Mateu-Ciscar, C.; Bellvis-Vázquez, E.; Planells-López, E.; Martínez-Ballester, A.; Gómez-Sánchez, M.J.; Espuig-Sebastian, R. What is the best anthropometric indicator for the pregnancy control? *Nutr. clínica y dietética Hosp.* **2016**, *36(3)*, 87–96, doi:10.12873/363vilacandel.

2. Gao, X.; Yan, Y.; Xiang, S.; Zeng, G.; Liu, S.; Sha, T.; He, Q.; Li, H.; Tan, S.; Chen, C.; et al. The mutual effect of pre-pregnancy body mass index,

waist circumference and gestational weight gain on obesity-related adverse pregnancy outcomes: A birth cohort study. *PLoS One* **2017**, *12*, 1–13, doi:10.1371/journal.pone.0177418.

3. Yu, Z.; Han, S.; Zhu, J.; Sun, X.; Ji, C.; Guo, X. Pre-Pregnancy Body Mass Index in Relation to Infant Birth Weight and Offspring Overweight/Obesity: A Systematic Review and Meta-Analysis. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0061627.

4. Liao, X.P.; Yu, Y.; Marc, I.; Dubois, L.; Abdelouahab, N.; Bouchard, L.; Wu, Y.T.; Ouyang, F.; Huang, H.F.; Fraser, W.D. Prenatal determinants of childhood obesity: A review of risk factors1. *Can. J. Physiol. Pharmacol.* **2019**, *97*, 147–154, doi:10.1139/cjpp-2018-0403.

5. Widen, E..; Kahn, L.G.; Cirillo, P.; Cohn, B.; Kezios, K.L.; Factor-Litvak, P. Prepregnancy overweight and obesity are associated with impaired child neurodevelopment. *Matern. Child Nutr.* **2018**, *14*, doi:10.1111/mcn.12481.

6. Campos, C.A.S.; Malta, M.B.; Neves, P.A.R.; Lourenço, B.H.; Castro, M.C.; Cardoso, M.A. Gestational weight gain, nutritional status and blood pressure in pregnant women. *Rev. Saude Publica* **2019**, *53*, 57, doi:10.11606/S1518-8787.2019053000880.

7. Concina, F.; Pani, P.; Carletti, C.; Rosolen, V.; Knowles, A.; Parpinel, M.; Ronfani, L.; Mariuz, M.; Brumatti, L.V.; Valent, F.; et al. Nutrient intake during pregnancy and adherence to dietary recommendations: The mediterranean phime cohort. *Nutrients* **2021**, *13*, 1434, doi:10.3390/nu13051434.

8. Vivian Ukah, U.; Bayrampour, H.; Sabr, Y.; Razaz, N.; Chan, W.S.; Lim, K.I.; Lisonkova, S. Association between gestational weight gain and severe adverse birth outcomes in Washington State, US: A population-based retrospective cohort study, 2004-2013. *PLoS Med.* **2019**, *16*, 2004–2013, doi:10.1371/journal.pmed.1003009.

9. Bodnar, L.M.; Siminerio, L.L.; Himes, K.P.; Hutcheon, J.A.; Lash, T.L.;

Parisi, S.M.; Abrams, B. Maternal obesity and gestational weight gain are risk factors for infant death. *Obesity* **2016**, *24*, 490–498, doi:10.1002/oby.21335.

10. Widen, E.M.; Gallagher, D. Body composition changes in pregnancy: Measurement, predictors and outcomes. *Eur. J. Clin. Nutr.* 2014, *68*, 643–652.

11. Bzikowska, A.; Czerwonogrodzka-Senczyna, A.; Weker, H.; Wesołowska, A. Correlation between human milk composition and maternal nutritional status. *Rocz. Panstw. Zakl. Hig.* **2018**, *69*, 363–367, doi:10.32394/rpzh.2018.0041.

12. Hernández-Díaz, D.; Sarasa-Muñoz, N.L.; Cañizares-Luna, O.; Orozco-Muñoz, C.; Lima-Pérez, Y.; Machado-Díaz, B. Maternal anthropometry and trophic condition of the newborn. *Rev. Arch Med Camagüey* **2016**, *20(5)*, 477–487.

13. Kannieappan, L.M.; Deussen, A.R.; Grivell, R.M.; Yelland, L.; Dodd, J.M. Developing a tool for obtaining maternal skinfold thickness measurements and assessing inter-observer variability among pregnant women who are overweight and obese. *BMC Pregnancy Childbirth* **2013**, *13*, 1–6, doi:10.1186/1471-2393-13-42.

14. Dodd, J.M.; Kannieappan, L.M.; Grivell, R.M.; Deussen, A.R.; Moran, L.J.; Yelland, L.N.; Owens, J.A. Effects of an antenatal dietary intervention on maternal anthropometric measures in pregnant women with obesity. *Obesity* **2015**, *23*, 1555–1562, doi:10.1002/oby.21145.

15. Orozco-Muñoz, C.; Sarasa-Muñoz, N.L.; Hernández-Díaz, D.; Cañizares-Luna, O.; Álvarez-Guerra González, E.; Artiles-Santana, A. Anthropometric indicators for the characterization of body adiposity in healthy pregnant women at the beginning of pregnancy. *Cuba. Soc. Cardiol.* **2009**, *10(4)*, 274–285.

16. Aktaç, S.; Sabuncular, G.; Kargin, D.; Gunes, F.E. Evaluation of Nutrition

Knowledge of Pregnant Women before and after Nutrition Education according to Sociodemographic Characteristics. *Ecol. Food Nutr.* **2018**, *57*, 441–455, doi:10.1080/03670244.2018.1544561.

17. Ülger, T.G.; Akçali, Ç.; Yardimci, H. *Evaluation of Nutrition Education during Pregnancy and Nutritional Knowledge of Pregnant Women*;

18. Bach-Faig, A.; Berry, E.M.; Lairon, D.; Reguant, J.; Trichopoulou, A.; Dernini, S.; Medina, F.X.; Battino, M.; Belahsen, R.; Miranda, G.; et al. Mediterranean diet pyramid today. Science and cultural updates. *Public Health Nutr.* **2011**, *14*, 2274–2284, doi:10.1017/S1368980011002515.

19. Álvarez, I.Á.; Ontoso, I.A.; Fernández, B.M.; Grima, F.G.; Niu, H. Estudio transversal de los factores que influyen en la adhesión a la dieta mediterránea en el embarazo. *Nutr. Hosp.* **2015**, *31*, 1845–1852, doi:10.3305/nh.2015.31.4.8420.

20. Biagi, C.; Di Nunzio, M.; Bordoni, A.; Gori, D.; Lanari, M. Effect of Adherence to Mediterranean Diet during Pregnancy on Children's Health: A Systematic Review. *Nutrients* **2019**, *11*, 1–25.

21. Schuindt da Silva, V.; Soares-Vieira, M.F. International society for the advancement of kinanthropometry (Isak) global: International accreditation scheme of the competent anthropometrist. *Rev. Bras. Cineantropometria e Desempenho Hum.* **2020**, *22*, 1–6, doi:10.1590/1980-0037.2020v22e70517.

22. Ngel Martínez-González, M.A.´; Corella, D.; Salas-Salvadó, J.; Ros, E.; Covas, M.I.; Fiol, M.; Wärnberg, J.; Arós, F.; Ruíz-Gutiérrez, V.; María Lamuela-Raventós, R.; et al. Cohort Profile: Design and methods of the PREDIMED study. *Int. J. Epidemiol.* **2012**, *41*, 377–385, doi:10.1093/ije/dyq250.

23. Vioque, J.; Navarrete-Muñoz, E.-M.; Gimenez-Monzó, D.; García-De-La-Hera, M.; Granado, F.; Young, I.S.; Ramón, R.; Ballester, F.; Murcia, M.; Rebagliato, M. Reproducibility and validity of a food frequency questionnaire among pregnant women in a Mediterranean area. *Nutr. J.*

2013, *12:26*, doi:10.1186/1475-2891-12-26.

24. Dietowin® 8.0. ©1991-2015 Dietowin SL, Barcelona, España.

25. Dietética, A. Ingestas Dietéticas de Referencia (IDR) para la Población Española, 2010. *Act. Diet.* **2010**, *14*, 196–197, doi:10.1016/S1138-0322(10)70039-0.

26. Fattah, C.; Farah, N.; Barry, S.C.; O'Connor, N.; Stuart, B.; Turner, M.J. Maternal weight and body composition in the first trimester of pregnancy. *Acta Obstet. Gynecol. Scand.* **2010**, *89*, 952–955, doi:10.3109/00016341003801706.

27. Rode, L.; Kjærgaard, H.; Ottesen, B.; Damm, P.; Hegaard, H.K. Association between gestational weight gain according to body mass index and postpartum weight in a large cohort of Danish women. *Matern. Child Health J.* **2012**, *16*, 406–413, doi:10.1007/s10995-011-0775-z.

28. Gilmore, L.A.; Redman, L.M. Weight gain in pregnancy and application of the 2009 IOM guidelines: Toward a uniform approach. *Obesity* **2015**, *23*, 507–511, doi:10.1002/oby.20951.

29. Martínez-Hortelano, J.A.; Cavero-Redondo, I.; Álvarez-Bueno, C.; Garrido-Miguel, M.; Soriano-Cano, A.; Martínez-Vizcaíno, V. Monitoring gestational weight gain and prepregnancy BMI using the 2009 IOM guidelines in the global population: a systematic review and meta-analysis. *BMC Pregnancy Childbirth* **2020**, *20*, doi:10.1186/s12884-020-03335-7.

30. Ramon-Arbues, E.; Abadia, B.M.; Gomez, S.M. Ganancia de peso gestacional y retención de peso posparto en una cohorte de mujeres en aragón (España). *Nutr. Hosp.* **2017**, *34*, 1138–1145, doi:10.20960/nh.749.

31. Ma, D.; Szeto, I.M.Y.; Yu, K.; Ning, Y.; Li, W.; Wang, J.; Zheng, Y.; Zhang, Y.; Wang, P. Association between gestational weight gain according to prepregnancy body mass index and short postpartum weight retention in postpartum women. *Clin. Nutr.* **2015**, *34*, 291–295,

doi:10.1016/j.clnu.2014.04.010.

32. Kac, G.; Benício, M.H.D.A.; Velásquez-Meléndez, G.; Valente, J.G.; Struchiner, C.J. Gestational Weight Gain and Prepregnancy Weight Influence Potpartum Weight Retention in a Cohort of Brazilian Women. *J. Nutr.* **2004**, *134*, 661–666, doi:10.1093/jn/134.3.661.

33. Templin, T.; Hashiguchi, T.C.O.; Thomson, B.; Dieleman, J.; Bendavid, E. The overweight and obesity transition from the wealthy to the poor in low-And middleincome countries: A survey of household data from 103 countries. *PLoS Med.* **2019**, *16*, e1002968, doi:10.1371/journal.pmed.1002968.

34. Norte, A.; Sospedra, I.; Ortíz-Moncada, R. Influence of economic crisis on dietary quality and obesity rates. *Int. J. Food Sci. Nutr.* **2019**, *70*, 232–239, doi:10.1080/09637486.2018.1492523.

35. Llorente-Romero, Z.P.; López-Marín, B.E.; Deossa-Restrepo, G.C.; Arboleda-Montoya, L.M. Cross-sectional study to determine the relationship between anthropometric nutritional status of a group of lactating women of Medellin and the fatty acids profile of their mature breast milk. *Rev. Esp. Nutr. Humana y Diet.* **2021**, *24*, 292–310, doi:10.14306/RENHYD.24.4.797.

36. Organización Mundial de la Salud (OMS). El estado físico: uso e interpretación de la antropometría 2000.

37. Pérez, A.; Murillo, C.; Hernández, R.; Herrera, H.A.; Simón, U.; Básico, B.E. Circumferences to evaluate changes in body mass and amount of total fat of women in second and third trimester of pregnancy. *Nutr Hosp* **2010**, *25*, 662–668, doi:10.3305/nh.2010.25.4.4467.

38. López, L.B.; Calvo, E.B.; Poy, M.S.; del Valle Balmaceda, Y.; Cámera, K. Changes in skinfolds and mid-upper arm circumference during pregnancy in Argentine women. *Matern. Child Nutr.* **2011**, *7*, 253–262, doi:10.1111/j.1740-8709.2009.00237.x.

39. Durnin, J.V.G.A.; Womersley, J. Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 Years. *Br. J. Nutr.* **1974**, *32*, 77–97, doi:10.1079/bjn19740060.

40. Henriksson, P.; Sandborg, J.; Söderström, E.; Leppänen, M.H.; Snekkenes, V.; Blomberg, M.; Ortega, F.B.; Löf, M. Associations of body composition and physical fitness with gestational diabetes and cardiovascular health in pregnancy: Results from the HealthyMoms trial. *Nutr. Diabetes* **2021**, *11*, 16, doi:10.1038/s41387-021-00158-z.

41. World Health Organisation (WHO) WHO | Waist Circumference and Waist–Hip Ratio. Report of a WHO Expert Consultation. Geneva, 8-11 December 2008. **2008**, 8–11.

42. Tran, N.T.; Nguyen, L.T.; Berde, Y.; Low, Y.L.; Tey, S.L.; Huynh, D.T.T. Maternal nutritional adequacy and gestational weight gain and their associations with birth outcomes among Vietnamese women. *BMC Pregnancy Childbirth* **2019**, *19*, 468, doi:10.1186/s12884-019-2643-6.

43. Tomaino, L.; Suárez, D.R.; Domínguez, A.R.; Cruz, L.G.; Díaz, M.R.; Majem, L.S. Adherence to mediterranean diet is not associated with birthweight – results form a sample of canarian pregnant women. *Nutr. Hosp.* **2020**, *37*, 86–92, doi:10.20960/nh.02780.

44. Grillone, L.; Castriotta, L.; Antinolfi, F.; Fasan, J.; Cortelazzo, F.; Madia, A.; Righini, M.; Savoia, A.; Brusaferro, S.; Parpinel, M. Mediterranean diet adherence in pregnant women: results of a survey in North East of Italy. *Eur. J. Public Health* **2020**, *30*, doi:10.1093/eurpub/ckaa166.214.

45. Fernández-Barrés, S.; Romaguera, D.; Valvi, D.; Martínez, D.; Vioque, J.; Navarrete-Muñoz, E.M.; Amiano, P.; Gonzalez-Palacios, S.; Guxens, M.; Pereda, E.; et al. Mediterranean dietary pattern in pregnant women and offspring risk of overweight and abdominal obesity in early childhood: The INMA birth cohort study. *Pediatr. Obes.* **2016**, *11*, 491–499,

doi:10.1111/ijpo.12092.

46. Kominiarek, M.A.; Rajan, P. Nutrition Recommendations in Pregnancy and Lactation. *Med. Clin. North Am.* 2016, *100*, 1199–1215.

47. Mousa, A.; Naqash, A.; Lim, S. Macronutrient and micronutrient intake during pregnancy: An overview of recent evidence. *Nutrients* 2019, *11*, 443.

48. Blumfield, M.L.; Hure, A.J.; MacDonald-Wicks, L.; Smith, R.; Collins, C.E. Systematic review and meta-analysis of energy and macronutrient intakes during pregnancy in developed countries. *Nutr. Rev.* 2012, *70*, 322–336.

49. Rodríguez-Bernal, C.L.; Ramón, R.; Quiles, J.; Murcia, M.; Navarrete-Muñoz, E.M.; Vioque, J.; Ballester, F.; Rebagliato, M. Dietary intake in pregnant women in a Spanish Mediterranean area: As good as it is supposed to be? *Public Health Nutr.* **2013**, *16*, 1379–1389, doi:10.1017/S1368980012003643.

50. Serra Majem, L. Objetivos nutricionales para la población española: consenso de la Sociedad Española de Nutrición Comunitaria 2011. *Rev. española Nutr. comunitaria = Spanish J. community Nutr.* **2011**, *17*, 178–199.

51. Elango, R.; Ball, R.O. Protein and Amino Acid Requirements during Pregnancy. *Adv. Nutr.* 2016, *7*, 839S-844S.

52. Herring, C.M.; Bazer, F.W.; Johnson, G.A.; Wu, G. Impacts of maternal dietary protein intake on fetal survival, growth, and development. *Exp. Biol. Med.* 2018, *243*, 525–533.

53. Brown, L.S. Nutritional requirements during pregnancy. *Nurs. Made Incred. Easy* **2011**, *9*, 21–24, doi:10.1097/01.NME.0000403193.68168.d0.

54. Bartels, Ä.; O'Donoghue, K. Cholesterol in pregnancy: a review of knowns and unknowns. *Obstet. Med.* **2011**, *4*, 147–151,

doi:10.1258/om.2011.110003.

55. Wu, Y.; Sun, G.; Zhou, X.; Zhong, C.; Chen, R.; Xiong, T.; Li, Q.; Yi, N.; Xiong, G.; Hao, L.; et al. Pregnancy dietary cholesterol intake, major dietary cholesterol sources, and the risk of gestational diabetes mellitus: A prospective cohort study. *Clin. Nutr.* **2020**, *39*, 1525–1534, doi:10.1016/j.clnu.2019.06.016.

56. Evans, C.E.L.; Greenwood, D.C.; Threapleton, D.E.; Cleghorn, C.L.; Nykjaer, C.; Woodhead, C.E.; Gale, C.P.; Burley, V.J. Effects of dietary fibre type on blood pressure: A systematic review and meta-analysis of randomized controlled trials of healthy individuals. *J. Hypertens.* 2015, *33*, 897–911.

57. Hutchinson, A.L.O. Requerimientos nutricionales en el embarazo y de dónde suplirlos. *Rev. clínica la Esc. Med.* **2016**, *6*.

58. Aguilar-Cordero, M.J.; Lasserrot-Cuadrado, A.; Mur-Villar, N.; León-Ríos, X.A.; Rivero-Blanco, T.; Pérez-Castillo, I.M. Vitamin D, preeclampsia and prematurity: A systematic review and meta-analysis of observational and interventional studies. *Midwifery* 2020, *87*, 102707.

59. Lee-Sarwar, K.; Litonjua, A.A. As You Eat It: Effects of Prenatal Nutrition on Asthma. *J. Allergy Clin. Immunol. Pract.* **2018**, *6*, 711–718, doi:10.1016/j.jaip.2018.01.026.

60. Allan, K.M.; Prabhu, N.; Craig, L.C.A.; McNeill, G.; Kirby, B.; McLay, J.; Helms, P.J.; Ayres, J.G.; Seaton, A.; Turner, S.W.; et al. Maternal vitamin D and E intakes during pregnancy are associated with asthma in children. *Eur. Respir. J.* **2015**, *45*, 1027–1036, doi:10.1183/09031936.00102214.

61. Ley, S.H.; Hanley, A.J.; Sermer, M.; Zinman, B.; O'Connor, D.L. Lower dietary vitamin E intake during the second trimester is associated with insulin resistance and hyperglycemia later in pregnancy. *Eur. J. Clin. Nutr.* **2013**, *67*, 1154–1156, doi:10.1038/ejcn.2013.185.

62. Domínguez, L.; Fernández-Ruiz, V.; Morales, P.; Sánchez-Mata, M.C.; Cámara, M. Assessment of health claims related to folic acid in food supplements for pregnant women according to the european regulation. *Nutrients* **2021**, *13*, 1–10, doi:10.3390/nu13030937.

63. Olmedo-Requena, R.; Gómez-Fernández, J.; Mozas-Moreno, J.; Lewis-Mikhael, A.M.; Bueno-Cavanillas, A.; Jiménez-Moleón, J.J. Factors associated with adherence to nutritional recommendations before and during pregnancy. *Women Heal.* **2018**, *58*, 1094–1111, doi:10.1080/03630242.2017.1388332.

64. Hu, E.A.; Toledo, E.; Diez-Espino, J.; Estruch, R.; Corella, D.; Salas-Salvado, J.; Vinyoles, E.; Gomez-Gracia, E.; Aros, F.; Fiol, M.; et al. Lifestyles and Risk Factors Associated with Adherence to the Mediterranean Diet: A Baseline Assessment of the PREDIMED Trial. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0060166.

65. Kritsotakis, G.; Chatzi, L.; Vassilaki, M.; Georgiou, V.; Kogevinas, M.; Philalithis, A.E.; Koutis, A. Social capital, tolerance of diversity and adherence to Mediterranean diet: The Rhea Mother-Child Cohort in Crete, Greece. *Public Health Nutr.* **2015**, *18*, 1300–1307, doi:10.1017/S136898001400144X.


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Publicación II

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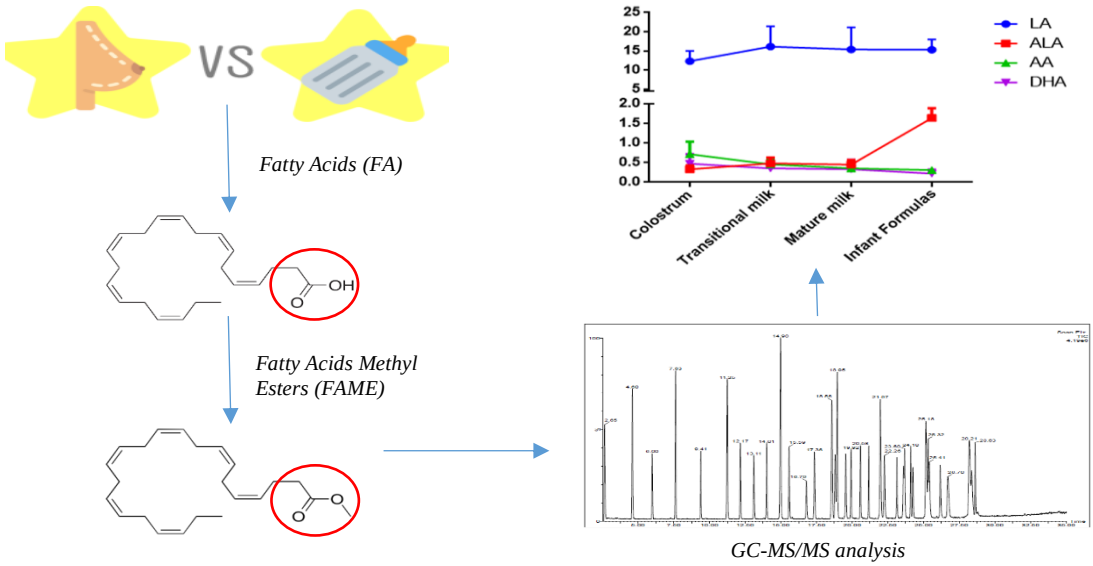


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A Comparison of Changes in the Fatty Acid Profile of Human Milk of Spanish Lactating Women during the First Month of Lactation Using Gas Chromatography-Mass Spectrometry. A Comparison with Infant Formulas

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Abstract

Breastfeeding is the ideal way to provide infants with the nutrients they need for healthy growth and development. Milk composition changes throughout lactation, and fat is one of the most variable nutrients in human milk. The aim of this study was to determine the main differences between the fatty acid (FA) profile of human milk samples (colostrum, transitional, and mature milk group) and infant formulas. Human milk samples were provided by lactating women from Granada. Moreover, different commercial infant formulas were analyzed. FAs were determined using gas chromatography coupled with mass spectrometry. According to the results, oleic acid was the predominant monounsaturated fatty acid (41.93% in human milk and 43.53% in infant formulas), While palmitic acid was the most representative saturated fatty acid (20.88% in human milk and 23.09% in infant formulas). Significant differences were found between human milk groups and infant formulas, mainly in long-chain polyunsaturated FAs (LC-PUFAs). The content of araquidonic acid (AA) and docosahexaenoic acid (DHA) was higher in

human milk (0.51% and 0.39%, respectively) than in infant formulas (0.31% and 0.22%, respectively). Linoleic acid (LA) percentage (15.31%) in infant formulas was similar to that found in human milk (14.6%). However, α-linolenic acid (ALA) values were also much higher in infant formulas than in human milk (1.64% and 0.42%, respectively).

Keywords: fatty acids; human milk; infant formula; GC-MS/MS; LC-PUFA

1. Introduction

Breastfeeding is the ideal way to provide young children with the nutrients they need for healthy growth and development [1]. The World Health Organization (WHO) and the United Nations International Emergency Fund for Children (UNICEF) adopted measures in 2002 to promote global health. As a part of this, the global strategy for optimal food use in infants and young children recommended breastfeeding from the first hour of life, continuing with exclusive breastfeeding during the first 6 months of life and further breastfeeding up to 2 years of age which is supplemented with other foods [2,3]. Data published in 2016 by UNICEF indicates that, overall, only 43% (2 out of 5) of children continue to receive exclusive breastfeeding at 6 months of age [4]. This is because some mothers cannot or choose not to breastfeed and instead use infant formulas (IF) as a substitute. IF are manufactured foodstuffs for feeding newborns and babies that attempts to match, as much as possible, the composition of human milk (HM), especially the lipid profile [5]. Lipids are the largest source of energy in human milk. Triacylglycerols (TAGs) represent 98–99% of total fats and their properties are determined by the length of and degree of unsaturation of fatty acids (FAs) esterified to the glycerol backbone [6]. Milk composition changes throughout lactation, and fat is one of the most variable nutrients in human milk. Lipid content changes according to the stage of lactation and time of day, and during feeding. Whilst in human milk the composition of fatty acids is dynamic and modulated by maternal diet, infant formulas have a much less complex composition tan human milk fat [6–8]. This can provide challenges in attempting to ensure normal or

typical fatty acid intake in breastfed infants and in establishing fatty acid targets when developing infant formulas [8].

Human milk contains the parent essential fatty acids (EFA) linoleic acid (LA, C18:2 n-6) and α-linolenic acid (ALA, C18:3 n-3), and n-3/n-6 very long-chain polyunsaturated fatty acids (LC-PUFA) [9]. A balanced amount of these fatty acids is required for normal maturation and functioning of the nervous system [10]. These fatty acids are also associated with the development of allergic diseases and inflammatory responses [11]. They regulate growth, alongside visual, cognitive, and motor development during the first year of life [12–15].

LC-PUFA are present in human milk in concentrations greater than in other commercially available milks [16]. This is biologically relevant since the two major LC-PUFA are arachidonic acid (AA, C20:4 n-6) and docosahexaenoic acid (DHA, C22:6 n-3). These accumulate in the fetal retina and brain during the last trimester of pregnancy and the early period of postnatal development when milk represents the only source of fat [13,16]. Infant formulas provide all of these essential nutrients for adequate growth and development. These nutrients include the LA and ALA, as required by regulatory agencies [17], although the addition of LC-PUFA as AA and DHA is not mandated [18].

In recent years, variability of the fatty acid profile in human milk and in infant feeding has become very important. For this, the aim of the present study was: (1) To determine the main differences between the fatty acid profile of human milk samples at three stages of lactation (colostrum, transitional, and mature milk group) in women from Granada and compare the fatty acid profile of human milk with different commercially available infant formulas in Spain. This will provide new fatty acid data through use of novel analytical techniques. (2) To study the relationship between different milk samples according to their FA composition and determine which fatty acids are mostly responsible for the differences found between human milk samples and infant formulas.

2. Materials and Methods

2.1. Chemicals and Reagents

Fatty acid methyl esters (Supelco 35 Component FAME Mix) were obtained from Sigma-Aldrich SL (Madrid, Spain). N-hexane, isopropanol, anhydrous sodium sulfate, undecanoic acid (C11:0), methyl acetate, sodium methoxide, methanol, oxalic acid, and diethyl ether were acquired from Panreac Química SL (Panreac AppliChem, Barcelona, Spain) and Sigma-Aldrich (Merck, Munich, Germany). All reagents were of analytical grade.

2.2. Subject

The present research was carried out in the obstetrics department of one of the six regional hospitals of Andalusia "Hospital Universitario Virgen de las Nieves". Of all of the participants who formed part of this study, the three samples of human milk (colostrum, transitional, and mature milk) were donated by only thirteen lactating mothers due to the complexity of obtaining these samples. Details of the study were explained to all mothers who voluntarily gave written consent to participate. The characteristics of the study sample were as follows: women aged 18–40, who had given birth to healthy babies in the "Hospital Universitario Virgen de las Nieves". This also constituted the inclusion criteria.

Ethical approval for this study was provided by the "Hospital Virgen de las Nieves" Ethics and Scientific Committee and the trial was registered at ClinicalTrials.gov.

The mean age of participating mothers was 32 ± 4.7 years, of which eight reported this being their first lactation/child (N1, N2, N3, N5, N6, N10, N11, and N12). The main characteristics of sampled mothers are shown in **Table 1**.

Table 1. Characteristics the sampled mothers.

	Mean	SD	Minimum	Maximum
Age (years)	32	4.7	26	40
Height (cm)	163.6	7.00	150.0	172.0
Weight (kg)	69.6	13.50	47.4	90.8
BMI (kg/cm^2)	26.2	5.33	18.4	33.4
Birth weight (kg)	2.9	0.79	1.2	3.8
Parity	1.6	0.92	1	3

BMI: body mass index; SD: standard deviation.

2.3. Milk Samples

Thirty-eight milk samples were categorized according to the length of time post-partum. Samples obtained between the 1st and 5th day post-delivery were assigned to the colostrum group (n = 13, 1.75 ± 0.53 days) with the Marmet manual extraction technique being used for delivery [19]; samples obtained between the 6th and 15th day post-delivery were assigned to the transitional group (n = 13, 12.83 ± 4.43 days); and samples obtained after the 15th day post-delivery were assigned to the mature milk group (n = 12, 24.75 ± 9.77 days). For both of the latter groups, milk extraction was achieved by means of a mechanical breast pump (Medela®, Medela, Switzerland) following the manufacturer's instructions. Milk from each breast was obtained at both the beginning and end of each feed.

All human milk samples collected from study participants were aliquoted and immediately stored at - 70 ºC until extraction.

In addition to the human milk samples, seven different initiation formulas (0–6 months) for full-term infants were also analyzed. These are the most commonly consumed infant formulas and include brands such as Nestlé, Combiotik, Blemil, Nutribén, and Almirón, and were purchased in different commercial and agricultural areas of the market. They were randomly coded as IF 1–7 (**Table S1**). All samples were analyzed in triplicate.

2.4. Total lipid Content and Fat Extraction

Infant formulas were reconstituted in water following the manufacturer's instructions. The human milk samples and infant formulas were extracted with a mixture of solvents according to the method of Hara and Radin [20].

First, 500 µL of milk was mixed with 1.8mL of n-hexane, isopropanol (3:2, v/v), then and homogenized. Next, 1.2mL of aqueous sodium sulfate was added and centrifuged to separate the layers. The organic layer was evaporated using nitrogen (N2). The aqueous phase was re-extracted, and the lipid recovered and stored at -18 ºC in n-hexane: isopropanol (4:1, v/v) until the fatty acid methyl esters (FAME) were prepared.

2.5. Preparation of Methyl Esters for Gas Chromatographic Analysis

Fatty acid methyl esters (FAME) were obtained after following the base-catalyzed transesterification method described by Christie WW. [21]. The previously evaporated sample was dissolved in 0.5 mL of n-hexane. 40 µL of methyl acetate and 80 µL of sodium methoxide in methanol (0.5M) were added. The solution was mixed for 30 s and left for 15 min at room temperature, at which time the reaction was stopped by adding 30 µL of a saturated solution of oxalic acid in diethyl ether. After a brief agitation, the mixture was centrifuged at 1500x g for 2 min and the supernatant, containing FAME, was collected into chromatography vials.

2.6 Chromatographic and Mass Operating Conditions

Determination of fatty acids was conducted using a mass spectrometer with tandem quadrupole model QUATTRO micro GC (WATERS, Milford, MA, USA), equipped with a split/splitless injector. A Flame Ionization Detector (FID) type detector with ionization mode EI+ at 300 ºC was used, measuring a mass range of 45 to 450 and a 35 min chromatogram, and a capillary column with a length of 30.0 m and a diameter of 250 µm. The temperature of the initial oven was 100 ºC

with a maximum of 350 ºC. The injection volume was 1 µL and the split ratio was set at 10.0 and split flow 10.0 mL/min.

All data was collected using MassLynx V4.1 software (Waters Inc., 2010, Milford, MA, USA). Peaks were identified by comparing retention times with standard mixtures. Fatty acids were quantified by comparing the peak area of each compound with that of the standard.

2.7. Analytical Validation

Validation was carried out by studying the parameters of linearity, linear range, limit of detection (LOD), and limit of quantification (LOQ), following the guidelines for the validation of analytical methods (AOAC, 2012). Linearity in all fatty acids was achieved in their dynamic range of between 250–1000 ppm. A total of 35 fatty acids were identified and quantified in the different milk samples.

This is a highly sensitive and precise analytical method based on mass spectrometry combined with high resolution separation methods.

2.8. Statistical Analyses

The homogeneity of variance was assessed using the Levene test and normality of data distribution of the samples was examined with the Shapiro–Wilk test.

Results of individual FA content of the different human milk samples and infant formulas were analyzed using one-way ANOVA followed by the Tukey test in order to compare significant variations between means ($p < 0.05$). Moreover, in order to verify the capacity of the FA analysis as a tool for human milk characterization, a multivariate discriminant analysis was performed. Graphical representation of this analysis allows the similarity of samples to be assessed according to their FA composition.

The significance level was set at 5% ($p < 0.05$) in all tests. SPSS 15.0 for Windows (IBM SPSS Inc., Chicago, IL, USA) was used for data analyses.

In order to complete the examination of the fatty acid profile of human milk and infant formulas, the results obtained were subjected to discriminant analysis.

This analysis is aimed at supporting the interpretation of complex multivariate data. It considers all observations as a single group with the aim of uncovering the variables with the greatest influence, so that observations can be grouped and predictive groups formed. The data obtained were expressed as means ± standard deviations (SD).

3. Results

3.1. Analytical Validation

A chromatogram of the standards of these fatty acids over a period of 35 min is shown in **Figure 1**. **Table 2** shows the linear range, retention time, adjusted linear equations, correlation coefficients, detection limits, and quantification limits of the standards. A favorable correlation between the experimental data and the theoretical values was obtained with good linearity in the ranges evaluated and correlation coefficients (R2) greater than 0.998.

The highest LOD and LOQ corresponded to α-linolenic acid (C18:3 n-3) (1.8403 and 6.1344) and the lowest to arachidonic acid (C20:4 n-6) (0.1345 and 0.4482). These LOD and LOQ were compared with values previously reported by other authors for fatty acids using GC-MS/MS methods.

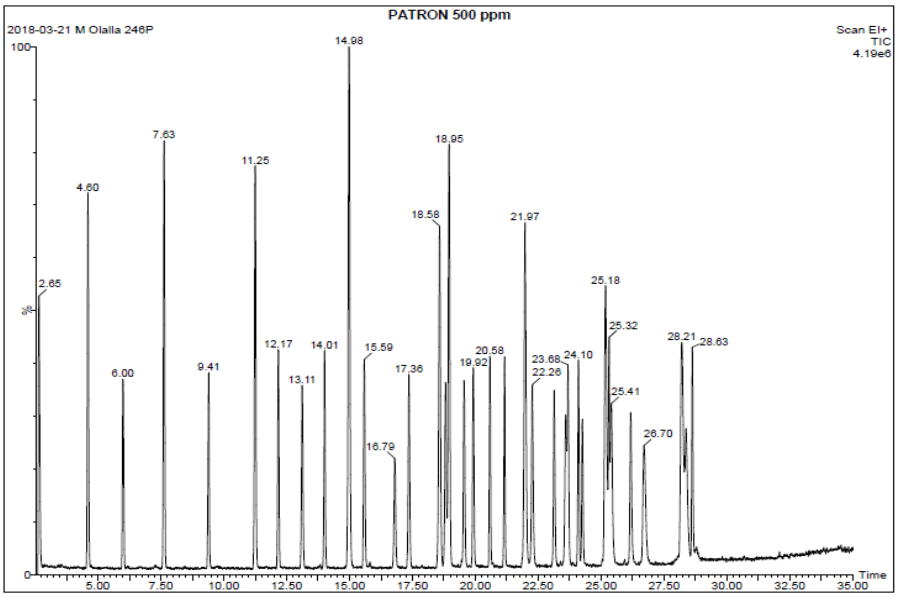

Figure 1. Chromatogram of a 37 Component FAME Mix of standards

Table 2. Quality parameters for the chromatographic determination of fatty acids.

Fatty Acids	Linear range (ppm)	$t_r \pm SD$	Linear eq
Caprylic acid (C8:0)	250 – 1000	2.647 ± 0.006	y = 271.7940x
Capric acid (C10:0)	250 – 1000	4.597 ± 0.006	y = 362.0136x
Undecanoic acid (C11:0)	250 – 1000	6.000 ± 0.000	y = 183.0014x
Lauric acid (C12:0)	250 – 1000	7.623 ± 0.006	y = 403.2163x
Tridecanoic acid (C13:0)	250 – 1000	9.400 ± 0.010	y = 197.6763x
Myristic acid (C14:0)	250 – 1000	11.247 ± 0.006	y = 414,5987x
Myristoleic acid (C14:1)	250 – 1000	12.167 ± 0.006	y = 217,1785x
Pentadecanoic acid (C15:0)	250 – 1000	13.117 ± 0.012	y = 211,0244x
Cis-10-pentadecenoic acid (C15:1)	250 – 1000	14.010 ± 0.010	y =221,6763 x
Palmitic acid (C16:0)	250 – 1000	14.980 ± 0.010	y = 663,2947x
Palmitoleic acid (C16:1 n-9 Z)	250 – 1000	15.587 ± 0.006	y = 227,0054x
Margaric acid (C17:0)	250 – 1000	16.793 ± 0.006	y = 147,9136x
Cis-10-heptadecenoic acid (C17:1)	250 – 1000	17.357 ± 0.006	y = 229,9246x
Stearic acid (C18:0)	250 – 1000	18.577 ± 0.006	y = 464,1352x
Elaidic acid (C18:1 n-9 E)	250 – 1000	18.817 ± 0.006	y = 158.1385x
Oleic acid (C18:1 n-9 Z)	250 – 1000	18.953 ± 0.006	y = 425.2101x
Linolelaidic acid (C18:2 n-6 E)	250 – 1000	19.553 ± 0.006	y = 230.1367x
Linoleic acid (C18:2 n-6 Z)	250 – 1000	19.920 ± 0.000	y = 223.0317x
γ-linolenic acid (C18:3 n-6, GLA)	250 – 1000	20.580 ± 0.000	y = 212.2357x
α-linolenic acid (C18:3 n-3, ALA)	250 – 1000	21.160 ± 0.000	y = 202.2944x
Arachidic acid (C20:0)	250 – 1000	21.973 ± 0.015	y = 494.0689x
Cis-11-eicosenoic acid (C20:1)	250 – 1000	22.263 ± 0.006	y = 241.1562x
Cis-11,14-eicosadienoic acid (C20:2)	250 – 1000	23.130 ± 0.010	y = 211.9830x
Heneicosanoic acid (C21:0)	250 – 1000	23.590 ± 0.010	y = 150.6156x
Cis-8,11,14-eicosatrienoic acid (C20:3 n-6, DGLA)	250 – 1000	23.687 ± 0.006	y = 197.8801x
Arachidonic acid (C20:4 n-6, AA)	250 – 1000	24.103 ± 0.006	y = 194.4791x

Cis-11,14,17-eicosatrienoic acid (C20:3)	250 – 1000	24.253 ± 0.006	y = 141.3401x -
Behenic acid (C22:0)	250 – 1000	25.180 ± 0.010	y = 408.6182x -
Erucic acid (C22:1 n-9)	250 – 1000	25.313 ± 0.006	y = 221.7645x -
5,8,11,14,17-eicosapentadienoic acid (C20:5 n-3, EPA)	250 – 1000	25.407 ± 0.006	y = 199.8515x -
Cis-13,16-docosadienoic acid (C22:2)	250 – 1000	26.173 ± 0.006	y = 213.4314x -
Tricosanoic acid (C23:0)	250 – 1000	26.710 ± 0.010	y = 228.7863x -
Lignoceric acid (C24:0)	250 – 1000	28.213 ± 0.006	y = 443.1121x -
Nervonic acid (C24:1)	250 – 1000	28.373 ± 0.006	y = 228.3534x -
Cis-4,7,10,13,16,19-docosahexadienoic acid (C22:6 n-3, DHA)	250 – 1000	28.630 ± 0.000	y = 215.8342x -

3.2. Fatty Acid Profile

The fatty acid composition of colostrum, transitional, and mature milk samples of women from Granada, as well as that of infant formulas marketed are presented in **Tables 3–5**. Of the thirty-five standard fatty acids, a total of twenty-eight fatty acids were identified and quantified in our human milk and infant formula samples using GC-MS/MS analysis. These were grouped into saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated (PUFAs) fatty acids. **Figure 2** shows the distribution of the main fatty acid groups in the different samples of human milk and infant formulas.

Table 3. Saturated fatty acid (SFAs) composition of colostrum, transitional and mature milks, and infant formulas (% wt=wt).

Fatty Acids (FAs)	Colostrum	Transitional milk	Mature milk	Infant Formula
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C8:0	0.72±0.634	0.51±0.130	0.64±0.197	0.77±0.271
C10:0	$0.18^{a,b,d}$±0.160	1.06±0.268	1.06±0.173	1.04±0.424
C12:0	$1.43^{a,b,d}$±0.768	4.65^{e}±1.578	3.82^{f}±1.158	7.45±2.378
C13:0	0.00	0.01±0.020	0.00	0.01±0.010
C14:0	3.79±1.165	4.56±1.420	3.68±1.326	3.43±1.517
C15:0	0.15±0.140	0.22±0.009	0.18±0.093	0.15±0.282
C16:0	$23.75^{a,b}$±2.032	20.76^{c}±2.031	$18,13^{f}$±2.332	23.09±2.669
C17:0	0.09±0.133	0.07±0.104	0.08±0.109	0.05±0.109
C18:0	6.23^{d}±1.192	5.54^{e}±0.906	5.46^{f}±0.781	3.81±0.516
C20:0	0.04^{d}±0.096	0.02^{e}±0.074	0.03^{f}±0.075	0.33±0.110
C24:0	0.04±0.144	0.00	0.00	0.00
SFA	36.05±5.266	37.49±3.153	33.19±4.899	37.31±4.522
SC-SFA (C8-C10)	$0.97^{a,b,d}$±0.622	1.51±0.346	1.70±0.266	2.01±0.465
MC-SFA (C12-C16)	27.71^{d}±3.305	30.38^{c}±2.856	26.05^{f}±4.370	33.76±4.219
LC-SFA (> C17)	$6.41^{b,d}$±1.153	5.35±0.901	5.55^{f}±0.735	4.15±0.451

SD: standard deviation; SFA: saturated FAs; SC-SFA: short-chain SFA; MC-SFA: medium-chain SFA; LC-SFA: long-chain SFA. (a) Significant differences ($p < 0.05$) between colostrum and transitional milk groups, (b) Significant differences ($p < 0.05$) between colostrum and mature milk groups, (c) Significant differences ($p < 0.05$) between transitional and mature milk groups, (d) Significant differences ($p < 0.05$) between colostrum and infant formula groups, (e) Significant differences ($p < 0.05$) between transitional and infant formula groups, (f) Significant differences ($p < 0.05$) between mature and infant formula groups.

Table 4. Monounsaturated fatty acid (MUFAs) composition of colostrum, transitional, mature milk and infant formulas (% wt=wt).

Fatty Acids (FAs)	Colostrum	Transitional milk	Mature milk	Infant Formula
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C14:1	0.02[a]±0.064	0.08±0.085	0.06±0.0796	0,18±0.328
C15:1	0.00	0.00	0.00	0,01±0.034
C16:1 n-9 Z	1.52[d]±0.320	1.23[e]±0.667	1.45[f]±0.416	0,37±0.159
C18:1 n-7 E	1.42[a,b,d]±0.148	1.12[e]±0.161	1.11[f]±0.099	0,47±0.103
C18:1 n-9 Z	42.11±5.228	39.81±4.608	43.88±7.041	43.54±3.428
C20:1 n-9	0.97[a,b,d]±0.253	0.52[e]±0.173	0.38±0.097	0,36±0.046
C22:1 n-9	0.00	0.03±0.066	0.01±0.035	0,00
MUFA	45.99±5.750	43.34±4.754	46.99±6.796	44.86±3.689

SD: standard deviation; MUFA: monounsaturated fatty acids. (a) Significant differences ($p < 0.05$) between colostrum and transitional milk groups, (b) Significant differences ($p < 0.05$) between colostrum and mature milk groups, (c) Significant differences ($p < 0.05$) between transitional and mature milk groups, (d) Significant differences ($p < 0.05$) between colostrum and infant formula groups, (e) Significant differences ($p < 0.05$) between transitional and infant formula groups, (f) Significant differences ($p < 0.05$) between mature and infant formula groups.

Table 5. Polyunsaturated fatty acid (PUFAs) composition of colostrum, transitional and mature milk, and infant formulas (% wt=wt).

Fatty Acids (FAs)	Colostrum	Transitional milk	Mature milk	Infant Formula
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C16:2 n-4	0.00	0.00	0.01±0.032	0.00
C18:2 n-6 E	0.01±0.047	0.00	0.00	0.03±0.072
C18:2 n-6 Z (LA)	12.32±2.643	16.10±5.325	15.38±5.754	15.31±2.667
C18:3 n-6 (GLA)	0.00[a,b]	0.04[e]±0.064	0.07[f]±0.087	0.00
C18:3 n-3 (ALA)	0.33[d]±0.103	0.48[e]±0.149	0.45[f]±0.121	1.64±0.247
C20:2	0.98[a,b,d]±0.282	0.64[e]±0.332	0.40[f]±0.084	0.00
C20:3 n-6 (DGLA)	0.74[a,b,d]±0.274	0.49[e]±0.139	0.34[f]±0.098	0.00
C20:4 n-6 (AA)	0.72[a,b,d]±0.321	0.46±0.114	0.36±0.073	0.31±0.078
C22:2	0.03±0.084	0.00	0.00	0.00
C22:6 n-3 (DHA)	0.47±0.240	0.36±0.140	0.33±0.240	0.22±0.099
PUFA	15.94±2.504	17.80±5.038	17.48±5.828	17.55±2.667
UFA	62.68±6.83	63.07±3.62	65.78±5.88	60.94±6.22
SFA/UFA	0.62±0,20	0.59±0,09	0.53±0,14	0.64±0.17
n-3 PUFA	0.77[d]±0.308	0.84[e]±0.165	0.79[f]±0.350	1.81±0.232
n-6 PUFA	14.11[a]±2.333	16.38±4.979	16.14±5.907	14.83±1.756
LC-PUFA (C20-C24)	3.06[a,b,d]±0.641	1.85[c,e]±0.381	1.43[f]±0.260	0.54±0.158
LA/ALA	38.15[d]±17.980	32.03[e]±10.030	31.30[f]±15.370	9.53±0.667
AA/DHA	1.74±0.812	1.54±0.683	1.43±1.016	1.33±0.361

SD: standard deviation; PUFA: polyunsaturated fatty acids; UFA: unsaturated FAs; SFA/UFA: saturated FAs / unsaturated FAs; LC-PUFA: long-chain PUFA; LA/ALA: linoleic acid / α-linolenic acid; AA/DHA: araquidonic acid / docoxahexaenoic acid. (a) Significant differences ($p < 0.05$) between colostrum and transitional milk groups, (b) Significant differences ($p < 0.05$) between colostrum and mature milk groups, (c) Significant differences ($p < 0.05$) between transitional and mature milk groups, (d) Significant differences ($p < 0.05$) between colostrum and infant formula groups, (e) Significant differences ($p < 0.05$) between transitional and infant formula groups, (f) Significant differences ($p < 0.05$) between mature and infant formula groups.

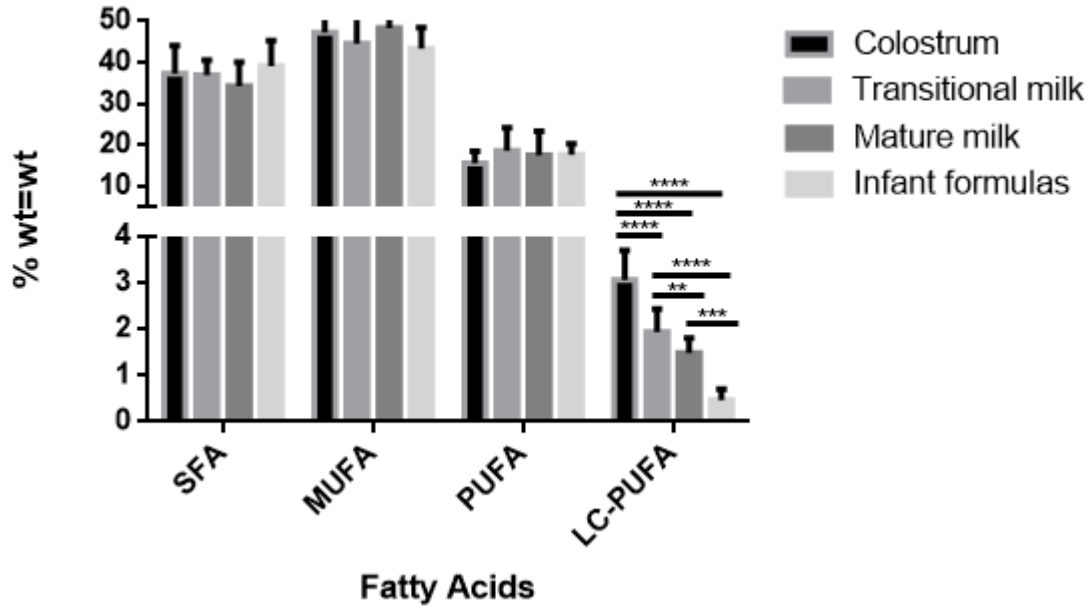

Figure 2. Fatty acid profile in human milk and infant formulas. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids and LC-PUFA: long-chain polyunsaturated fatty acids. Significant differences (** $p < 0.01$), (*** $p < 0.001$) and (**** $p < 0.0001$).

3.2.1. Saturated Fatty Acids (SFAs)

The saturated fatty acids identified are shown in **Table 3.** The major SFA was C16:0, which is seen to decrease significantly when comparing the colostrum group to the transitional group ($p < 0.05$) and the mature group ($p < 0.0001$) within the milk samples (23.75%, 20.76% to 18.13% respectively). Nevertheless, a significant increase was observed between the colostrum group and the transitional

and mature milk group ($p < 0.0001$) for C10:0 (0.18%; 1.06%, and 1.06%, respectively) and C12:0 (1.43%; 4.65%, and 3.82%, respectively).

Infant formulas showed significantly lower values of C18:0 than the colostrum ($p < 0.0001$), transitional ($p < 0.01$), and mature ($p < 0.01$) groups. In contrast, higher values of C12: 0 were found in infant formulas than the colostrum ($p < 0.0001$), transitional ($p < 0.01$), and mature ($p < 0.001$) groups. In addition, higher levels of C10:0 were found relative to colostrum group ($p < 0.0001$), and higher C16: 0 relative to the transitional ($p < 0.05$) and mature ($p < 0.001$) groups.

Thus, infant formulas have a higher proportion of SC-SFA (0.97% for colostrum, 1.51% for transitional milk, 1.70% for mature milk, and 2.01% for infant formulas) and MC-SFA (27.71% for colostrum, 30.38% for transitional milk; 26.05% for mature milk and 33.76% for infant formulas) tan human milk. In contrast, they have a lower percentage of LC-SFA (6.41% for colostrum, 5.35% for transitional milk, 5.55% for mature milk, and 4.15% for infant formulas).

The other saturated fatty acids and overall SFA did not show significant differences between the milk groups.

3.2.2. Monounsaturated Fatty Acids (MUFAs)

The monounsaturated fatty acids identified are shown in **Table 4**. The major MUFA was C18:1 n-9 Z, with similar values being found between human milk groups and infant formulas.

There was a decreasing trend between the colostrum group to the transitional and mature milk groups for C16:1 n-9 Z (1.52%, 1.23%, and 1.45%, respectively); C18:1 n-7 E (1.42%, 1.12%, and 1.11%, respectively); and C20:1 n-9 (0.97%, 0.52%, and 0.38%, respectively).

In addition, significantly higher percentages were observed for these three fatty acids in human milk samples than in infant formulas ($p < 0.01$) (0.37% for C16:1 n-9 Z; 0.47% for C18:1 n-7 E; and 0.36% for C20:1 n-9).

The other monounsaturated fatty acids and the total MUFA did not show significant differences between the milk groups and infant formulas.

3.2.3. Polyunsaturated Fatty Acids (PUFAs)

The polyunsaturated fatty acids identified are shown in Table 5. The major PUFA was C18:2 n-6 Z, (LA), with similar values being found between human milk groups and infant formulas. In contrast, C18:3 n-3 (ALA) showed significantly higher values in infant formula than in the different human milk groups ($p < 0.0001$). Thus, the LA/ALA ratio showed statistically significant differences between the human milk group and infant formulas ($p < 0.001$).

C20:2 and C20:3 n-6 showed a significant decrease between the colostrum group and the transitional ($p < 0.01$) and mature ($p < 0.0001$) milk groups. Whilst measurements for C22:6 n-3 (DHA) did not show statistically significant differences, instead presenting similar trends between groups.

With regards to LC-PUFA (C20–24), greater variability was seen between the human milk and infant formula groups. **Figure 3** shows the variability of the main PUFAs in human milk and infant formulas. The latter group did not contain C20:2, GLA, and DGLA, whilst the percentages of AA and DHA were significantly lower than that found in human milk.

The remaining polyunsaturated fatty acids and overall PUFA did not show significant differences between the milk groups and infant formulas.

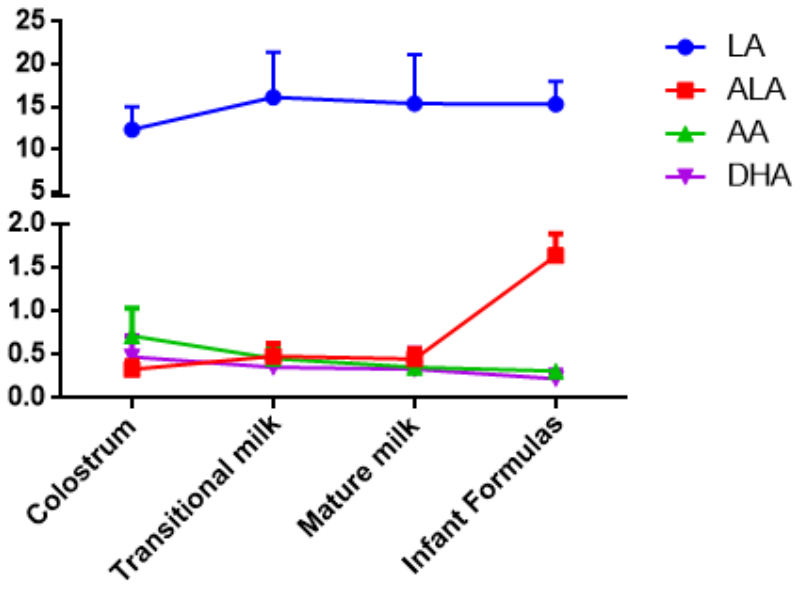

Figure 3. Trend of the majority of long-chain polyunsaturated fatty acids (LC-PUFAs) in human milk (colostrum, transitional and mature milk) and infant formulas. LA: linoleic acid; ALA: α-linolenic acid; AA: araquidonic acid; DHA: docosahexaenoic acid.

3.3. Discriminant Analysis

Forty-five cases were used to develop a model that discriminates between the human milk and infant formula samples. Thirty-six predictor variables were introduced. **Table 6** displays the discriminant functions obtained in the present analysis.

Table 6. Discriminant functions that predict the types of milks analyzed based on fatty acid levels.

Discriminant functions	Eigenvalue	Relative percentage (%)	Canonical correlation	Lambda de Wilks	Chi-Cuadrada	p-value
1	126.3990	87.58	0.9961	0.0002	210.5987	0.0000*
2	15.9167	11.03	0.9610	0.0197	94.2629	0.0282*
3	2.0021	1.39	0.8166	0.3331	26.3837	0.8214

*: Significant differences ($p < 0.05$)

In this case, our first discriminant function has a relative variance percentage close to 100% (87.58%), while the second and third discriminant function is only able to explain 11.03% and 1.39% of the variance of the data, respectively. The p-value of discriminant functions 1 and 2 are less than 0.05, whereby both discriminant functions are statistically significant with a 95.0% confidence level.

In addition, the canonical correlation of the first and second functions is closer to 1. These functions allowed 91.1% of groups to be classified (100% for infant formulas versus 83.3% of mature human milk). Finally, Wilk's Lambda is also closer to 0 in the first two discriminant functions.

Taken together, results indicate that our first two discriminant functions are capable of separating the data much better than the third function.

A graphical representation of the two dimensions is shown in **Figure 4**, according to the first and second discriminating functions.

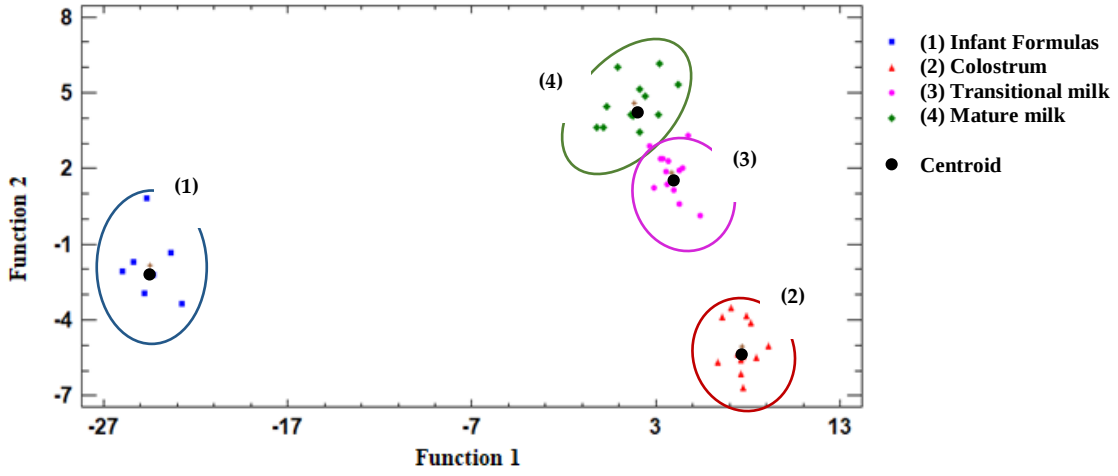

Figure 4. Representation of the different groups of milks studied (colostrum, transitional milk, mature milk and infant formulas) based on the two significant functions according to the discriminant analysis performed. Centroid: average value of the discriminant function for each one of the samples.

4. Discussion

The results of the present study show that the average characteristics relating to the fatty acid composition of human milk can be summarized as follows. Palmitic acid (≈20%), oleic acid (≈44%), and linoleic acid (≈15%) were the predominant fatty acids in the colostrum, transitional, and mature milk groups, and in the infant formulas. These represented between 76.67% and 81.94% of the total fatty acids. This trend is similar to that found by Yang et al., which was between 77.98% and 78.57% [22]. Next, we consider the results relating to myristic acid (≈4%) and stearic acid (≈6%).

All of these values were very similar to those described by other authors. Yang et al. [23] found similar values in palmitic acid (21.77%), myristic acid (3.91%), and stearic acid (5.14%); but found lower values in oleic acid (33.32%) and higher values in linoleic acid (22.54%). However, Zou et al. [24] reported more similar values with respect to palmitic acid (20.3%), linoleic acid (17.1%), myristic acid (4.9%), and stearic acid (6.0%). On the other hand, they also identified lower percentages of oleic acid (34.9%).

4.1. Saturated Fatty Acids (SFAs)

Total saturated fatty acid (SFA) content remained stable across all of the milk groups [24]. Human milk typically contains approximately 34% to 47% saturated fatty acids, mainly palmitic acid (17–25%) [7,22]. Similar values were found in other studies [10,13,25]. The colostrum and infant formula groups presented similar percentages of this SFA (23.75% and 23.09%, respectively). However, in human milk, palmitic acid is esterified with triglycerides in position 2 (position β), whereas unmodified milk fat of infant formulas is esterified in positions 1 and 3. The specific distribution of fatty acids in the triglyceride plays a key role in the digestion and absorption of lipids. It appears that this modification of fat decreases the stability and quantity of calcareous soaps in feces, thus decreasing its consistency [24–26].

Other fatty acids, such as C10.0 and C12:0 increased from one lactation stage to the next. This trend was very similar to that found in another Spanish study

[10] (0.66%, 1.66%, and 1.63%) for C10:0 and (3.49%, 6.97%, and 6.28%) for C12:0 in colostrum, transitional, and mature milk, respectively.

Therefore, infant formulas have a higher proportion of SC-SFA and MC-SFA tan human milk, possibly due to the addition of vegetable oils [26]. MC-SFA are commonly supplemented and incorporated into these infant formulas because they can be directly absorbed by the portal vein and rapidly generate energy for infant [27]. However, this has been related to an increase in the level of total and low density lipoprotein (LDL) cholesterol concentration in plasma leading to a high risk of cardiovascular disease. On the other hand, LC-SFA is reported to be neutral concerning its effects on lipoprotein cholesterol levels [28].

Despite this, overall lauric and myristic acid content is recommended to not exceed 20% of total fat in infant formulas, with content being 10.88% in the present case [29].

4.2. Monounsaturated Fatty Acids (MUFAs)

Total monounsaturated fatty acid (MUFA) remained stable in all milk samples (45.99% for colostrum, 43.34% for transitional, and 46.99% for mature milk group). This trend has also been described by other authors [30]. Barreito et al. reported a percentage of 44.1%.

Oleic acid (C18:1 n-9) constitutes more than 90% of the total MUFAs, finding similar values in human milk groups and infant formulas. This is fundamentally linked to the consumption of olive oil, representing levels greater than 40% [6,15].

These values are higher than those found in other studies, both Spanish and European studies, possibly because southern Spain shows higher levels of adherence to the Mediterranean diet [31]. This may be due to the potentially high adherence to the Mediterranean diet of our participants which is composed of foods rich in oleic acid, especially for the high consumption of extra virgin oil. According to the data offered by ministry of agriculture, food and environment (MAGRAMA) in 2018, Andalusia purchases and consumes more extra virgin olive oil than any

other autonomous community, accounting for 25.99% of the volume distributed across Spain (L) [32].

C16:1 n-9, C18:1 n-7, and C20:1 n-9 were the three most abundant MUFAs in human milk after oleic acid, with a percentage of around 3–4%, whilst the percentage present in infant formulas was 1.2%.

The results of a previous study indicate that the majority of commercially available IF do not contain scientifically recommended amounts of vaccenic acid, and that their fatty acid composition is deficient in comparison with human milk [33]. Vaccenic acid is the major trans-fatty acid in ruminant milk fat. It is unique in that it may provide cis-9, trans-11-octodecadienoic (cis 9, trans 11-C18:2; also known as rumenic acid) to the consumer through endogenous desaturation by the D-9 desaturase enzyme [34]. Rumenic acid is the most common conjugated linoleic acid (CLA) isomer and it has been shown to promote various beneficial health-related effects, including anti-carcinogenic, anti-atherosgenic, anti-diabetic, and immune-modulating effects, in addition to effects on body composition and fat metabolism [35–37].

4.3. Polyunsaturated Fatty Acids (PUFAs)

Concentrations of PUFA in human milk are relatively stable during the first year of life. AA typically constitutes 1% (0.72%) in colostrum and 0.5% in mature milk (0.36%); DHA is approximately equivalent to 0.5% in colostrum (0.47%) and 0.25% in mature milk (0.33%) [16]. Also, it typically contains approximately 12% to 26% n-6 PUFA and 0.8% to 3.6% n-3 PUFA [7].

Moreover, essential fatty acid (EFA) content varies depending on the stage of lactation, particularly LA levels (12.32% for colostrum; 16.10% for transitional; and 15.38% for mature milk). Zou et al. Showed this trend in the results of their study (21.01%, 21.05%, and 25.58% for colostrum, transitional, and mature milk, respectively) [23], although the values of our study were lower. Values, however, were similar to those reported by Ribeiro et al. [38] (15.46% for samples 7 days postpartum and 16.20% for samples at 4 weeks postpartum).

DHA, AA, and DGLA content also showed a decrease according to the stage of lactation [38,39], finding higher values in colostrum than in transitional and mature milk groups [23]. Only a slight increase in GLA was observed, correlated with a reduction in inflammation after childbirth. GLA is the substrate for DGLA synthesis, another anti-inflammatory fatty acid [36,37,40] Fu et al. Evaluated the DHA and arachidonic acid (AA) levels in human milk according to country and region, reporting similar values (0.42% for DHA and 0.71% for AA) for Spain to those found in the present study [41].

However, higher levels of ALA were observed in mature milk compared to the colostrum and transitional groups, as has also been described by other authors [15].

In infant formulas, LA percentage (15.31%) was similar to that found in human milk (14.6%). However, the content of AA (0.31%) and DHA (0.22%) was lower than in human milk (0.51% and 0.39%, respectively), especially when considering the colostrum stage. Conversely, ALA values were also much higher in infant formulas than in human milk (1.64% and 0.42%, respectively). DHA experiences a longer and more complicated synthesis which limits the conversion rate of ALA/DHA [42]. This trend is shown in **Figure 2**.

For this reason, infant formulas present a significantly higher percentage of n-3 PUFA than that seen in the different groups of human milk. This is due to their high amount of α-linolenic acid.

Codex Alimentarius stipulate that the AA and DHA content of infant formulas should, at least, have the same concentration [43]. The content of these fatty acids in our infant formulas was 0.31% and 0.22%, respectively. These values being similar to those described by Chen et al. [27], which reported values of 0.41% for AA and 0.23% for DHA.

These values are below estimated averages for AA and DHA in human milk samples studied by Brenna et al. [42]. These authors included 84 studies and reported that the world wide mean concentration in human milk was 0.47% ± 0.13% for AA and 0.32% ± 0.22% for DHA. These values are very similar to those uncovered in the present sample (0.51% for AA and 0.39% for DHA).

The AA/DHA ratio did not vary with increasing milk maturation, however the LA/ALA ratio was significantly higher in the colostrum, transitional, and mature milk groups than in infant formulas (38.15%, 32.03%, 31.30%, and 9.53%, respectively). It is important to note that this ratio is within the guideline range of 5:1 and 15:1 suggested by the ESPHGAN Committee on Nutrition (European Society for Pediatric Gastroenterology Hepatology and Nutrition) [29].

4.4. Discriminant Analysis

An eigenvalue in discriminant analysis is the characteristic root of each function, that is, it is an indication of how well that function differentiates the groups, and the larger the eigenvalue is, the better the function differentiates the groups [44].

In the present study, the infant formula group shows left-handside displacement on the graph, while the human milk groups are found to the right of the graph. Differences between the colostrum, transitional, and mature human milk groups can be established.

This indicates that all of the present samples can be grouped and differentiated from each other according to their fatty acid profile. Infant formulas are distinct from the human milk groups, largely due to the differences described in their LC-PUFAs. In addition, it is interesting to observe that the different human milk samples can be assigned to independent groups, following the observation that samples belonging to the colostrum group have a different composition than the other samples (transitional and mature groups).

In conclusion, this method was proven to be a useful tool for studying the relationships between oils according to their fat composition [44].

4.5. Limitations and Strengths

Some limitations should be acknowledged. The small sample size used in the present study is a limitation, although we did not observe significant differences in the fatty acid profile of lactating women according to the anthropometric variables studied, such as weight, height, or BMI (normal BMI and

those with a BMI above 25 kg/m2); this also being the case for the other variables described.

On the other hand, data on human milk has been previously reported for many European countries and cultures but no recent data about milk from lactating women relates to southern Spain [10,22]. This means there is an interest in carrying out this study with lactating women in Granada. It also constitutes a strength of the present study that it provides new fatty acid data in human milk, as well as infant formulas that are currently commercial, through the use of novel analytical techniques (GC-MS/MS).

5. Conclusions

The outcome of the present study showed that the fatty acid profile of human milk samples varies throughout lactation. This variability in the profile of fatty acids between the different samples of human milk justifies that is not a static fluid and changes over time, adapting to the nutritional needs of the infant. However, infant formulas are rather uniform with respect to their composition.

According to the results, despite the fatty acid profile being similar in infant formulas and in human milk in terms of total SFA, MUFA, and PUFA; significant differences were found in some important fatty acids (such as ALA, GLA, DHA, or AA) between different human milk groups and infant formulas. Further, more evident statistically significant differences were observed between the colostrum group and the other samples.

Nevertheless, although infant formulas are enriched with the main LC-PUFAs, typically rich in LA, and in some cases with sufficient contributions of ALA, they tend to have low DHA, AA, and GLA content with respect to human milk. These fatty acids are important for fetal growth, and brain and retina development during pregnancy and the early years of life. Scientific evidence has shown that non-breastfed children suffer from a greater prevalence, severity, and longevity of diseases, not only during the time of breastfeeding but many years later.

Furthermore, the distribution of palmitic acid in infant formulas should resemble that of human milk as the specific distribution of fatty acids in triglyceride plays a key role in the digestion and absorption of lipids.

In conclusion, this experimental work can be used to ensure that infant formulas are as similar as possible to human milk and reproduces as close as possible the complexity of human milk composition. This is important in circumstances where breastfeeding is impossible, insufficient, or undesired.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/12/3055/s1, Table S1: Basic formulation details of Infant Formulas (IF).

Author Contributions: The author's responsibilities were as follows: Conceptualization, M.O.-H.; data curation, R.G.-M.; investigation, S.S.-H.; methodology and writing-original draft, S.S.-H. and A.E.-M.; supervision, M.O.-H. and B.M.-B.; writing-review and editing, M.O.-H. and M.J.A.-C.; project administration, M.J.A.-C.

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Conflicts of Interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

References

1. Organización Mundial de la Salud (OMS). Lactancia Materna Exclusiva; WHO: Geneva, Switzerland, 2019.

2. Binns, C.; Lee, M.; Low, W.Y. The Long-Term Public Health Benefits of Breastfeeding. Asia Pac. J. Public Health 2016, 28, 7–14. [CrossRef] [PubMed].

3. Organización Mundial de la Salud (OMS). Organización Panamericana de la Salud (OPS) La Alimentación del Lactante y del Niño Pequeño; OMS: Geneva, Switzerland, 2009; Volume 1, ISBN 9789275330944.

4. Comité de Lactancia Materna de la Asociación Española de Pediatría (AEPED). LACTANCIA MATERNA EN CIFRAS: TASAS DE INICIO Y DURACIÓN DE LA LACTANCIA EN ESPAÑA Y EN OTROS PAÍSES; AEPED: Madrid, Spain, 2016.

5. Lopes, T.I.B.; Cañedo, M.C.; Oliveira, F.M.P.; Alcantara, G.B. Toward Precision Nutrition: Commercial Infant Formulas and Human Milk Compared for Stereospecific Distribution of Fatty Acids Using Metabolomics. OMICS J. Integr. Biol. 2018, 22, 484–492. [CrossRef] [PubMed]

6. Mazzocchi, A.; D'Oria, V.; Cosmi, V.; Bettocchi, S.; Milani, G.; Silano, M.; Agostoni, C.; Mazzocchi, A.; D'Oria, V.; De Cosmi, V.; et al. The Role of Lipids in Human Milk and Infant Formulae. Nutrients 2018, 10, 567. [CrossRef] [PubMed]

7. Delplanque, B.; Gibson, R.; Koletzko, B.; Lapillonne, A.; Strandvik, B. Lipid Quality in Infant Nutrition. J. Pediatr. Gastroenterol. Nutr. 2015, 61, 8. [CrossRef] [PubMed]

8. Long, A.C.; Kaiser, J.L.; Katz, G.E. Lipids in infant formulas: Current and future innovations. Lipid Technol. 2013, 25, 127–129. [CrossRef]

9. Sioen, I.; van Lieshout, L.; Eilander, A.; Fleith, M.; Lohner, S.; Szommer, A.; Petisca, C.; Eussen, S.; Forsyth, S.; Calder, P.C.; et al. Systematic Review on

N-3 and N-6 Polyunsaturated Fatty Acid Intake in European Countries in Light of the Current Recommendations—Focus on Specific Population Groups. Ann. Nutr. Metab. 2017, 70, 39–50. [CrossRef] [PubMed]

10. López-López, A.; López-Sabater, M.; Campoy-Folgoso, C.; Rivero-Urgell, M.; Castellote-Bargalló, A. Fatty acid and sn-2 fatty acid composition in human milk from Granada (Spain) and in infant formulas. Eur. J. Clin. Nutr. 2002, 56, 1242–1254. [CrossRef]

11. Van Elten, T.M.; Van Rossem, L.; Wijga, A.H.; Brunekreef, B.; de Jongste, J.C.; Koppelman, G.H.; Smit, H.A. Breast milk fatty acid composition has a long-term effect on the risk of asthma, eczema, and sensitization. Allergy 2015, 70, 1468–1476. [CrossRef]

12. Hurtado, J.A.; Iznaola, C.; Peña, M.; Ruíz, J.; Peña-Quintana, L.; Kajarabille, N.; Rodriguez-Santana, Y.; Sanjurjo, P.; Aldámiz-Echevarría, L.; Ochoa, J.; et al. Effects of Maternal W-3 Supplementation on Fatty Acids and on Visual and Cognitive Development. J. Pediatr. Gastroenterol. Nutr. 2015, 61, 472–480. [CrossRef]

13. Barreiro, R.; Regal, P.; López-Racamonde, O.; Cepeda, A.; Fente, C.A. Comparison of the fatty acid profile of Spanish infant formulas and Galician women breast milk. J. Physiol. Biochem. 2018, 74, 127–138. [CrossRef]

14. Juber, B.A.; Jackson, K.H.; Johnson, K.B.; Harris, W.S.; Baack, M.L. Breast milk DHA levels may increase after informing women: A community-based cohort study from South Dakota USA. Int. Breastfeed. J. 2016, 12, 7. [CrossRef] [PubMed]

15. De Souza Santos da Costa, R.; da Silva Santos, F.; de Barros Mucci, D.; de Souza, T.V.; de Carvalho Sardinha, F.L.; Moutinho de Miranda Chaves, C.R.; das Graças Tavares do Carmo, M. trans Fatty Acids in Colostrum, Mature Milk and Diet of Lactating Adolescents. Lipids 2016, 51, 1363–1373. [CrossRef] [PubMed]

16. Marangoni, F.; Agostoni, C.; Lammard, A.M.; Giovannini, M.; Galli, C.; Riva, E. Polyunsaturated fatty acid concentrations in human hindmilk are stable

throughout 12-months of lactation and provide a sustained intake to the infant during exclusive breastfeeding: An Italian study. Br. J. Nutr. 2000, 84, 103–109. [PubMed]

17. EU. Commission Directive 2006/141/EC of 22 December 2006 on Infant Formulae and Follow-on Formulae and Amending Directive 1999/21/EC, 30. 12. 2006; EU: Brussels, Belgium, 2006; pp. 1–33.

18. Lien, E.L.; Richard, C.; Hoffman, D.R. DHA and ARA addition to infant formula: Current status and future research directions. Prostaglandins Leukot. Essent. Fat. Acids 2018, 128, 26–40. [CrossRef] [PubMed]

19. Ministerio de Sanidad, Servicios Sociales e Igualdad. Guía de Práctica Clínica sobre lactancia materna 2017, 321. Available online: https://www.aeped.es/sites/default/files/guia_de_lactancia_materna.pdf (accessed on 19 June 2019).

20. Hara, A.; Radin, N. Lipid Extraction of Tissues. Evaluation 1978, 426, 420-426.

21. Christie, W.W. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J. Lipid Res. 1982, 23, 1072–1075.

22. He, Y.-B.; Ren, H.-W.; Cao, Y.-T.; Li, H.-J.; Zhang, Z.; Liu, N. Comparing the composition and trend of fatty acid in human milk with bovine milk and infant formula in northeast region of China. CyTA J. Food 2016, 14, 632–638. [CrossRef]

23. Zou, L.; Pande, G.; Akoh, C.C. Infant Formula Fat Analogs and Human Milk Fat: New Focus on Infant Developmental Needs. Annu. Rev. Food Sci. Technol. 2016, 7, 139–165. [CrossRef]

24. Gardner, A.S.; Rahman, I.A.; Lai, C.T.; Hepworth, A.; Trengove, N.; Hartmann, P.E.; Geddes, D.T. Changes in Fatty Acid Composition of Human Milk in Response to Cold-Like Symptoms in the Lactating Mother and Infant. Nutrients 2017, 9, 1034. [CrossRef]

25. Innis, S.M. Palmitic Acid in Early Human Development. Crit. Rev. Food Sci. Nutr. 2016, 56, 1952–1959. [CrossRef]

26. Garcia, C.; Innis, S. Structure of the human milk fat globule. Lipid Technol. 2013, 25, 223–226. [CrossRef]

27. Chen, Y.; Zhang, X.; Li, D.; Yi, H.; Xu, T.; Li, S.; Zhang, L. Fatty acid and triacylglycerol comparison of infant formulas on the Chinese market. Int. Dairy J. 2019, 95, 35–43. [CrossRef]

28. Di, D.; In, R.; Agrarie, S.E.T. Milk and dairy products: Evaluation of bioactive components. 2014. Available online: https://pdfs.semanticscholar.org/7145/03282aa7de66a3c8d0c224daf32d865d4a.pdf (accessed on 19 June 2019).

29. Koletzko, B.; Baker, S.; Cleghorn, G.; Neto, U.F.; Gopalan, S.; Hernell, O.; Hock, Q.S.; Jirapinyo, P.; Lonnerdal, B.; Pencharz, P.; et al. Global Standard for the Composition of Infant Formula: Recommendations of an ESPGHAN Coordinated International Expert Group. J. Pediatric Gastroenterol. Nutr. 2005, 41, 584–599. Available online: https://pdfs.semanticscholar.org/b165/c33c861705c790a4e9620e7d99334e96cd3b.pdf (accessed on 24 July 2019). [CrossRef] [PubMed]

30. Barreiro, R.; Díaz-Bao, M.; Cepeda, A.; Regal, P.; Fente, C.A. Fatty acid composition of breastmilk inGalicia (NW Spain): A cross-country comparison. Prostaglandins Leukot. Essent. Fat. Acids 2018, 135, 102–114. [CrossRef]

31. Kreši´c, G.; Dujmovi´c, M.; Mandi´c, M.L.; Delaš, I. Relationship between Mediterranean diet and breast milk fatty acid profile: A study in breastfeeding women in Croatia. Dairy Sci. Technol. 2013, 93, 287–301. [CrossRef]

32. Ministerio de Agricultura, P.y.A. Informe del Consumo Alimentario en España 2017. Gob. España 2018, 242.

33. Martysiak-Z˙ urowska, D.; Kiełbratowska, B.; Szlagatys-Sidorkiewicz, A. The content of conjugated linoleic acid and vaccenic acid in the breast milk of

women from Gdansk and the surrounding district, as well as in, infant formulas and follow-up formulas. nutritional recommendation for nursing women. Dev. Period Med. 2018, 22, 128–134.

34. Mosley, E.E.; McGuire, M.K.; Williams, J.E.; McGuire, M.A. Cis-9, Trans-11 Conjugated Linoleic Acid Is Synthesized from Vaccenic Acid in Lactating Women. J. Nutr. 2006, 136, 2297–2301. [CrossRef]

35. Turpeinen, A.M.; Mutanen, M.; Aro, A.; Salminen, I.; Basu, S.; Palmquist, D.L.; Griinari, J.M. Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am. J. Clin. Nutr. 2002, 76, 504–510. [CrossRef]

36. Rist, L.; Mueller, A.; Barthel, C.; Snijders, B.; Jansen, M.; Simões-Wüst, A.P.; Huber, M.; Kummeling, I.; von Mandach, U.; Steinhart, H.; et al. Influence of organic diet on the amount of conjugated linoleic acids in breast milk of lactating women in the Netherlands. Br. J. Nutr. 2007, 97, 735–743. [CrossRef]

37. Daud, A.Z.; Mohd-Esa, N.; Azlan, A.; Chan, Y.M. The trans fatty acid content in human milk and its association with maternal diet among lactating mothers in Malaysia. Asia Pac. J. Clin. Nutr. 2013, 22, 431–442. [PubMed]

38. Ribeiro, M.; Balcao, V.; Guimaraes, H.; Rocha, G.; Moutinho, C.; Matos, C.; Almeida, C.; Casal, S.; Guerra, A. Fatty Acid Profile of Human Milk of Portuguese LactatingWomen: Prospective Study from the 1st to the 16th Week of Lactation. Ann. Nutr. Metab. 2008, 53, 50–56. [CrossRef] [PubMed]

39. Sala-Vila, A.; Castellote, A.I.; Rodriguez-Palmero, M.; Campoy, C.; López-Sabater, M.C. Lipid composition in human breast milk from Granada (Spain): Changes during lactation. Nutrition 2005, 21, 467–473. [CrossRef] [PubMed]

40. Importancia del Ácido Gamma-Linolénico en la Salud y en la Nutrición. Available online: https://w3.ual.es/~{}jlguil/gamma.htm (accessed on 15 October 2019).

41. Fu, Y.; Liu, X.; Zhou, B.; Jiang, A.C.; Chai, L. An updated review of worldwide levels of docosahexaenoic and arachidonic acid in human breast milk by region. Public Health Nutr. 2016, 19, 2675–2687. [CrossRef] [PubMed]

42. Brenna, J.T.; Varamini, B.; Jensen, R.G.; Diersen-Schade, D.A.; Boettcher, J.A.; Arterburn, L.M. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. Am. J. Clin. Nutr. 2007, 85, 1457–1464. [CrossRef] [PubMed]

43. FAO. Standard for infant formula and formulas for special medical purposes intended for infants. Codex Aliment. 2016, 72–1981, 1–21.

44. Rueda, A.; Seiquer, I.; Olalla, M.; Giménez, R.; Lara, L.; Cabrera-Vique, C. Characterization of fatty acid profile of argan oil and other edible vegetable oils by gas chromatography and discriminant analysis. J. Chem. 2014, 2014. [CrossRef]

2.3.1 Material Suplementario Publicación II

Table S1. Basic formulation details of Infant Formulas (IF).

	Fat Total	SFA	MUFA	PUFA	Palmitic Acid	LA (w-6)	ALA (w-3)	AA (w-6)	DHA (w-3)	Added ingredients
	g/100mL					mg/100mL				
Infant Formula 1	3.4	1.5	-	-	-	388	48	13.1	13.1	w-3 y w-6 LC_PUFA, FOS, Nucleotidos
Infant Formula 2	3.5	1.3	-	-	-	454	52	6.9	6.9	GOS, Nucletidos, DHA
Infant Formula 3	3.4	1.5	-	-	-	400	80	10	10	GOS/FOS (9:1), DHA/AA, Nucleotidos
Infant Formula 4	3.5	1.2	1.6	0.7		600	70	12	7	GOS, PCL (w-6 y w-3)
Infant Formula 5	3.5	1.5	-	-	0.8	418	46	7	7	w-3 y w-6 AGPI-CL, FOS, Nucleotidos
Infant Formula 6	3.6	1.4	-	-	-	573	69	-	-	Nucleotidos
Infant Formula 7	3.5	1.3	-	-	-	429	52	7.4	6.7	GOS, Nucleotidos, DHA

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; LA: LA: linoleic acid; ALA: α-linolenic acid; AA: arachidonic acid; DHA: docoxahexaenoic acid; LC-PUFA: long-chain polyunsaturated fatty acids; FOS: frutooligosaccharides; GOS: galactooligosaccharides.


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Publicación III

Protein Profile and Simulated Digestive Behavior of Breast Milk from Overweight and Normal Weight Mothers

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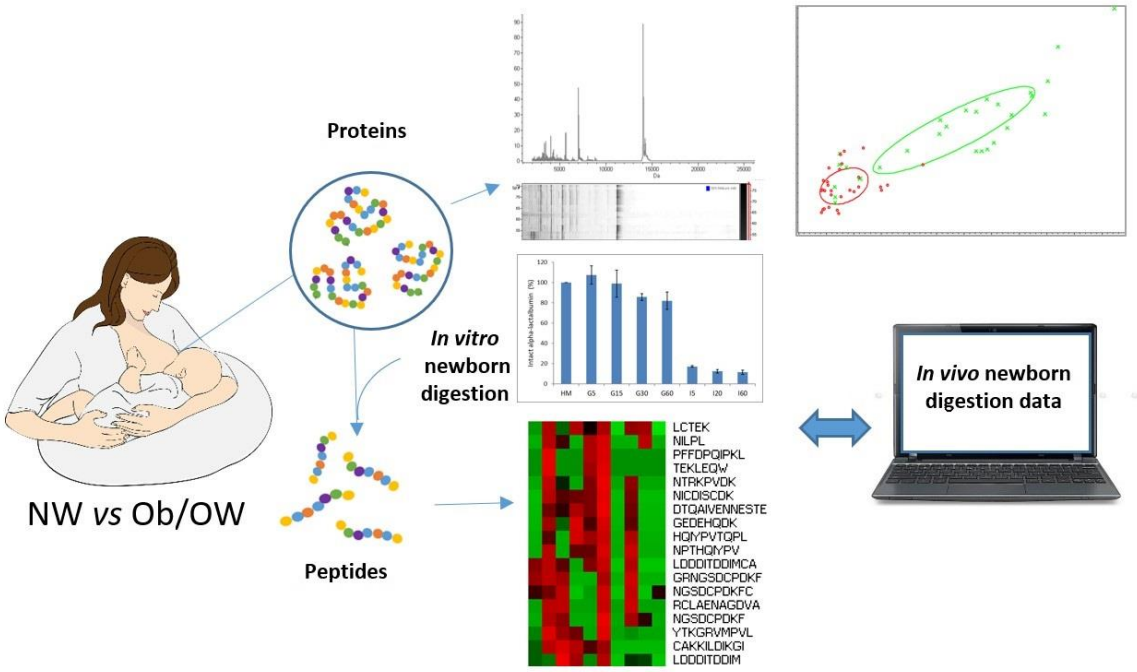


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Protein Profile and Simulated Digestive Behavior of Breast Milk from Overweight and Normal Weight Mothers

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Abstract

Human milk proteins have shown to vary in concentration and distribution through lactation. However, while some regulatory components, such as hormones, have shown associations with regard to the mothers' body mass index, there is limited information on the possible influence of this condition on the whole protein distribution. The objective of this study was to evaluate the protein profile of human milk from normal weight and overweight or obese mothers to identify differences in protein expression in colostrum, transitional and mature milk. The mass spectrometry analysis showed the ability to class with a high degree of confidence the lactation state and the milk profile according to the mother's condition. Individual milk samples were subjected to a digestion in vitro model that takes into account the specificities of the gastrointestinal conditions of full-term newborn infants. The digestion products were compared with available data from the digestive contents in newborns. The behavior of the most abundant proteins and the overall peptide generation and survival, showed good correspondence with in vivo data.

Keywords: human milk; MALDI mass spectrometry; body mass index; in vitro infant digestion; bioactive proteins

1. Introduction

Human milk presents a combination of components ideal for infant nutrition, allowing optimal growth and providing several short and long-term health benefits. While lipids are the largest source of energy in breast milk, contributing 40–55% of the total energy, proteins from breast milk provide approximately 8% of the energy required by the infant [1]. However, they constitute an essential fraction from the point of view of biological implications, including antimicrobial and immunomodulatory activities, and stimulating the absorption of nutrients [2]. Hormones (such as insulin or adipokines) are contained within the peptide components and may influence energy intake, weight gain, growth and development of infants directly or indirectly [3].

Milk proteins can be classified into caseins, whey proteins and the milk fat globule membrane (MFGM) fraction. Breast milk composition, and concretely the protein fraction, varies during lactation to adapt to the newborn's protein requirements. The total content of casein, suspended in casein micelles, and whey proteins, soluble, change profoundly over time; whey proteins dominate the profile in the first days of lactation compared to virtually undetectable casein [4]. In mature milk, an estimated average whey/casein ratio of 60:40 has been reported, although quantification with proteomics reveals a median whey/casein ratio even lower, 73:27 [5]. Regarding protein composition, the levels of α- lactalbumin (α-LA), lactoferrin and β-casein decrease throughout lactation while, lysozyme concentration increases [6]. Milk composition can also vary with mother´s phenotype and health status. Increased levels of glucose and insulin are reported in obese and overweight mothers [7]. Maternal body mass index (BMI) is positively correlated with leptin levels in milk [8]. Maternal adiposity has also been associated with increased amounts of non- glucose monosaccharides [9] and with higher milk fat and lactose, whereas total protein concentration seems to be unaffected [10,11]. However, there is limited information about a possible influence on protein or peptide distribution.

Digestion of nutrients is an essential function step for the newborn to allow normal growth and development. Both, the supply of essential amino acids and the bioactivities of milk proteins are dependent on their digestibility: some proteins act only in the intact form, others in the form of larger or small peptides formed during digestion, and some are completely digested and serve as a source of amino acids [4]. Interesting data about healthy full term infant digestion have been reported in the last years. Some works point to the role of digestion kinetics on the distinct effect of breastfed and infant formula fed infants [12]. Many factors, such as the type of food matrix (liquid/solid), pH, the enzyme secretion, peristaltic movements and emptying of the stomach need to be considered in mimicking digestion [13]. In order to set relevant infant digestive conditions, outcomes from these models should be contrasted with in vivo data, for example, with regards to protein regions resistant to digestion. Peptidomics has allowed the identification of peptides deriving from many milk proteins in the gastric and intestinal aspirates of the newborns after their mother's milk ingestion [14–16].

The objective of this study was to evaluate the protein profile of a set of human milks from normal weight (NW) and overweight/obese (OW/Ob) mothers to identify differences in protein expression in colostrum, transitional and mature milk. In addition, these milk samples were subjected to an in vitro static digestion model that takes into account the specificities of the gastrointestinal conditions of full-term newborn infants. The protein digestion products have been compared with available data from the digestive contents in newborns. The protein fraction of milk at diverse stages of lactation and the peptides released after digestion have been used to evaluate the influence of the mother's condition on its composition.

2. Materials and Methods

2.1. Subjects and Samples

Human milk samples were provided by participating mothers in a study conducted in the Obstetrics and Gynecology Department of "Hospital

Universitario Virgen de las Nieves" from Granada (Spain). Ethics were approved by the relevant scientific committee and the trial was registered at ClinicalTrials.gov (accessed on 15 April 2019) (number NCT): NCT02811172. Details of the study were explained to all mothers who voluntarily gave written consent to participate. Eighteen lactating women were recruited for HM collection, with each mother donating three samples (colostrum, transitional and mature milk). The main characteristics of sampled mothers are shown in **Table 1**. Colostrum corresponded to milk from the 3rd to the 5th post-partum day, transitional milk to days 9–13 and mature milk from day 17 thereafter. Mothers with BMI in the 18.5–24.9 kg m−2 range were included in the NW group while those with a BMI greater than 25.0 kg m−2 were included in the OW/Ob group. All human milk samples collected from the participants in sterile tubes were aliquoted and immediately stored at −80 ◦C until used.

Table 1. Demographic description of study mothers and delivery characteristics by group.

Characteristics	Normal Weight [a] (*N* = 9)	Overweight/Obese [a] (*N* = 9)	*p*-Value [b]
Age, years	33.0 (5.5)	32.0 (5.0)	0.8642
Colostrum collection, days postpartum	4.0 (1.0)	5.0 (1.0)	0.1340
Transitional milk collection, days postpartum	9.5 (1.0)	10.3 (1.3)	0.1970
Mature milk collection, days postpartum	19.0 (3.0)	19.0 (0.8)	0.6445
Gestational age, days	278.0 (14.0)	282.0 (10.8)	0.2162
BMI early pregnancy, kg m^{-2}	21.45 (1.36)	30.53 (7.88)	0.0012
BMI at delivery, kg m^{-2}	25.95 (2.39)	34.23 (3.64)	0.0047
Weight gain, kg	12.0 (4.5)	11.5 (4.5)	0.7576
Newborn weight, kg	3.23 (0.40)	3.44 (0.38)	0.2343
Gender distribution, %			
Female	43	50	
Male	57	50	

BMI: Body Mass Index; a Median (Interquartile range) b Mann–Whitney test.

2.2. MALDI-TOF Protein Profile

Milk was centrifuged at 11,000 xg for 20 min at 4 ◦C for skimming. The skimmed milk was subsequently diluted 1:100 with ultrapure water (Milli-Q Millipore) and subjected to mass spectrometry analysis. An aliquot (1 µL) of skimmed milk was directly spotted onto an MSP 96 polished steel target (Bruker Daltonics, Bremen, Germany), overlaid with 1 µL of sinapinic acid matrix, in 50% acetonitrile, containing 0.1% trifluoroacetic acid (v/v) and allowed to dry at room temperature, and analyzed on an Autoflex speed instrument (Bruker Daltonics, Bremen, Germany) using previously reported conditions [17]. Three independent spectra for each skimmed milk's fraction were collected in the automated mode, externally calibrated by using the Bacterial Test Standard (Bruker Daltonics, Bremen, Germany) and subsequently analyzed with the FlexAnalysis version 3.3 (Bruker Daltonics, Bremen, Germany) for the quality checking on raw data.

MALDI-TOF spectra were analyzed with ClinProTools (Version 2.2, Bruker Daltoics, Bremen, Germany) for statistical treatment. Baseline was subtracted using the Top Hat algorithm with 10% of minimal baseline width, and spectra were smoothed using the Savitsky–Golay algorithm with one cycle of 5 m/z width. Peak picking was calculated on total average spectrum with a signal-to-noise of 3 and 5% of relative threshold base peak. Principal component analysis (PCA) was calculated to visualize samples projection on the score plot and the variables explaining the projection of the loading plot. Supervised analysis consisted in model calculation with three complementary algorithms. The quick classifier (QC) algorithm uses the statistical differences between classes to discriminate them. The peaks included in the calculation were thus selected according to their p-value after an automatic detection of peak number. The supervised neural network (SNN) algorithm is an iterative method based on the characteristics of data distribution [18]. The number of peaks involved in the calculation and the number of prototype detection were determined automatically by the algorithm. The genetic algorithm (GA) is a random algorithm that mimics the natural evolution [19]. Each spectrum is considered as a chromosome and each

peak as a gene, and the overall principle is to recover the family belonging, meaning the class discrimination. For these three models, internal validation was calculated to ensure the calculation reliability, using 20% of the spectral data selected randomly and 10 iterations.

2.3. In Vitro Simulated Gastrointestinal Digestion

The infant gastrointestinal static in vitro model was carried out following the protocol described by Ménard et al. [20]. The gastrointestinal in vitro simulation included two consecutive steps: a gastric phase (60 min at 37 ºC with pepsin and lipase at pH 5.3) and an intestinal phase (60 min at 37 ºC with porcine pancreatin and bile at pH 6.6). Enzyme activity and bile concentration were measured according to the assays described by Brodkorb et al. (2019) [21]. Pepsin and gastric lipase were added as rabbit gastric extract (RGE). The added amount of RGE covered 100% of lipase activity and 120% of pepsin activity (320 U/mL). Samples were withdrawn at 5, 15, 30 and 60 min during gastric digestion and the reaction was stopped by adjusting the pH at 7.0 with NaOH 1 M and snap freezing in liquid nitrogen. The intestinal phase was carried out by mixing the final gastric volume with an appropriate amount of simulated intestinal fluid, containing pancreatin from porcine pancreas, which covered the lipase (90 U/mL) and trypsin (16 U/mL) activity required of intestinal content, and porcine bile extract 3.1 mM and the pH was adjusted to the intestinal pH of 6.6 using HCl 1 M. Samples were withdrawn at 5, 20 and 60 min during intestinal digestion and the reaction was stopped by snap freezing in liquid nitrogen. All samples were lyophilized and stored at -20 ºC until analysis. Nitrogen contents at the end of the gastric and intestinal phase were determined by elemental analysis.

2.4. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Identification of Bands by In-Gel Digestion

Undigested (control) and digested human milk samples were dissolved at 1 mg of protein/mL in sample buffer and analyzed on 12% Bis-Trispolyacrilamide gels (Crite- rion_XT, Bio-Rad, Hercules, CA, USA). In gel digestion and analysis on an Autoflex speed MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) instrument was performed as previously reported [22]. MASCOT v2.4 software (MatrixScience, Boston, MA, USA) was used to carry out protein identification searches against a homemade database of human milk proteins selected from the Uniprot database (https://www.uniprot.org/ accessed on 15 April 2019).

2.5. Analysis by Ultra-Performance Liquid Chromatography (UPLC)

Human milk proteins were separated by reverse-phase UPLC following an adaptation from Visser et al. (1991) [23] on an Aquity UPLC (Waters Technologies, Cerdanyola del Vallès, Spain). Solvent A was acetonitrile-water-trifluoroacetic acid (100:900:1 v/v/v) and solvent B was the same mixture with the proportions 900:100:0.1 (v/v/v). The analytical column was a Phenomenex AerisTM 3.6 µm Widepore XB-C18 (150 mm x 2.1 mm). Briefly, 100 µL of human milk or digested sample were mixed with 100 µL of 0.02 M 1,3-bis[tris(hydroxymethyl)methylamino] propane (Bis-Tris) buffer (pH 7) containing 8 M urea and 0.3% of 2-mercaptoethanol. After standing at room temperature for 1 h, samples were diluted (four-fold for human milk/gastric samples and two-fold for intestinal samples) with solvent A containing 6 M urea, and centrifuged at 13,000 xg 5 min before injection of the supernatant.

2.6. Analysis of Digests by HPLC–Tandem Mass Spectrometry (HPLC–MS/MS)

Freeze-dried samples were reconstituted in ammonium carbonate (NH4CO3) 25 mM and treated for 60 min at 37 ºC with 1,4-dithiothreitol 140 mM (1:1, v/v) (Sigma-Aldrich, St. Louis, MO, USA) to reduce disulfide linkages in order to improve the identification. Samples were analyzed by HPLC–MS/MS in duplicate using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranean Sea18 column (150 mm x 2.1 mm, Teknokroma, Barcelona, Spain) connected to an Esquire 3000 linear ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) as previously described [24]. The spectra were recorded over the m/z ranges 100–700, 100–1700 and 100–2000, selecting 500, 750 and 1200 as target mass, respectively. A homemade database of most abundant human milk proteins was used for the peptide sequencing in MASCOT v2.4 software. No specific enzyme cleavage was used. Peptide mass tolerance was set to 0.1% and 0.5 Da for MS and MS/MS analysis, respectively. Biotools version 3.2 (Bruker Daltonics, Bremen, Germany) was used for the interpretation of the matched MS/MS spectra. The list of peptides appearing in the selected number of subjects was compiled through Venn diagrams using the Venny tool [25]. The representation of the peptide profile was performed with Peptigram [26]. Bioactive sequences were searched in the MPDB (Nielsen, New York, NY, USA). With the identified peptides after simulated digestion, hierarchical clustering trees were built and optimized using the PermutMatrix software version 1.9.3.0 [27]. The mean center rows were used as datasets, and Ward's dissimilarity aggregation procedure based on Pearson distance was used.

3. Results and Discussion

3.1. Protein Profile of Human Milks by MALDI-TOF Mass Spectrometry

The profile of individual milks counting large peptides and small proteins was examined by MALDI-TOF mass spectrometry. The spectra from colostrum, transitional milk and mature milk from mothers corresponding to the different BMI groups, NW **(Figure 1)** and OW/Ob subjects **(Figure 2)** were first contrasted. In the gel-like view of human colostrum, transitional milk and mature milk of all individual spectral data are shown as lines and the grey level represents the peak intensity, which permits to visualize individual variability. In contrast, the spectrum view represents the average profile from all samples. In the protein profile, the main peak around 14 kDa corresponds to α-LA, although adjacent small signals can be ascribed to lysozyme C and other less abundant proteins in human milk. Besides, many peaks in the range 2000–10,000 Da characterize all samples, although species between 3000 and 4500 Da were more intense in colostrum and transitional milk. In mature milks, peak intensity was comparatively lower, which is compatible with inferior relative protein content. It is known that the protein content of human milk decreases rapidly during the first month of lactation, and declines more slowly after that [4,28]. In spite of the use of sinapinic acid matrix, which favors the ionization of high molecular weight proteins, the intensity of peaks above m/z ratio of 15,000, where caseins should appear, was very low. An impaired detection of higher mass-range species in human milk under 30 days post-partum was previously reported under similar conditions [17].

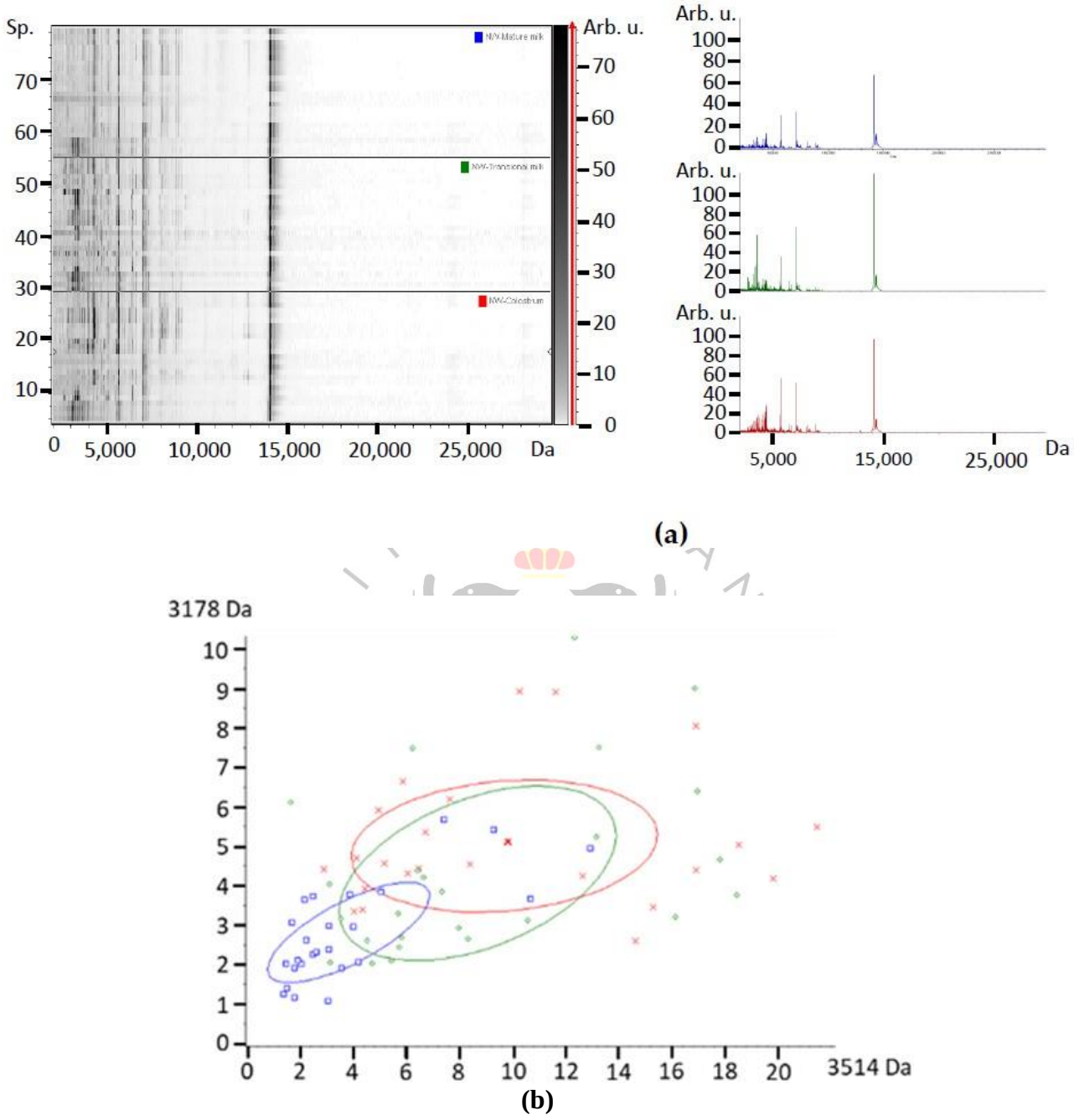


Figure 1. (a) Gel-like view and average spectrum. **(b)** 2D-plot protein spectra according to the intensities of m/z 3178 and m/z 3514 from colostrum (red), transitional milk (green) and mature milk (blue) from NW mothers. Number of points = 72 (8 subjects, 3 milk types, 3 replicates).

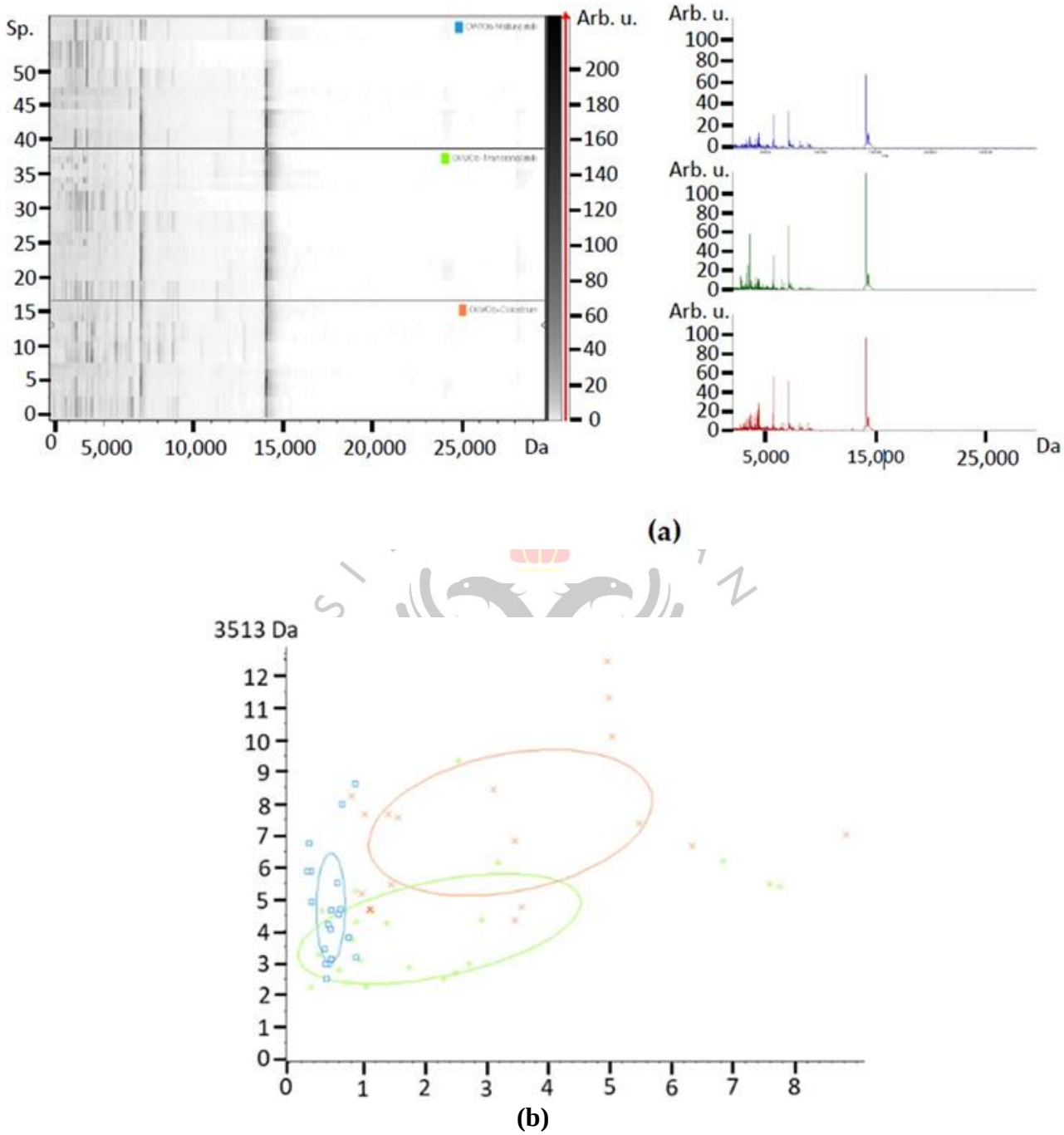
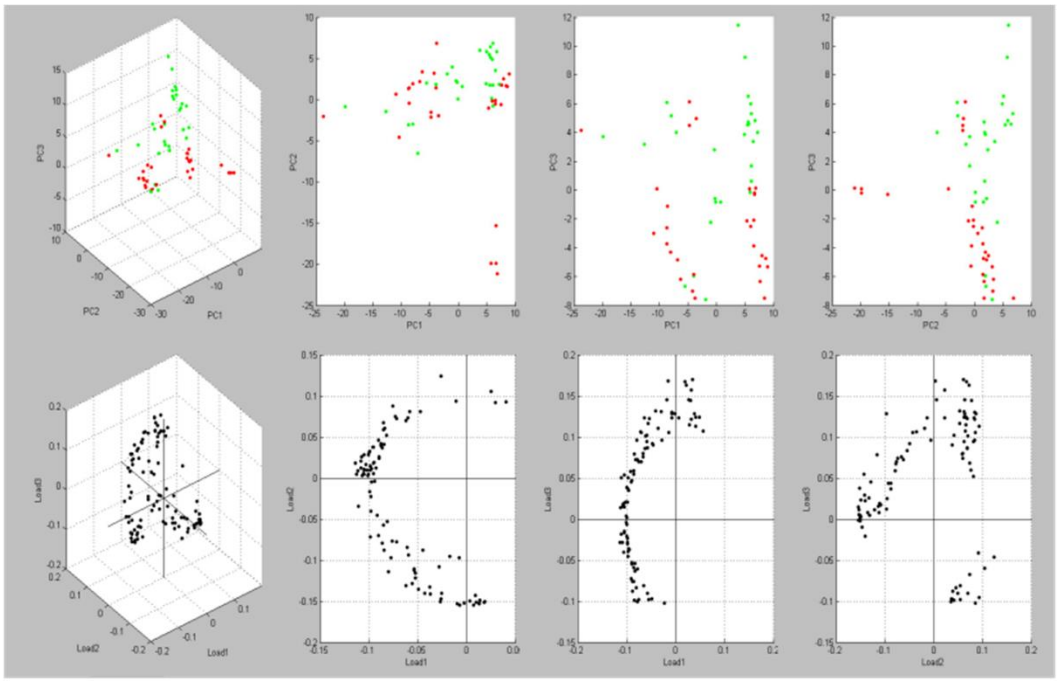


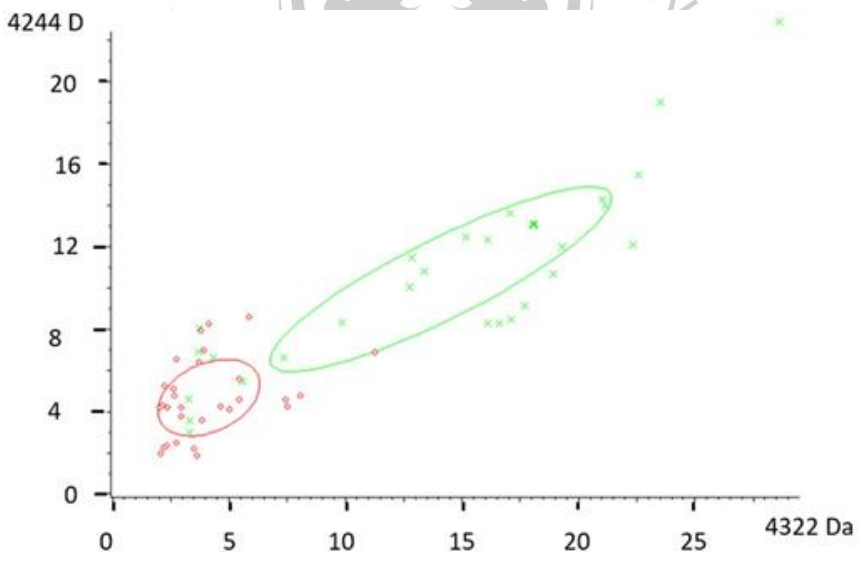
Figure 2. (a) Gel-like view and average spectrum. **(b)** 2D-plot protein spectra according to the intensities of m/z 3513 and m/z 9123 from colostrum (light red), transitional milk (light green) and mature milk (light blue) from OW/Ob mothers. Number of points = 54 (6 subjects, 3 milk types, 3 replicates).

The effect of the lactation time in the protein profile was assessed by studying samples of colostrum, transitional milk and mature milk from 8 NW and 6 OW/Ob mothers. A representation of the three milk types is shown for each group in the form of 2D-plot according to the two peaks with highest variance **(Figure 1b or Figure 2b)**. Consistent with other measurements, the interindividual variation is considerable, although dispersion was lower for mature milk compared to both colostrum and transitional milk. However, an evolution from colostrum to mature milk can be observed in both groups. Less spreading was observed in the OW/Ob group, in spite of a lower number of subjects.

A second design was used to study the effect of the mother BMI condition in the protein profile of the expressed mature milk. Combined spectra from 9 NW and 9 OW/Ob subjects were used to determine the state of classification in the first three principal components (PC) **(Figure 3)**. The PCA analysis showed that the mature milk profiles clustered together, with certain individuals from both classes being slightly separated. The contributions of PC1, PC2 and PC3 to the generation of profile explained less than 30% of the variance. However, the 2D-plot representation according to the intensities of m/z 4244 and m/z 4322 grouped OW/Ob and NW separately, with a tighter grouping in the case of OW/Ob mothers. These two masses corresponded to well defined signals present in all samples and merit attention as possible biomarkers. A predictive model generation with different algorithms was used to evaluate the discrimination potential of the protein profile. The QC algorithm provided 83.44% recognition capability with a cross validation of 76.61%, the signal corresponding to mass 4322 being selected in the integration region for classification. The application of the models based on the SNN and GA provided higher rates, with cross validation values of 79.77 and 78.98%, and recognition capability percent- ages of 96.49 and 98.21%, respectively, the GA providing 100% recognition capability for class 2 (OW/Ob). The list of masses involved in the model generation and their weight are shown in **Table S1**.



(**a**)



(**b**)

Figure 3. (a) PCA analysis contrasting spectral data of mature milks from NW (green) and OW/Ob (red) subjects. 3D view and combinations of the first three principal components. **(b)** 2D-plot protein spectra according to the intensities of m/z 4244 and 4322. Number of points = 54 (9 NW subjects, 9 OW/Ob subjects, 3 replicates).

This supervised analysis showed the ability of MALDI-TOF analysis to class with a high degree of confidence mature milks according to the mothers BMI. Recent studies relating breast milk with body composition have indicated that individual bioactive components of human milk may regulate different compartments of infant weight gain, i.e., adiposity or accumulation of lean mass, separately [29]. In fact, some studies correlate the adipokines content in breast milk with the respective levels in maternal and infant blood [30]. In this regard, it is still unknown how alterations in breast milk composition will subsequently impact later health outcomes. Delivered components as different as proteins, fatty acids or insulin have shown associations with infant fat mass gain [31]. The used strategy has allowed to evidence specific signals characterizing the mothers by BMI. Therefore, they might be useful to identify new biomarkers and complement these analyses in the way to discern the role of human milk composition in the infants' development.

3.2. Protein Degradation of Human Milk during Infant In Vitro Gastrointestinal Digestion

Ten mature milk samples, six from NW subjects and four from OW/Ob subjects, were submitted to in vitro gastrointestinal digestion under conditions that simulate those of the newborn. The SDS-PAGE electrophoretic pattern during gastrointestinal digestion of a milk sample from a NW donor is presented in Figure 4a. Digested samples from the gastric (G5, G15, G30 and G60) and intestinal (I5, I30 and I60) phases were compared with the undigested human milk (HM). The analysis of the gel bands by MALDI–MS/MS allowed identification of bile salt-activated lipase (BAL), lactoferrin, immunoglobulin heavy constant alpha 2 (IgHA2), β-casein and α-LA. The whey proteins α-LA and lactoferrin are major proteins in human milk, comprising 25–35% and 15–20% of the total protein content, respectively [32], as observed in the band size. Despite similar molecular weight than lactoferrin, 78 vs. 79 kDa, BAL shows a lower electrophoretic mobility, probably due to its mucin-like structure with extended conformation

[33]. α-LA and lactoferrin resisted gastric digestion at G60 due to the globular and compact structure. The caseins, especially β-casein, were hydrolyzed in some measure over gastric digestion, in accordance with their loose and flexible structure that makes them susceptible to pepsinolysis. All proteins were detected after 60 min, consistent with the high gastric pH in infants (5.3) compared to the adult (3.0) that causes pepsin only be at 10% of its maximal activity. By contrast, intestinal digestion resulted in a rapid hydrolysis of the intact proteins remaining after the gastric step. After five min, the bands visible on the SDS-PAGE gel corresponded to the proteins added in the pancreatin, as previously observed [20].

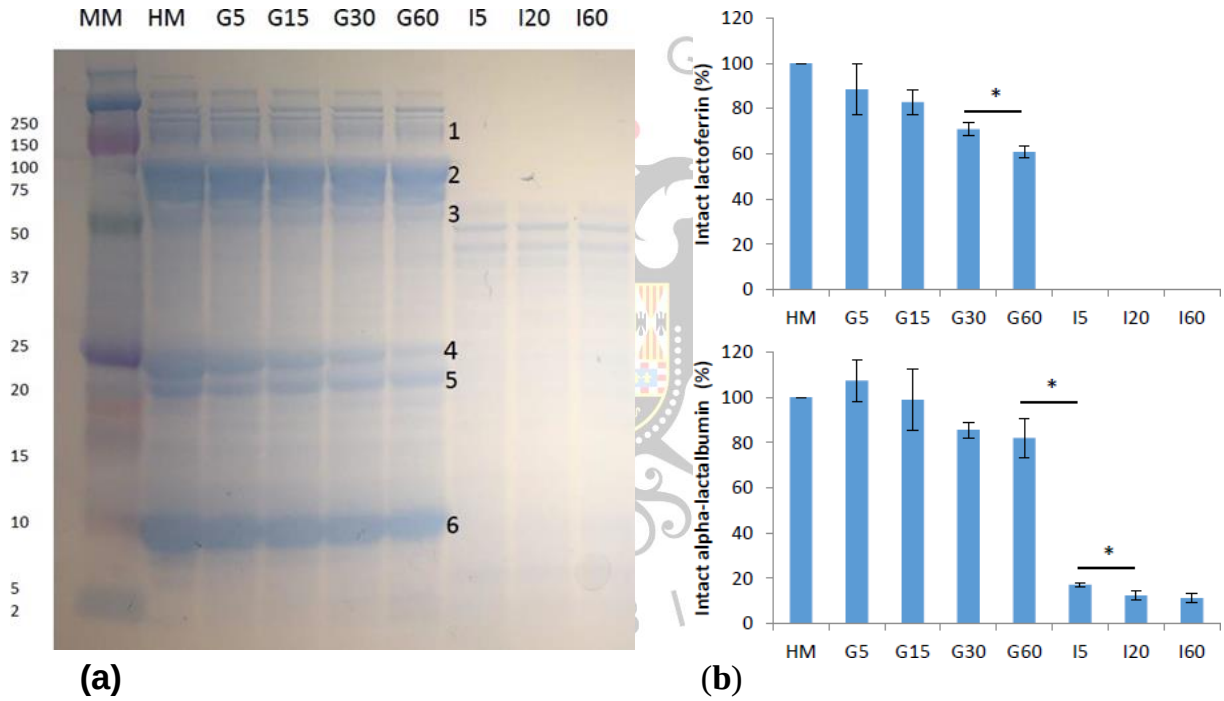


Figure 4. (a) SDS-PAGE profile of a mature milk sample submitted to in vitro gastrointestinal digestion. Band identification by tandem MS: 1. Bile salt-activated lipase. 2. Lactoferrin. 3. Immunoglobulin heavy constant alpha 4. β-casein. 5. β- and αs1-casein. 6. α-LA. **(b)** Quantification of lactoferrin and α-LA by UPLC (n = 4). * $p < 0.05$ (t-test) MM: molecular marker; HM: human milk; gastric digestion time 5 (G5), 15 (G15), 30 (G30) and 60 (G60) min; intestinal digestion time 5 (I5), 20 (I20) and 60 (I60) min.

Human milk samples and in vitro digests were analyzed by UPLC to quantitatively determine the protein changes over digestion. Lactoferrin remained as 61% intact protein during the gastric phase with no protein being detected in the intestinal phase. 82% intact α-LA was observed, while around 11% of the initial concentration was determined at the end of the intestinal digestion **(Figure 4b)**. These results are similar to the percentage of α-LA reported to be resistant to hydrolysis in the development of this digestion model (86.7%), although an infant formula with bovine proteins was used [20]. Interestingly, De Oliveira et al. (2017) [34] determined the proteolysis of human milk proteins during gastric digestion of human milk by preterm infants. In that study, ca. 40% and 75% of lactoferrin and α-LA, respectively, was found in the infant stomach after 90 min of ingestion, in good correspondence with the present values. The observed protein resistance might well mimic the full-term infant digestion, which is intended by this protocol, although full-term infant data of intact protein resistance should be contrasted when available.

Lactoferrin and IgA have a structure that makes them comparatively resistant to digestion. Furthermore, intact lactoferrin and IgA have been observed in stools of both preterm and term exclusively breastfed infants by immunological methods [35]. It is considered that only partial digestion of human lactoferrin takes place in the infant and this supports the beneficial effect of this abundant iron-binding protein in terms of bacteriostatic, bactericidal and antiviral activity. This report shows resistance of human lactoferrin under these gastric digestive conditions, in good correspondence with previous in vivo data. On the other hand, when comparing digestion of different human Ig in a dynamic simulated model, higher resistance of IgA and IgM has been reported compared to IgG, by means of proteomic techniques [36].

3.3. Peptidomic Characterization of In Vitro Digests

Human milk gastrointestinal digests were analyzed under equal conditions by HPLC– MS/MS. The peptide trace from α-LA, β-casein and lactoferrin was represented using the Peptigram tool. **Figure 5** represents the peptides identified after the application of the gastric phase and the intestinal phase at both 5 and 60 min. After the gastric digestion, only peptides from β-casein were identified, consistent with the resistance of α-LA and lactoferrin. Gastrointestinal digests produced a pattern with higher number of peptides, with great similarity between 5 and 60 min. However, more intense color in specific areas denotes greater number of overlapping identified peptides after 60 min for the three proteins. Calculated coverages reached 86, 52 and 48% for β-casein, α-LA and lactoferrin, respectively.

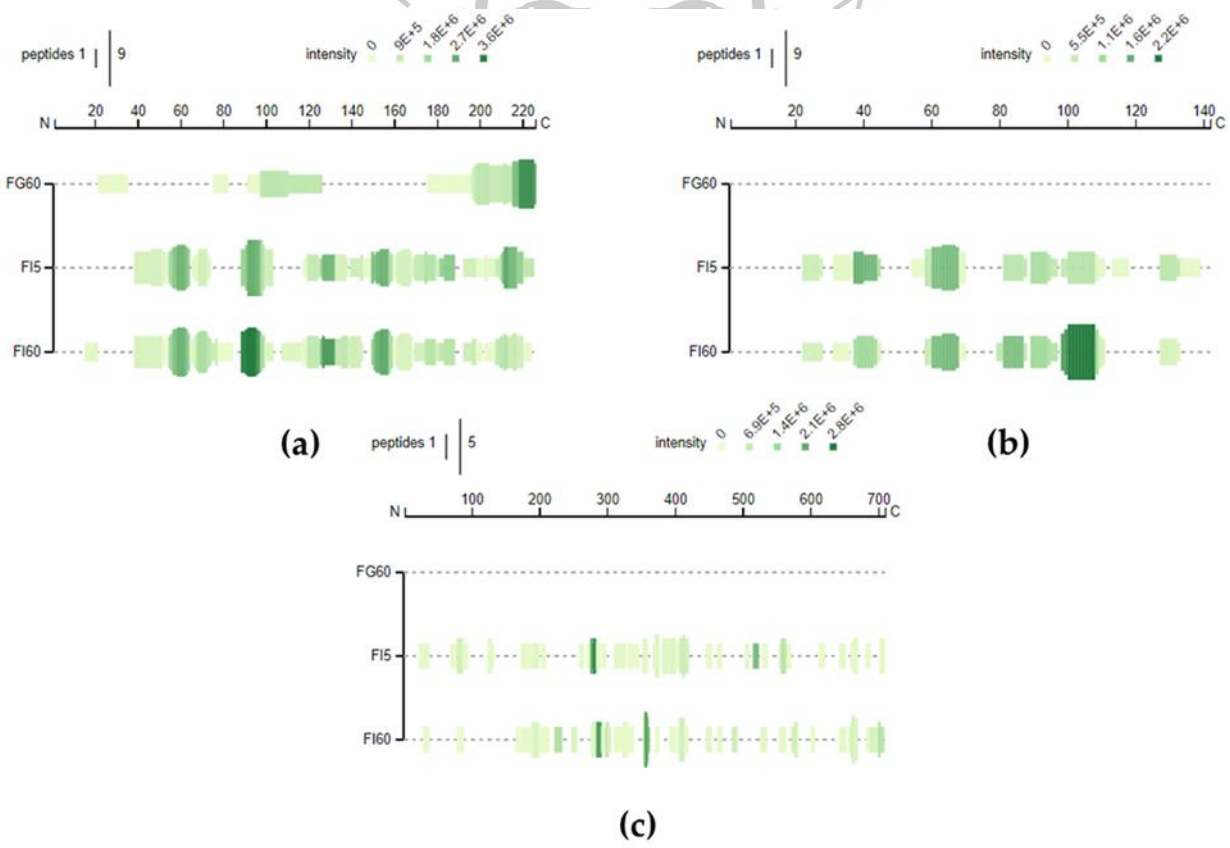


Figure 5. Peptigram profile of β-casein **(a)**, α-LA **(b)** and lactoferrin **(c)** derived peptides in digests of human milk (N = 10) after 60 min gastric digestion (FG60), and 5 min (FI5) and 60 min (FI60) intestinal digestion.

The peptides identified in the human milk gastrointestinal digests (endpoint at 60 min) consistently derived from 20 proteins **(Table 2)**. These proteins were considered represen- tative because a similar number of released peptides were found in at least four subjects. Identified peptides arose, for the most part, from β-casein, α-LA and lactoferrin, in accordance with their abundance in milk. Other predominantly precursor proteins were butyrophilin subfamily 1 member A1, BAL and IgAH2. From the human milk fat globule membrane, mucin-1, lactadherin and butyrophilin are three major components that gave rise to peptides under the used digestive conditions. It has been reported that mucin and lactadherin resist digestion in the stomach of milk-fed infants, while butyrophilin is rapidly degraded [37]. The number of peptides from the last protein in the digests can be ascribed to this rapid degradation.

The identified sequences were contrasted to those previously found upon analysis of the gastrointestinal tract content in infants [14,15,38] or in a suckling rat pup model [39], after human milk consumption. Ninety sequences from seven proteins found upon in vitro digestion were coincident with those reported in vivo. The complete list of identical peptides to those found in vivo is shown in **Table S2**.

Table 2. Number identified of peptides and corresponding proteins after individual in vitro digestion of breast milk from NW (N = 6) and OW/Ob (N = 4) subjects.

Protein name	Accession number	Peptides identified in two or more subjects (n)	Mol. Weight, Parent Protein [kDa]	Sequence length, Parent Protein	Score (Digest Matches)
β-casein	W5RWE1	123	25.38	226	332.54
αs1-casein	P47710	26	21.67	185	46.73
κ-casein	P07498	11	20.31	182	36.41
Lactotransferrin	P02788	121	78.18	710	103.31
α-lactalbumin	P00709	61	16.23	142	277.99
Bile Salt-activated lipase (BAL)	P19835	19	79.32	753	30.53
Immunoglobulin heavy constant alpha 2 (IGHA2)	P01877	18	36.59	340	27.96
Butyrophilin subfamily 1 member A1 (BT1A1)	Q13410	23	58.96	526	28.90
Polymericimmunoglobulin receptor (PIGR)	P01833	25	83.28	764	37.73
Tenascin	P24821	59	240.85	2201	29.50
Mucin-4	Q99102	56	231.52	2169	38.42
Xanthine dehydrogenase/oxidase	P47989	44	146.42	1333	23.27
Receptor tyrosine-proteinkinase erbB-4	Q15303	29	146.81	1308	28.30
Osteopontin	P10451	19	35.42	314	24.96
Cadherin-1	P12830	12	97.46	882	28.02
Clusterin	P10909	11	52.50	449	25.23
Galectin-3-binding protein	Q08380	9	65.33	585	24.74
Mucin-1	P15941	8	122.10	1255	28.77
Zinc-alpha-2-glycoprotein	P25311	4	34.26	298	20.67
Plateletglycoprotein 4	P16671	3	53.05	472	22.34

The results from the individual digestions in terms of sequence and intensity of iden- tified peptides were analyzed with software permitting the visualization of the possible associations. Identified peptides derived from α-LA, lactoferrin and β-casein were used to construct a dendrogram **(Figure 6)**. The peptide fingerprint from digests did not discriminate the samples by the donor BMI, indicating that the peptide profile after in vitro digestion reduces the differences observed in the protein fraction. On one side, this can be attributed to digestion bringing closer the protein features of differing milk classes. On the other side, it might also be the case that resistant peptides are not those responsible for the differences.

Discriminating peptides might have been degraded, or be too large to be detected by the proteomic approach employed. Lastly, identical (in vitro) gastrointestinal conditions have been applied to the samples but the impact of differing milk composition on the individual digestive behavior is still unknown and is worthy of future studies.

Although the number of subjects is acknowledged as a limitation, this study provides novel tools to depict the protein/peptide fraction of human milk, which can be used in nutritional studies intended to associate milk composition with the physiological status while nursing.

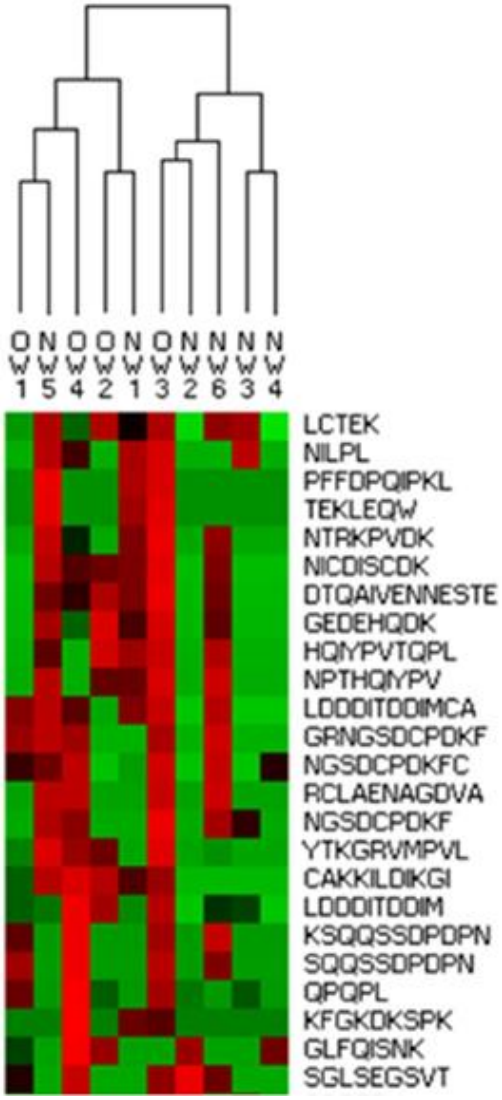


Figure 6. Heat map representation (fragment) of the hierarchical clustering analysis of peptides identified after in vitro digestion of breast milk. Columns correspond to subjects included in the overweight/obese group or normal weight group. Rows correspond to intensity of sequences derived from β-casein, α-lactalbumin and lactoferrin identified in at least 3 subjects. Complete figure in **Figure S1**.

4. Conclusions

Human milk contains a multitude of proteins, with a dynamic composition varying along lactation time. The applied MALDI-TOF analysis has proved effective as a tool to associate the effect of the lactation stage with the protein profile of the expressed milk in both NW and OW/Ob subjects, lower dispersion being found in the second group. Moreover, the mothers BMI could be predicted using the variations in the 4322 m/z signal. The study of a larger amount of subjects might contribute to identify biomarkers making it possible to associate the breast milk composition with metabolic features in the mother and later health outcomes in the offspring. The newborn digestion model results in a resistance of most abundant human whey proteins, α-LA and lactoferrin, similar to the observed in vivo. Likewise, proteins ascribable to the undegraded peptides and a large number of specific sequences closely resemble those previously found in newborn digests after human milk consumption. Therefore, important outcomes after passage of human milk through the gastrointestinal tract can be predicted using the infant model. It remains to elucidate if the different protein composition might have physiological significance for the newborn.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10040887/s1, **Table S1:** MALDI-TOF signals provided by the predictive model generation. Effect of the mother condition (NW vs OW/Ob), **Table S2:** Identified peptides matching sequences previously observed in the gastrointestinal content of newborns or a rat pup model after human milk consumption, **Figure S1:** Complete heat map representation of the hierarchical clustering analysis of peptides identified after in vitro digestion of breast milk. Columns correspond to subjects included in the overweight/obese group or normal weight group. Rows correspond to intensity of sequences derived from β-casein, α-lactalbumin and lactoferrin identified in at least 3 subjects.

Author Contributions: The author's responsibilities were as follows: conceptualization B.M., I.R.; formal analysis S.S.-H., L.T., P.J.-B.; investigation

S.S.-H., P.J.-B.; writing—original draft preparation S.S.-H., B.M.; writing—review and editing B.M., I.R., L.T.; supervision B.M., I.R., M.O.-H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Ethics Committee of Hospital Universitario Virgen de las Nieves, Granada (SPID201600X080546IV0, 23 June 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest regarding the publication of this manuscript.

References

1. Butte, N.F.; Lopez-Alarcon, M.G.; Garza, C. Nutrient Adequacy of Exclusive Breastfeeding for the Term Infant During the First Six Months of Life; WHO: Geneva, Switzerland, 2002; Available online: https://apps.who.int/iris/handle/10665/42519 (accessed on 14 February 2018).

2. Lönnerdal, B. Bioactive Proteins in Human Milk: Health, Nutrition, and Implications for Infant Formulas. J. Pediatr. 2016, 173, S4–S9. [CrossRef]

3. Zepf, F.D.; Rao, P.; Moore, J.; Stewart, R.; Ladino, Y.M.; Hartmann, B.T. Human breast milk and adipokines—A potential role for the soluble leptin receptor (sOb-R) in the regulation of infant energy intake and development. Med. Hypotheses 2016, 86, 53–55. [CrossRef] [PubMed]

4. Lönnerdal, B. Infant formula and infant nutrition: Bioactive proteins of human milk and implications for composition of infant formulas. Am. J. Clin. Nutr. 2014, 99, 712S–717S. [CrossRef] [PubMed]

5. Liao, Y.; Weber, D.; Xu, W.; Durbin-Johnson, B.P.; Phinney, B.S.; Lönnerdal, B. Absolute Quantification of Human Milk Caseins and the Whey/Casein Ratio during the First Year of Lactation. J. Proteome Res. 2017, 16, 4113–4121. [CrossRef]

6. Ren, Q.; Zhou, Y.; Zhang, W.; Tian, Y.; Sun, H.; Zhao, X.; Xu, Y.; Jiang, S. Longitudinal changes in the bioactive proteins in human milk of the Chinese population: A systematic review. Food Sci. Nutr. 2021, 9, 25–35. [CrossRef] [PubMed]

7. Ahuja, S.; Boylan, M.; Hart, S.L.; Román-Shriver, C.; Spallholz, J.E.; Pence, B.C.; Sawyer, B.G. Glucose and Insulin Levels are Increased in Obese and Overweight Mothers' Breast-Milk. Food Nutr. Sci. 2014, 2, 201–206. [CrossRef]

8. Andreas, N.J.; Hyde, M.J.; Gale, C.; Parkinson, J.R.C.; Jeffries, S.; Holmes, E.; Modi, N. Effect of Maternal Body Mass Index on Hormones in Breast Milk: A Systematic Review. PLoS ONE 2014, 9, e115043. [CrossRef]

9. Saben, J.L.; Sims, C.R.; Piccolo, B.D.; Andres, A. Maternal adiposity alters the human milk metabolome: Associations between nonglucose monosaccharides and infant adiposity. Am. J. Clin. Nutr. 2020, 112, 1228–1239. [CrossRef]

10. Young, B.E.; Patinkin, Z.; Palmer, C.; De La Houssaye, B.; Barbour, L.A.; Hernandez, T.; Friedman, J.E.; Krebs, N.F. Human milk insulin is related to maternal plasma insulin and BMI: But other components of human milk do not differ by BMI. Eur. J. Clin. Nutr. 2017, 71, 1094–1100. [CrossRef]

11. Leghi, G.E.; Netting, M.J.; Middleton, P.F.; Wlodek, M.E.; Geddes, D.T.; Muhlhausler, B.S. The impact of maternal obesity on human milk macronutrient composition: A systematic review and meta-analysis. Nutrients 2020, 12, 934. [CrossRef]

12. Bourlieu, C.; Deglaire, A.; de Oliveira, S.C.; Ménard, O.; Le Gouar, Y.; Carrière, F.; Dupont, D. Towards infant formula biomimetic of human milk structure and digestive behaviour. OCL 2017, 24, D206. [CrossRef]

13. Devle, H.; Ulleberg, E.K.; Naess-Andresen, C.F.; Rukke, E.O.; Vegarud, G.; Ekeberg, D. Reciprocal interacting effects of proteins and lipids during exvivo digestion of bovine milk. Int. Dairy J. 2014, 36, 6–13. [CrossRef]

14. Dallas, D.C.; Guerrero, A.; Khaldi, N.; Borghese, R.; Bhandari, A.; Underwood, M.A.; Lebrilla, C.B.; German, J.B.; Barile, D. A Peptidomic Analysis of Human Milk Digestion in the Infant Stomach Reveals Protein-Specific Degradation Patterns. J. Nutr. 2014, 144, 815–820. [CrossRef]

15. Nielsen, S.D.; Beverly, R.L.; Underwood, M.A.; Dallas, D.C. Release of functional peptides from mother's milk and fortifier proteins in the premature infant stomach. PLoS ONE 2018, 13, e0208204. [CrossRef]

16. Beverly, R.L.; Woonnimani, P.; Scottoline, B.P.; Lueangsakulthai, J.; Dallas, D.C. Peptides from the Intestinal Tract of Breast Milk-Fed Infants Have Antimicrobial and Bifidogenic Activity. Int. J. Mol. Sci. 2021, 22, 2377. [CrossRef] [PubMed]

17. Di Francesco, L.; Di Girolamo, F.; Mennini, M.; Masotti, A.; Salvatori, G.; Rigon, G.; Signore, F.; Pietrantoni, E.; Scapaticci, M.; Lante, I.; et al. A MALDI-TOF MS approach for mammalian, human, and formula milks' profiling. Nutrients 2018, 10, 1238. [CrossRef] [PubMed]

18. Hammer, B.; Strickert, M.; Villmann, T. Supervised neural gas with general similarity measure. Neural Process. Lett. 2005, 21, 21–44. [CrossRef]

19. Holland, J.H. Adaptation in Natural and Artificial Systems; University of Michigan Press: Ann Arbor, MI, USA, 1975.

20. Ménard, O.; Bourlieu, C.; De Oliveira, S.C.; Dellarosa, N.; Laghi, L.; Carrière, F.; Capozzi, F.; Dupont, D.; Deglaire, A. A first step towards a consensus static in vitro model for simulating full-term infant digestion. Food Chem. 2018, 240, 338–345. [CrossRef]

21. Brodkorb, A.; Egger, L.; Alminger, M.; Alvito, P.; Assunção, R.; Ballance, S.; Bohn, T.; Bourlieu-Lacanal, C.; Boutrou, R.; Carrière, F.; et al. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 2019, 14, 991–1014. [CrossRef] [PubMed]

22. Miralles, B.; Sanchón, J.; Sánchez-Rivera, L.; Martínez-Maqueda, D.; Le Gouar, Y.; Dupont, D.; Amigo, L.; Recio, I. Digestion of micellar casein in duodenum cannulated pigs. Correlation between in vitro simulated gastric digestion and in vivo data. Food Chem. 2021, 343, 128424. [CrossRef] [PubMed]

23. Visser, S.; Slangen, C.J.; Rollema, H.S. Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chro- matography. J. Chromatogr. A 1991, 548, 361–370. [CrossRef]

24. Sánchez-Rivera, L.; Diezhandino, I.; Gómez-Ruiz, J.Á.; Fresno, J.M.; Miralles, B.; Recio, I. Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion. Electrophoresis 2014, 35, 1627–1636. [CrossRef]

25. Oliveros, J.C. Venny 2.1.0. Available online: https://bioinfogp.cnb.csic.es/tools/venny/index.html (accessed on 25 March 2021).

26. Manguy, J.; Jehl, P.; Dillon, E.T.; Davey, N.E.; Shields, D.C.; Holton, T.A. Peptigram: A Web-Based Application for Peptidomics Data Visualization. J. Proteome Res. 2017, 16, 712–719. [CrossRef] [PubMed]

27. Caraux, G.; Pinloche, S. PermutMatrix: A graphical environment to arrange gene expression profiles in optimal linear order. Bioinformatics 2005, 21, 1280–1281. [CrossRef] [PubMed]

28. Jose'stam, J.; Sauer, P.J.; Boehm, G. Can we define an infant's need from the composition of human milk? Am. J. Clin. Nutr. 2013, 98, 521S–528S. [CrossRef]

29. Young, B.E.; Levek, C.; Reynolds, R.M.; Rudolph, M.C.; MacLean, P.; Hernandez, T.L.; Friedman, J.E.; Krebs, N.F. Bioactive components in human milk are differentially associated with rates of lean and fat mass deposition in infants of mothers with normal vs. elevated BMI. Pediatr. Obes. 2018, 13, 598–606. [CrossRef]

30. Kratzsch, J.; Bae, Y.J.; Kiess, W. Adipokines in human breast milk. Best Pract. Res. Clin. Endocrinol. Metab. 2018, 32, 27–38. [CrossRef]

31. Young, B.E.; Johnson, S.L.; Krebs, N.F. Biological determinants linking infant weight gain and child obesity: Current knowledge and future directions. Adv. Nutr. 2012, 3, 675–686. [CrossRef]

32. Lönnerdal, B. Bioactive Proteins in Human Milk: Mechanisms of Action. J. Pediatr. 2010, 156, S26–S30. [CrossRef]

33. Baba, T.; Downs, D.; Jackson, K.W.; Tang, J.; Wang, C.S. Structure of Human Milk Bile Salt Activated Lipase. Biochemistry 1991, 30, 500–510. [CrossRef]

34. de Oliveira, S.C.; Deglaire, A.; Ménard, O.; Bellanger, A.; Rousseau, F.; Henry, G.; Dirson, E.; Carrière, F.; Dupont, D.; Bourlieu, C. Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under in vitro dynamic term newborn digestion. Food Res. Int. 2016, 88, 263–275. [CrossRef]

35. Davidson, L.A.; Lonnerdal, B. Persistence of human milk proteins in the breast-fed infant. Acta Paediatr. Scand. 1987, 76, 733–740. [CrossRef] [PubMed]

36. Zhang, Q.; Cundiff, J.K.; Maria, S.D.; McMahon, R.J.; Wickham, M.S.J.; Faulks, R.M.; Van Tol, E.A.F. Differential digestion of human milk proteins in a simulated stomach model. J. Proteome Res. 2014, 13, 1055–1064. [CrossRef] [PubMed]

37. Peterson, J.A.; Hamosh, M.; Scallan, C.D.; Ceriani, R.L.; Henderson, T.R.; Mehta, N.R.; Armand, M.; Hamosh, P. Milk fat globule glycoproteins in human milk and in gastric aspirates of mother's milk-fed preterm infants. Pediatr. Res. 1998, 44, 499–506. [CrossRef] [PubMed]

38. Beverly, R.L.; Underwood, M.A.; Dallas, D.C. Peptidomics Analysis of Milk Protein-Derived Peptides Released over Time in the Preterm Infant Stomach. J. Proteome Res. 2019, 18, 912–922. [CrossRef]

39. Wada, Y.; Phinney, B.S.; Weber, D.; Lönnerdal, B. In vivo digestomics of milk proteins in human milk and infant formula using a suckling rat pup model. Peptides 2017, 88, 18–31. [CrossRef]


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2.4.1 Material Suplementario Publicación III

Table S1. MALDI-TOF signals provided by the predictive model generation. Effect of the mother condition (NW vs OW/Ob).

Algorithm	m/z	weight
Quick classifier	4322.43	1.895
Supervised neural network	4041.4	0.123
	14281.32	0.103
	4162.71	0.097
	10516.64	0.089
	8131.44	0.066
	8838.71	0.058
	9127.24	0.052
	15203.42	0.039
	5867.56	0.038
	6294.35	0.032
	7144.01	0.028
	8558.49	0.025
	11301.63	0.025
	8420.52	0.024
	8459.07	0.024
	8245.42	0.021
	14488.11	0.021
	26114.9	0.017
Genetic algorithm	4162.71	0.687
	8838.71	1.035
	10308.53	0.432
	11960.16	0.440
	6021.39	0.129
	4162.71	0.687

Table S2. Identified peptides matching sequences previously observed in the gastrointestinal model after human milk consumption.

Sequence	Range[1]	Protein[2]	*In vivo* occurrence
RETIESL	1 - 7	CASB_HUMAN	Nielsen et al (2018)
VKHEDQQQGEDEHQDK	24 - 39	CASB_HUMAN	Nielsen et al (2018)
VKHEDQQQGEDEHQDKIYPS	24 - 43	CASB_HUMAN	Nielsen et al (2018)
HEDQQQGEDEHQDK	26 - 39	CASB_HUMAN	Nielsen et al (2018)
GEDEHQDKIYP	32 - 42	CASB_HUMAN	Nielsen et al (2018)
VEPIPY	54 - 59	CASB_HUMAN	Wada et al (2014)
GFLPQNILP	60 - 68	CASB_HUMAN	Su et al (2017), Nielsen et al (2018)
AQPAVVLPVPQPE	70 - 82	CASB_HUMAN	Wada et al (2017)
QPAVVLPVPQPE	71 - 82	CASB_HUMAN	Wada et al (2017)
QPAVVLPVPQPEIMEVPK	71 - 88	CASB_HUMAN	Dallas et al (2014), Nielsen et al (2018).
VVLPVPQP	74 - 81	CASB_HUMAN	Nielsen et al (2018)
VVLPVPQPE	74 - 82	CASB_HUMAN	Nielsen et al (2018), Wada et al (2017)
VVLPVPQPEIM	74 - 84	CASB_HUMAN	Dallas et al (2014), Nielsen et al (2018), Wad
VVLPVPQPEIMEVPK	74 - 88	CASB_HUMAN	Nielsen et al (2018), Wada et al (2017)
VVLPVPQPEI	74 - 83	CASB_HUMAN	Dallas et al (2014), Nielsen et al (2018), Wad
LPVPQPE	76 - 82	CASB_HUMAN	Wada et al (2017)
LPVPQPEIM	76 - 84	CASB_HUMAN	Dallas et al (2014), Nielsen et al (2018)
LPVPQPEIMEVPK	76 - 88	CASB_HUMAN	Dallas et al (2014), Nielsen et al (2018), Ding
PVPQPEIM	77 - 84	CASB_HUMAN	Dallas et al (2014), Nielsen et al (2018)
PQPEIMEVPK	79 - 88	CASB_HUMAN	Nielsen et al (2018)
PEIMEVPK	81 - 88	CASB_HUMAN	Nielsen et al (2018)
MEVPK	84 - 88	CASB_HUMAN	Wada et al (2017)

MPVLKSPTIPFFD	100 - 112	CASB_HUMAN	Nielsen et al (2018)
VLKSPTIPFFD	102 - 112	CASB_HUMAN	Nielsen et al (2018)
APVHNPI	203 - 209	CASB_HUMAN	Wada et al (2017)
CAVSQPEAT	9 - 17	TRFL_HUMAN	Wada et al (2017)
GPPVSC	31 - 36	TRFL_HUMAN	Wada et al (2017)
FPNLC	166 - 170	TRFL_HUMAN	Wada et al (2017)
EDLSDEAERDE	216 - 226	TRFL_HUMAN	Dallas et al (2014), Su et al (2017), Nielsen et
ELLCPDNT	228 - 235	TRFL_HUMAN	Wada et al (2017)
FKDSAIGF	300 - 307	TRFL_HUMAN	Dallas et al (2014), Su et al (2017), Nielsen et
CVDRPVEG	427 - 434	TRFL_HUMAN	Wada et al (2017)
CIGDEQGEN	507 - 515	TRFL_HUMAN	Wada et al (2017)
CLAENAGDVA	534 - 543	TRFL_HUMAN	Wada et al (2017)
NGSDCPDKF	623 - 631	TRFL_HUMAN	Wada et al (2017)
NGSDCPDKFC	623 - 632	TRFL_HUMAN	Wada et al (2017)
TKCELSQ	4 - 10	LALBA_HUMAN	Wada et al (2017)
KDIDGYGGIAL	13 - 23	LALBA_HUMAN	Dallas et al (2014)
GGIALPELI	19 - 27	LALBA_HUMAN	Wada et al (2017)
DTQAIVENNESTE	37 - 49	LALBA_HUMAN	Nielsen et al (2018)
AIVENNESTEY	40 - 50	LALBA_HUMAN	Dallas et al (2014). Nielsen et al (2018)
AIVENNESTEYG	40 - 51	LALBA_HUMAN	Nielsen et al (2018)
IVENNESTEY	41 - 50	LALBA_HUMAN	Dallas et al (2014), Su et al (2017), Nielsen et
IVENNESTEYG	41 - 51	LALBA_HUMAN	Su et al (2017), Nielsen et al (2018)
GLFQISNK	51 - 58	LALBA_HUMAN	Nielsen et al (2018)
NICDISCD	71 - 78	LALBA_HUMAN	Wada et al (2017)
NICDISCDK	71 - 79	LALBA_HUMAN	Wada et al (2017)
CDISCDK	73 - 79	LALBA_HUMAN	Wada et al (2017)
DDDITDDIM	82 - 90	LALBA_HUMAN	Wada et al (2017)
ALCTEKLEQ	109 - 117	LALBA_HUMAN	Wada et al (2017)
CTEKLEQ	111 - 117	LALBA_HUMAN	Wada et al (2017)
CTEKLEQWL	111 - 119	LALBA_HUMAN	Wada et al (2017)
TEKLEQW	112 - 118	LALBA_HUMAN	Wada et al (2017)
LQNPSESSEPIPL	12 - 24	CASA1_HUMAN	Nielsen et al (2018)
CAEQFC	84 - 89	CASA1_HUMAN	Wada et al (2017)

SHVQVPFQQL	115 - 124	CASA1_HUMAN	Nielsen et al (2018)
GGFVEGVNK	10 - 18	CEL_HUMAN	Nielsen et al (2018)
ASIDMPAINK	325 - 334	CEL_HUMAN	Nielsen et al (2018)
EDITSHME	171 - 178	OSTP_HUMAN	Nielsen et al (2018)
ELDSASSEVN	289 - 298	OSTP_HUMAN	Nielsen et al (2018)
NNPYVPR	52 - 58	CASK_HUMAN	Wada et al (2017)
LPNSHPPT	79 - 86	CASK_HUMAN	Wada et al (2017)

[1]Without signal peptide

[2]Uniprot entry name

References

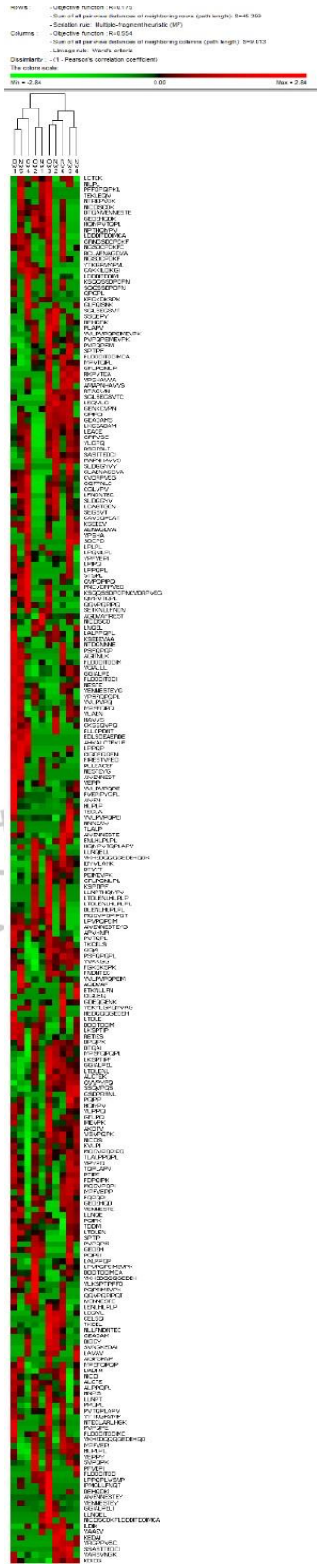
Dallas, D.C.; Guerrero, A.; Khaldi, N.; Borghese, R.; Bhandari, A.; Underwood, M.A.; Lebril
A Peptidomic Analysis of Human Milk Digestion in the Infant Stomach Reveals Protein-Specific De
144, 815–820, doi:10.3945/jn.113.185793.

Nielsen, S.D.; Beverly, R.L.; Underwood, M.A.; Dallas, D.C. Release of functional peptides
proteins in the premature infant stomach. PLoS One 2018, 13, e0208204, doi:10.1371/journal.pone.0

Beverly, R.L.; Woonnimani, P.; Scottoline, B.P.; Lueangsakulthai, J.; Dallas, D.C. Peptides f
Milk-Fed Infants Have Antimicrobial and Bifidogenic Activity. Int. J. Mol. Sci. 2021, 22, 2377, doi:

Wada, Y.; Phinney, B.S.; Weber, D.; Lönnerdal, B. In vivo digestomics of milk proteins in hur
a suckling rat pup model. Peptides 2017, 88, 18–31, doi:10.1016/j.peptides.2016.11.012.

Figure S1. Complete heat map representation of the hierarchical clustering analysis of peptides identified after in vitro digestion of breast milk. Columns correspond to subjects included in the overweight/obese group or normal weight group. Rows correspond to intensity of sequences derived from β-casein, α- lactalbumin and lactoferrin identified in at least 3 subjects.

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Publicación IV

Identification of bioactive peptides after in vitro digestion of human milk under relevant infant conditions

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Abstract

Over the course of human milk digestion, infant digestive proteases fragment proteins into peptides with potential bioactivity. In this study, ten mature human milks samples were subjected to a relevant infant gastrointestinal static *in vitro* digestion. Peptides released from human proteins were identified by tandem mass spectrometry. Numerous peptides with predomination of the three most abundant proteins, in the order β-casein > lactoferrin > α-lactoalbumin were identified. Twelve peptides, including antioxidative, antimicrobial and immunomodulatory peptides, were found to completely match the sequences of known bioactive peptides. Moreover, some of these sequences had been previously identified in the digestive content of infants after human milk intake, which supports their physiological significance. The present study shows that an in vitro model simulating infant gastrointestinal digestion can be a good alternative to *in vivo* digestion to predict the release of bioactive sequences.

Keywords: Human milk proteins, in vitro infant digestion, bioactive peptides, mass spectrometry

1. Introduction

Human milk (HM) is an excellent source of nutrients for infants, and breast-feeding is considered as the optimal mode to support their growth and development (Su et al., 2017). Numerous constituents of HM are responsible for these reported beneficial effects, and the protein fraction contained in human milk plays an important role for the development of the newborn. HM proteins contribute to the high degree of nutrients utilization in different ways. There are proteins that bind essential nutrients, keeping them soluble and readily available for their uptake by the digestive tract. Besides, protease inhibitors limit the activity of proteolytic enzymes and help the functional proteins to maintain their utility. Lastly, some enzymes decisively affect the effective use of macronutrients (Lönnerdal, 2003). Milk proteins with carrier and absorption activities include bile salt stimulated lipase, amylase or α_1-antitrypsin, caseins (β- and κ-casein), α-lactalbumin, or

lactoferrin. Some of these proteins, such as lactoferrin or κ-casein are also antimicrobial, as lysozyme, a major whey fraction component. There are also proteins involved in defence mechanisms of the organism such as the already mentioned lactoferrin, together with direct players such as secretory immunoglobulin A (Elwakiel et al., 2020; Lönnerdal, 2016).

Apart from exerting their effect in an intact form, HM proteins can have a functional role through the formation of biologically active peptides. It has been shown that milk proteases actively cleave milk proteins within the mammary gland, initiating the release of functional sequences (Ferranti et al., 2004). Thus, the breastfed infant receives partially predigested proteins including numerous physiologically active fragments (Nielsen, Beverly, & Dallas, 2017; Zhu & Dingess, 2019). However, some bioactive peptides are encrypted within the sequence of parental proteins and are only released by gastrointestinal digestive enzymes (Chatterton et al., 2004; Wada & Lönnerdal, 2020). Since the intestinal barrier of the newborn is quite permeable (Weaver & Walker, 1989), it can benefit from peptides that may be found in milk as well as those generated by digestion.

Milk proteins can be classified into caseins and whey proteins, that comprise the major part of the total protein fraction. In addition, mucins are present in the milk fat globule membrane (MFGM), they contribute a small percentage (1-4%) of the total protein content of HM and their biological importance is still poorly understood (Lönnerdal et al., 2017). Most of the bioactive peptides generated during gastrointestinal digestion are derived from the main proteins in HM: caseins, α-lactalbumin and lactoferrin, the amino acid sequence of the released peptides determining the exerted activity [9]. Biological activities of HM peptides that have been described include antioxidant, antimicrobial and antibacterial, opioid, immunomodulatory, antithrombotic, antihypertensive, probiotic, angiotensin-converting enzyme (ACE) inhibitory, calcium-binding and anti-inflammatory (Wada & Lönnerdal, 2014). However, it is important to demonstrate that the known biological sequences resist the gastrointestinal passage. Mass spectrometric tools permit to track the fate of food proteins during

gastrointestinal digestion and their selectivity allows the analysis of very complex digests where these sequences are to be found (Picariello et al., 2013). Although the peptidome analysis of HM is being the object of study in the last decade (Wan et al., 2013); and simulated digestion under highly complex models has been applied to this indispensable fluid (Zhang et al., 2014), there are still few reports on the presence of bioactive sequences from HM after digestion (Dall'Asta et al., 2015; Hernández-Ledesma et al., 2007; Su et al., 2017). In the last decade, *in vitro* digestion models have been refined to mimic the full term infant digestion (Ménard et al., 2018). On the other hand, some *in vivo* studies on the digestive contents after HM intake are contributing to unveil the HM digestome (Beverly et al., 2020; Dallas et al., 2014).

The aim of our study was to identify peptides with reported biological activity after gastrointestinal digestion under relevant infant digestion conditions in mature breast milk from mothers in the Granada region. The identified sequences have been contrasted to those present in the digestive contents after HM intake, both in infants and animal models.

2. Materials and methods

2.1 Human milk samples

Mature HM samples were donated from 10 Spain mothers in the Granada region and in a study conducted in the Obstetrics and Gynecology Department of "Hospital Universitario Virgen de las Nieves" from Granada (Spain). Ethics were approved by the relevant scientific committee and details of the study were explained to all mothers who voluntarily gave written consent to participate. The main characteristics of sampled mothers are shown in **Table 1**. All human milk samples collected from the lactating mothers in sterile tubes were aliquoted and immediately stored at -80ºC until used.

Table 1. Demographic description of study mothers and delivery characteristics by group.

Characteristics	Normal Weight (n=6)	Overweight (n=4)
Age, years	32.2 (4.2)	32.5 (3.4)
Mature milk collection, days postpartum	20.8 (3.0)	19.0 (1.2)
Gestational age, days	274.8 (8.6)	282.8 (6.7)
BMI early pregnancy, kg m^{-2}	21.0 (1.1)	28.7 (2.3)
BMI at delivery, kg m^{-2}	25.4 (1.6)	33.7 (2.9)
Weight gain, kg	12.2 (3.4)	13.3 (5.0)
Newborn weight, g	3040 (560)	3400 (500)
Gender distribution, %		
Female	33	50
Male	67	50

BMI: Body Mass Index

2.2 In Vitro Simulated Gastrointestinal Digestion

The infant gastrointestinal digestion was carried out following the protocol described by Ménard et al. (Ménard et al., 2018). The gastrointestinal in vitro simulation included two consecutive steps: a gastric phase (60 min at 37ºC with pepsin and lipase at pH 5.3) and an intestinal phase (60 min at 37ºC with porcine pancreatin and bile at pH 6.6). All samples were lyophilized and stored at -20ºC until analysis.

2.3 Analysis of Digests by HPLC–Tandem Mass Spectrometry (HPLC–MS/MS)

Freeze-dried digestion samples were reconstituted in ammonium carbonate (NH4CO3) 25 mM and treated for 60 min at 37ºC with 1,4-dithiothreitol 140 mM (1:1, v/v) (Sigma-Aldrich, St. Louis, MO, USA) to reduce disulfide linkages in order to improve the identification. Samples were analyzed by HPLC–MS/MS in duplicate using an Agilent 1100 HPLC system (Agilent Technologies, Waldbron, Germany), equipped with a Mediterranean Sea18 column (150 mm x 2.1 mm, Teknokroma, Barcelona, Spain) connected to an Esquire 3000 linear ion trap mass

spectrometer (Bruker Daltonics GmbH, Bremen, Germany) as previously described (Sánchez-Hernández et al., 2021). Bioactive sequences were searched using the milk bioactive peptide database (MBPDB) (Nielsen, Beverly, Qu, et al., 2017) and the database of bioactive peptides (BIOPEP) (Minkiewicz et al., 2019).

3. Results and discussion

3.1 Peptides generated after in vitro gastrointestinal digestion of HM

In vitro gastrointestinal digestion of HM under infant conditions resulted in the release of numerous peptides, with predomination of the three most abundant proteins, in the order β-casein > lactoferrin > α-lactalbumin. These three proteins accounted for 305 different sequences identified in at least two individual digestions (consensus sequences). In the preceding study, despite the fact that the HM samples were from normal weight or overweight subjects, no discrimination in the peptide profile after digestion could be observed regarding body mass index (BMI) (Sánchez-Hernández et al., 2021). A large percentage (70%) of the peptides derived from these three proteins after digestion were common between subject belonging to normal weight and overweight groups. In the case of β-casein, which is the protein where a higher number of bioactive sequences have been described, the percentage of common peptides was 80% **(Figure 1).**

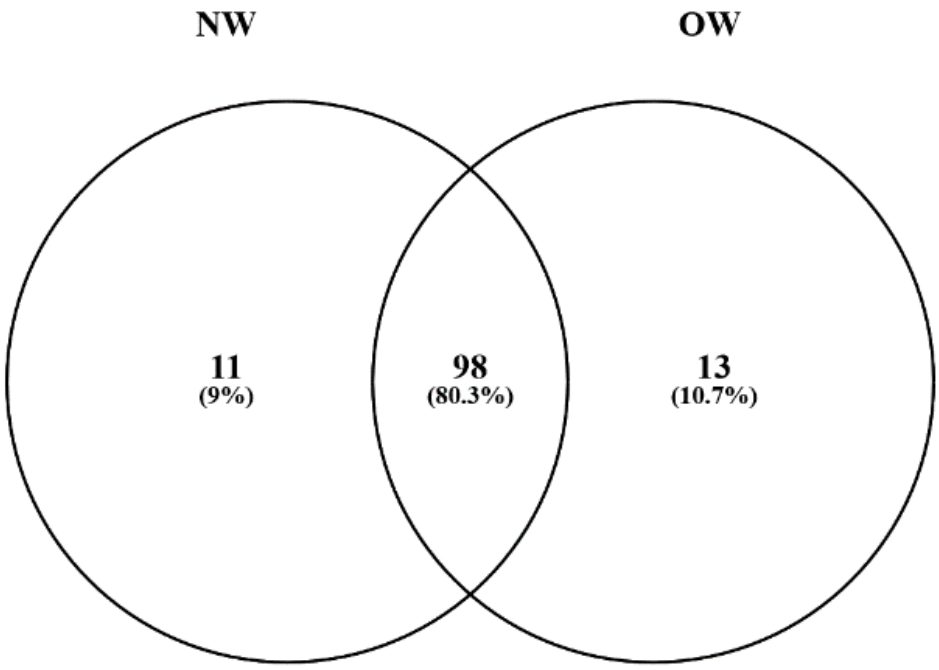

Figure 1. Peptides derived from β-casein, in the gastrointestinal digestion of milk from normal weight (NW) and overweight (OW) subjects.

3.3 Peptides released from HM with known biological activity

Twelve peptides identified in the digests were found to completely match the sequences of known bioactive peptides according to the literature, such as indexed by BIOPEP and MBPDB databases. In addition to the identical sequences, multiple peptides identified were highly homologous (≥ 80% sequence match) to known bioactive peptides, as shown in **Table 2**. The occurrence in the digestive contents of infants after HM intake is indicated for these sequences. Bioactivities annotated to peptides were antioxidative, antihypertensive or ACE inhibitory, opioid, immunomodulatory, prolyl-peptidase (PEP) inhibitory, antimicrobial and antibacterial. Several of these peptides were identified with more than one function.

Table 2. HM proteins-derived peptides identified after infant simulated digestion of HM (n
regions. In red, bioactive sequences found in simulated digests.

Protein fragment	Identified peptides after infant simulated digestion	Bioactive sequence	Reported activity
β-CN 41-58	YPS**FQPQPLIYP**FVEPIP	FQPQPLIYP	ACE inhibitor
β-CN 46-70	PQ**PLIYP**FVEPIPYGFLPQNILPLA	PLIYP	ACE inhibitor
β-CN 41-58	YPSFQPQ**PLIYP**FVEPIP	QPQPLIY	
β-CN 45-54	QPQ**PLIYP**FV	QPQPLIYP	
β-CN 46-70	PQ**PLIYPFVEPIP**YGFLPQNILPLA	IYPF	Antioxidative
β-CN 41-58	YPSFQPQ**PLIYPFVEPIP**	YPFVE	PEP inhibitor.
β-CN 45-54	QPQPL**IYPF**V	YPFVEPIP	Opioid Agonist
β-CN 50-58	I**YPFVEPIP**	PLIYPFVEPIP	
β-CN 50-57	**IYPFVE**PI		
β-CN 46-70	PQPLIY**PFVEPIPYG**FLPQNILPLA	PIPY	PEPi inhibitor
β-CN 53-62	**FVEPIPYG**FL	PFVEPIPY	
β-CN 53-60	**FVEPIPYG**		
β-CN 54-59	**VEPIPY**	VEPIPY	Immunomodulatin
		VEPIPYG	

		FVEPIPY	PEPi inhibitor Inmmunomodulatin
		YPFVEPIPY	PEPi Opioid
β-CN 83-111	IMEVPKAKD**TVYTKGRVMP**VLKSPTIPFF	TVYTKGRVMP	ACE inhibitor
β-CN 88-117	KAKD**TVYTKGRVMP**VLKSPT		
β-CN 105-117	**SPTIPFFDPQIPK**	SPTIPFFDPQIPK	Immunomodulatin
β-CN 118-130	LTD**LENLHLPLP**L	HLPLP	Antihypertensive
β-CN 118-129	LTD**LENLHLPLP**	LHLPLP	
β-CN 120-130	D**LENLHLPLP**L	LHLPLPL	
β-CN 121-129	**LENLHLPLP**		
β-CN 122-130	**ENLHLPLP**L	HLPLPL	Antiamnestic
β-CN 124-129	**LHLPLP**		Antihypertensive
β-CN 125-130	**HLPLPL**		
β-CN 125-129	**HLPLP**	ENLHLPLP	ACE inhibitor
		LENLHLPLP	
		NLHLPLPL	Antiproliferative ACE inhibitor

β-CN 150-160	PQPL**WSVPQPK**	WSVPQPK	ACE inhibitor
β-CN 154-160	**WSVPQPK**		Antioxidative
β-CN 161-180	**VLPIPQ**QVVPYPQRAVPVQA	VLPIPQ	ACE inhibitor
β-CN 160-167	K**VLPIPQ**Q		
β-CN 161-166	**VLPIPQ**		
β-CN 161-180	**VLPIPQQVVPYPQ**RAVPVQA	VPYPQ	Antioxidative
β-CN 167-173	**QVVPYPQ**	VVPYPQR	Antihypertensive
β-CN 169-173	**VPYPQ**		Antimicrobial
		QVVPYPQ	Antioxidative
		QVVPYPQR	
		VLPIPQQVVPYPQ	
β-CN 183-211	LNQELLLNPT**HQIYPVTQPLAP**VHNPISV		
β-CN 188-211	LLNPT**HQIYPVTQPLAP**VHNPISV		
β-CN 188-210	LLNPT**HQIYPVTQPLAP**VHNPIS		
β-CN 193-205	**HQIYPVTQPLAP**V	HQIYPV	Antioxidative
β-CN 188-198	LLNPT**HQIYPV**	HQIYPVTQPLAP	
β-CN 193-202	**HQIYPV**TQPL		
β-CN 189-198	LNPT**HQIYPV**		
β-CN 190-198	NPT**HQIYPV**		
β-CN 193-198	**HQIYPV**		
β-CN 183-211	LN**QELLLNPTHQIYPVTQPLAPVHNPISV**	PLAPVHNPISV	

β-CN 188-211 β-CN 188-210 β-CN 197-211 β-CN 201-211	LLNPTHQ**IYPVTQPLAPVHNPIS**V LLNPTHQ**IYPVTQPLAPVHNPIS** PVTQ**PLAPVHNPISV** **PLAPVHNPISV**	PVTQPLAPVHNPI IYPVTQPLAPVHNPI IYPVTQPLAPVHNPIS LLNQELLLNPTHQIYPV QELLLNPTHQIYPVTQPLAPVHNPISV	Antimicrobial Antibacterial
β-CN 183-211 β-CN 188-211 β-CN 188-210 β-CN 196-210 β-CN 197-211 β-CN 203-209 β-CN 206-209	LNQELLLNPTHQIYPVTQPLAPV**HNPI**SV LLNPTHQIYPVTQPLAPV**HNPI**SV LLNPTHQIYPVTQPLAPV**HNPI**S YPVTQPLAPV**HNPI**S PVTQPLAPV**HNPI**SV APV**HNPI** **HNPI**S	HNPI	Antioxidative
β-CN 1953-1967	GLL**YPFP**KDCSQAML	YPFP	Opioid agonist
κ-CN 31-36	**VPNSYP**	VPNSYP	Antioxidative
κ-CN 52-58	N**NPYVPR**	NPYVPR	Antioxidative
κ-CN 57-72	PRTY**YANPAVVRP**HAQ	YANPAVVRP	ACE inhibitor
κ-CN 96-106	SFIA**IPP**KKIQ	IPP IPPK PPKK	Antihypertensive Antithrombotic
α-LA 48-65 α-LA 48-60 α-LA 51-58	TEY**GLF**QISNKLWCKSSQ TEY**GLF**QISNKLW **GLF**QISNK	GLF	Immunomodulatin

α-LA 101-108	IDY**WLAHK**	WLAHK	ACE inhibitor
LF 311-326	PPRIDSGL**YLGSGY**FT	YLGSGY	Opioid antagonist
LF 543 – 577	AFVKDVTVLQNTDGNNNEAWAK**DLKLADFA**LLCLD	LKLADF	ACE inhibitor
LF 564 – 573	K**DLKLADFA**L	DLKLADFA	Antioxidative
		DLKLADFAL	
LF 659-670	YE**KYLGPQYV**AG	KYLGPQYV	Opioid

[a] Protein sequence including signal peptide

β-CN: β-casein, α-LA: α-lactalbumin; LF: lactoferrin; κ-CN: κ-casein, ACE-inhibitor: Angiotensin-Converting Enzyme inhibitor, F
inhibitor

Antioxidative peptides from digested HM can either prevent the formation of free radicals or scavenge existing free radicals or peroxides involved in the oxidation of membrane lipids, cellular proteins, DNA and enzymes (Nielsen, Beverly, Qu, et al., 2017). The studied digests were the source of five previously reported antioxidant peptides of small size, with a range from five to seven amino acids in length, four belonging to β-casein and one to κ-casein. The identified antioxidant peptides of β-casein have not only been described to be released under *in vitro* digestion conditions (Hernández-Ledesma et al., 2007), but have also been reported *in-vivo* after HM intake. Peptide β-casein ^{154}**WSVPQPK**^{160} was identified in the infant intestinal samples and in the premature infant stomach (Beverly et al., 2021; Nielsen et al., 2018). Wada et al. (2017) identified the peptide β-casein ^{169}**VPYPQ**^{173} and two homologous bioactive peptides of β-casein ^{193}**HQIYPV**^{198} and β-casein ^{167}**QVVPYPQ**^{173} in the small intestine of suckling rat pups. A peptide homologous to the latter was also found in the stool of infants after *in vivo* gastrointestinal digestion (Beverly et al., 2020).

Many of the query peptides matched bioactive peptides with antimicrobial activity against bacteria and fungi that can infect the infant gut, such as *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae and Candida albicans,* among others (Fu et al., 2017; Gan et al., 2019; Minervini et al., 2003). Antimicrobial effects following milk protein ingestion appear to mainly arise from lactoferrin, however peptides from caseins, particularly those originated from the β-casein C-terminal sequence, have also been shown to possess some antibacterial properties. In accordance to our results, Su et al. (2017) identified the peptide β-casein ^{201}**PLAPVHNPISV**^{211} after gastrointestinal digestion with reported antibacterial activity (Su et al., 2017). Other peptides at the C-terminal end of β-casein identified in the present study, i.e. β-casein fragments (183-211), (188-211), (188-210), and (197-211) are comprised in the antimicrobial sequence β-casein ^{185}QELLLNPTHQIYPVTQPLAPVHNPISV^{211}. This peptide has shown a broad spectrum of inhibition against Gram-positive and Gram-negative bacteria (Minervini et al., 2003) and has also been found in the digestive content *in vivo* (Beverly et al., 2020; Nielsen et al., 2018; Wada & Lönnerdal, 2020). Some of

these overlapping peptides could also exert antimicrobial functions after undergoing minor degradation.

One of the functions of immunomodulatory peptides include stimulation of lymphocyte activity and proliferation, and promotion of antibody formation (Migliore-Samour & Jollès, 1988). The hexapeptide β-casein ^{54}**VEPIPY**^{59}, first isolated from HM in 1984, had shown to stimulate the phagocytosis of opsonized sheep red blood cells by murine peritoneal macrophages and enhanced the resistance to infection with *Kebsiella pneumoniae* in mice (Parker et al., 1984). According to our results, this sequence might be released as such in the newborn's gut through gastrointestinal digestion. Another peptide identified with immunomodulatory activity was β-casein ^{105}**SPTIPFFDPQIPK**^{117}, which has also been described in the premature infant stomach and in the gastric samples from their infants 2h after ingestion of their mother's milk (Dallas et al., 2014; Nielsen et al., 2018).

Special attention has been paid to the β-casein region (118–130), where peptides with potent antihypertensive activity have been described (Miguel et al., 2010; Quirós et al., 2007), and have been found in premature infant gastric (Nielsen et al., 2018) and human jejunal aspirates (Boutrou et al., 2013) after human or cow's milk intake, respectively. It is a highly conserved region domain among different mammal species and hosts several active sequences such as the antihypertensive HLPLP and LHLPLP (Sánchez-Rivera et al., 2014). Most of the peptides described above were identified in all individual HM digests **(Table 3)**, suggesting that they could be representative peptides of the digested HM under infant conditions.

Table 3. Peptides in the digested HM with 100% homology to known bioactive peptides.

Peptide	Reported activity	NW1	NW2	NW3	NW4	NW5	NW6	OW1	OW2	OW3	OW4
VEPIPY	Immunomodulating	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
HLPLP	Antihypertensive	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
HLPLPL	Antihypertensive, antiamnestic,	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
VLPIPQ	ACE inhibitor	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
QVVPYPQ	Antioxidative	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
HQIYPV	Antioxidative	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
WSVPQPK	ACE inhibitor, antioxidative	✓	✓	✓	✓	✓	✓	✓	✓	✓	
LENLHLPLP	ACE inhibitor	✓	✓	✓	✓		✓	✓	✓	✓	✓
VPYPQ	Antioxidative	✓	✓	✓	✓	✓	✓	✓	✓	✓	
LHLPLP	Antihypertensive				✓				✓		

NW: Normal Weight; OW: Overweight

The composition of HM is often mirrored as the gold standard to estimate the nutritional needs of infants (Stam et al., 2013). Gastrointestinal formation of bioactive peptides derived from HM protein is a subject of growing interest in pediatric nutrition. It gives us not only a better understanding of the physiological significance of breast-feeding, but also might provide hints for improving the design of infant formulas. Peptides with biological activity reported in the present study overlap to a high extent those found in gastrointestinal samples from infants.

It is important to highlight that the use of a well-established *in vitro* digestion model may aid in profiling HM protein derived peptides in the small intestine of breastfed infants. As the number of peptides registered in databases such as BIOPEP and MBPDB is increasing, this will allow in the near future to ascribe new bioactivities for as yet undescribed sequences. However, it is important to note that the activity of the peptides has mainly been unraveled only *in vitro*, and it remains unclear in many cases whether the activity is physiologically significant (Wada & Lönnerdal, 2014). Further studies will be necessary to link the presence of these sequences in the neonate gastrointestinal tract with the observed health outcomes. Nevertheless, the present study shows

that an *in vitro* model simulating infant gastrointestinal digestion can be a good alternative to *in vivo* digestion for obtaining reliable data.

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References

Asano, M., Nio, N., & Ariyoshi, Y. (1991). Inhibition of Prolyl Endopeptidase by Synthetic Peptide Fragments of HumanjS-Casein. In *Biol. Chem* (Vol. 55, Issue 3).

Azuma, N., Nagaune, S., Yoko, I., Mori, H., Kaminogawa, S., & Yamauchi, K. (2014). *DNA-Synthesis Stimulating Peptides from Human β-Casein*. https://doi.org/10.1080/00021369.1989.10869716

Beverly, R. L., Huston, R. K., Markell, A. M., McCulley, E. A., Martin, R. L., & Dallas, D. C. (2020). Milk Peptides Survive in Vivo Gastrointestinal Digestion and Are Excreted in the Stool of Infants. *Journal of Nutrition*, *150*(4), 712–721. https://doi.org/10.1093/jn/nxz326

Beverly, R. L., Woonnimani, P., Scottoline, B. P., Lueangsakulthai, J., & Dallas, D. C. (2021). Peptides from the Intestinal Tract of Breast Milk-Fed Infants Have Antimicrobial and Bifidogenic Activity. *International Journal of Molecular Sciences*, *22*(5), 2377. https://doi.org/10.3390/ijms22052377

Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tomé, D., & Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *American Journal of Clinical Nutrition*, *97*(6), 1314–1323.

https://doi.org/10.3945/ajcn.112.055202

Boutrou, R., Henry, G., & Sanchez-Rivera, L. (2015). On the trail of milk bioactive peptides in human and animal intestinal tracts during digestion: A review. In *Dairy Science and Technology* (Vol. 95, Issue 6, pp. 815–829). Springer-Verlag France. https://doi.org/10.1007/s13594-015-0210-0

Brantl, V. (1984). Novel opioid peptides derived from human β-casein: Human β-casomorphins. *European Journal of Pharmacology*, *106*(1), 213–214. https://doi.org/10.1016/0014-2999(84)90702-7

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sørensen, E. S., & Petersen, T. E. (2004). In vitro digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. *Trends in Food Science and Technology*, *15*(7–8), 373–383. https://doi.org/10.1016/j.tifs.2003.12.004

Dall'Asta, C., Florio, P., Lammardo, A. M., Prandi, B., Mazzeo, T., Budelli, A., & Pellegrini, N. (2015). Development of an *in vitro* digestive model for studying the peptide profile of breast milk. *International Journal of Food Sciences and Nutrition*, *66*(4), 409–415. https://doi.org/10.3109/09637486.2015.1042846

Dallas, D. C., Guerrero, A., Khaldi, N., Borghese, R., Bhandari, A., Underwood, M. A., Lebrilla, C. B., German, J. B., & Barile, D. (2014). A Peptidomic Analysis of Human Milk Digestion in the Infant Stomach Reveals Protein-Specific Degradation Patterns. *The Journal of Nutrition*, *144*(6), 815–820. https://doi.org/10.3945/jn.113.185793

Deglaire, A., Oliveira, S. De, Jardin, J., Briard-Bion, V., Kroell, F., Emily, M., Ménard, O., Bourlieu, C., & Dupont, D. (2019). Impact of human milk pasteurization on the kinetics of peptide release during in vitro dynamic digestion at the preterm newborn stage. *Food Chemistry*, *281*, 294–303. https://doi.org/10.1016/j.foodchem.2018.12.086

Elwakiel, M., Boeren, S., Wang, W., Schols, H. A., & Hettinga, K. A. (2020).

Degradation of Proteins From Colostrum and Mature Milk From Chinese Mothers Using an in vitro Infant Digestion Model. *Frontiers in Nutrition*, *7*, 162. https://doi.org/10.3389/fnut.2020.00162

Ferranti, P., Traisci, M. V., Picariello, G., Nasi, A., Boschi, V., Siervo, M., Falconi, C., Chianese, L., & Addeo, F. (2004). Casein proteolysis in human milk: Tracing the pattern of casein breakdown and the formation of potential bioactive peptides. *Journal of Dairy Research*, *71*(1), 74–87. https://doi.org/10.1017/S0022029903006599

Fu, Y., Ji, C., Chen, X., Cui, X., Wang, X., Feng, J., Li, Y., Qin, R., & Guo, X. (2017). Investigation into the antimicrobial action and mechanism of a novel endogenous peptide β-casein 197 from human milk. *AMB Express*, *7*(1), 119. https://doi.org/10.1186/s13568-017-0409-y

Gan, J., Robinson, R. C., Wang, J., Krishnakumar, N., Manning, C. J., Lor, Y., Breck, M., Barile, D., & German, J. B. (2019). Peptidomic profiling of human milk with LC–MS/MS reveals pH-specific proteolysis of milk proteins. *Food Chemistry*, *274*, 766–774. https://doi.org/10.1016/j.foodchem.2018.09.051

Gobbetti, M., Minervini, F., & Rizzello, C. G. (2004). Angiotensin I-converting-enzyme-inhibitory and antimicrobial bioactive peptides. *International Journal of Dairy Technology*, *57*(2–3), 173–188. https://doi.org/10.1111/j.1471-0307.2004.00139.x

Goldman, A. S., Garza, C., Schanler, R. J., & Goldblum, R. M. (1990). *Molecular Forms of Lactoferrin in Stool and Urine from Infants Fed Human Milk* (Vol. 27, Issue 3).

Hayes, M., Stanton, C., Fitzgerald, G. F., & Ross, R. P. (2007). Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnology Journal*, *2*, 435–449. https://doi.org/10.1002/biot.200700045

Hernández-Ledesma, B., Quirós, A., Amigo, L., & Recio, I. (2007).

Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *International Dairy Journal*, *17*(1), 42–49. https://doi.org/10.1016/j.idairyj.2005.12.012

Kohmura, M., Nio, N., Kubo, K., Minoshima, Y., Munekata, E., & Yasuo Ariyoshi. (1989). Inhibition of angiotensin-converting enzyme by synthetic pep tides of human β-casein. *Agricultural and Biological Chemistry*, *53*(8), 2107–2114. https://doi.org/10.1080/00021369.1989.10869621

Lönnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. *The American Journal of Clinical Nutrition*, *77*(6), 1537S-1543S. https://doi.org/10.1093/ajcn/77.6.1537S

Lönnerdal, B. (2016). Human Milk: Bioactive Proteins/Peptides and Functional Properties. *Nestle Nutrition Institute Workshop Series*, *86*, 97–107. https://doi.org/10.1159/000442729

Lönnerdal, B., Erdmann, P., Thakkar, S. K., Sauser, J., & Destaillats, F. (2017). Longitudinal evolution of true protein, amino acids and bioactive proteins in breast milk: a developmental perspective. In *Journal of Nutritional Biochemistry* (Vol. 41, pp. 1–11). Elsevier Inc. https://doi.org/10.1016/j.jnutbio.2016.06.001

Meisel, H., & Fitzgerald, R. J. (2021). *Opioid peptides encrypted in intact milk protein sequences*. https://doi.org/10.1017/S000711450000221X

Ménard, O., Bourlieu, C., De Oliveira, S. C., Dellarosa, N., Laghi, L., Carrière, F., Capozzi, F., Dupont, D., & Deglaire, A. (2018). A first step towards a consensus static in vitro model for simulating full-term infant digestion. *Food Chemistry*, *240*, 338–345. https://doi.org/10.1016/j.foodchem.2017.07.145

Migliore-Samour, D., & Jollès, P. (1988). Casein, a prohormone with an immunomodulating role for the newborn? *Experientia*, *44*, 6.

Miguel, M., Gómez-Ruiz, J., Recio, I., & Aleixandre, A. (2010). Changes in

arterial blood pressure after single oral administration of milk-casein-derived peptides in spontaneously hypertensive rats. *Molecular Nutrition and Food Research*, *54*(10), 1422–1427. https://doi.org/10.1002/mnfr.200900448

Minervini, F., Algaron, F., Rizzello, C. G., Fox, P. F., Monnet, V., & Gobbetti, M. (2003). Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from Lactobacillus helveticus PR4 proteinase-hydrolyzed caseins of milk from six species. *Applied and Environmental Microbiology*, *69*(9), 5297–5305. https://doi.org/10.1128/AEM.69.9.5297-5305.2003

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. *International Journal of Molecular Sciences*, *20*(23). https://doi.org/10.3390/ijms20235978

Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T. (1995). Purification and Characterization of Angiotensin I-Converting Enzyme Inhibitors from Sour Milk. *Journal of Dairy Science*, *78*, 777–783. https://doi.org/10.3168/jds.S0022-0302(95)76689-9

Nielsen, S. D., Beverly, R. L., & Dallas, D. C. (2017). Milk Proteins Are Predigested Within the Human Mammary Gland. *Journal of Mammary Gland Biology and Neoplasia*, *22*(4), 251–261. https://doi.org/10.1007/s10911-018-9388-0

Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry*, *232*, 673–682. https://doi.org/10.1016/j.foodchem.2017.04.056

Nielsen, S. D., Beverly, R. L., Underwood, M. A., & Dallas, D. C. (2018). Release of functional peptides from mother's milk and fortifier proteins in the premature infant stomach. *PLOS ONE*, *13*(11), e0208204. https://doi.org/10.1371/journal.pone.0208204

Parker, F., Migliore-Samour, D., Floch, F., Zerial, A., Werner, G. H., JOLLÈS, J., Casaretto, M., Zahn, H., & Jolles, P. (1984). Immunostimulating

hexapeptide from human casein: amino acid sequence, synthesis and biological properties. *European Journal of Biochemistry*, *145*(3), 677–682. https://doi.org/10.1111/j.1432-1033.1984.tb08609.x

Picariello, G., Mamone, G., Nitride, C., Addeo, F., & Ferranti, P. (2013). Protein digestomics: Integrated platforms to study food-protein digestion and derived functional and active peptides. In *TrAC - Trends in Analytical Chemistry* (Vol. 52, pp. 120–134). Elsevier B.V. https://doi.org/10.1016/j.trac.2013.08.001

Pihlanto-lepp, A., Koskinen, I., Piilola, K., Tupasela, T., & Korhonen, H. (2021). *Angiotensin I-converting enzyme inhibitory properties of whey protein digests : concentration and characterization of active peptides*. https://doi.org/10.1017/S0022029999003982

Quirós, A., Contreras, M. del M., Ramos, M., Amigo, L., & Recio, I. (2009). Stability to gastrointestinal enzymes and structure-activity relationship of β-casein-peptides with antihypertensive properties. *Peptides*, *30*(10), 1848–1853. https://doi.org/10.1016/j.peptides.2009.06.031

Quirós, A., Ramos, M., Muguerza, B., Delgado, M. A., Miguel, M., Aleixandre, A., & Recio, I. (2007). Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. *International Dairy Journal*, *17*(1), 33–41. https://doi.org/10.1016/j.idairyj.2005.12.011

Sánchez-Hernández, S., Théron, L., Jiménez-Barrios, P., Olalla-Herrera, M., Recio, I., & Miralles, B. (2021). Protein Profile and Simulated Digestive Behavior of Breast Milk from Overweight and Normal Weight Mothers. *Foods*, *10*(4), 887. https://doi.org/10.3390/foods10040887

Sánchez-Rivera, L., Diezhandino, I., Gómez-Ruiz, J. Á., Fresno, J. M., Miralles, B., & Recio, I. (2014). Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion. *ELECTROPHORESIS*, *35*(11), 1627–1636. https://doi.org/10.1002/elps.201300510

Sánchez-Rivera, L., Santos, P. F., Miralles, B., Carrón, R., José Montero, M., & Recio, I. (2016). Peptide fragments from β-casein f(134–138), HLPLP, generated by the action of rat blood plasma peptidases show potent antihypertensive activity. *Food Research International*, *88*, 348–353. https://doi.org/10.1016/j.foodres.2015.12.007

Schmiedeberg', N.-, Koch, G., Wiedemann, K., & Teschemacher, H. (1985). Opioid activities of human fl-casomorphins. *Archives of Pharmacology*, *331*, 351–354.

Stam, J., Sauer, P. J., & Boehm, G. (2013). Can we define an infant's need from the composition of human milk? 1-3. *The American Journal of Clinical Nutrition*, *98(suppl)*, 521S-8S. https://doi.org/10.3945/ajcn.112.044370

Su, M. Y., Broadhurst, M., Liu, C. P., Gathercole, J., Cheng, W. L., Qi, X. Y., Clerens, S., Dyer, J. M., Day, L., & Haigh, B. (2017). Comparative analysis of human milk and infant formula derived peptides following in vitro digestion. *Food Chemistry*, *221*, 1895–1903. https://doi.org/10.1016/j.foodchem.2016.10.041

Svedberg, J., de Haas, J., Leimenstoll, G., Paul, F., & Teschemacher, H. (1985). Demonstration of β-casomorphin immunoreactive materials in in vitro digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. *Peptides*, *6*(5), 825–830. https://doi.org/10.1016/0196-9781(85)90308-0

Tani, F., Yoshkawa, M., Iio, K., & Chiba, H. (1990). Isolation and Characterization of Opioid Antagonist Peptides Derived from Human Lactoferrin. *Agricultural and Biological Chemistry*, *54*(7), 1803–1810. https://doi.org/10.1271/bbb1961.54.1803

Tsopmo, A., Romanowski, A., Banda, L., Lavoie, J. C., Jenssen, H., & Friel, J. K. (2011). Novel anti-oxidative peptides from enzymatic digestion of human milk. *Food Chemistry*, *126*(3), 1138–1143. https://doi.org/10.1016/j.foodchem.2010.11.146

Ukeda, H., Matsufuji, H., Matsui, T., Osajima, Y., Matsuda, H., & Osajima, K. (1992). Peptides from Peptic Hydrolyzate of Heated Sardine Meat That Inhibit Angiotensin I Converting Enzyme. *Nippon Nogeikagaku Kaishi*, *66*(1), 25–29. https://doi.org/10.1271/nogeikagaku1924.66.25

Wada, Y., & Lönnerdal, B. (2014). Bioactive peptides derived from human milk proteins - mechanisms of action. In *Journal of Nutritional Biochemistry* (Vol. 25, Issue 5, pp. 503–514). Elsevier Inc. https://doi.org/10.1016/j.jnutbio.2013.10.012

Wada, Y., & Lönnerdal, B. (2015). Bioactive peptides released from in vitro digestion of human milk with or without pasteurization. *Pediatric Research*, *77*(4), 546–553. https://doi.org/10.1038/pr.2015.10

Wada, Y., & Lönnerdal, B. (2020). Bioactive peptides derived from human milk proteins: An update. *Current Opinion in Clinical Nutrition and Metabolic Care*, *23*(3), 217–222. https://doi.org/10.1097/MCO.0000000000000642

Wada, Y., Phinney, B. S., Weber, D., & Lönnerdal, B. (2017). In vivo digestomics of milk proteins in human milk and infant formula using a suckling rat pup model. *Peptides*, *88*, 18–31. https://doi.org/10.1016/j.peptides.2016.11.012

Wan, J., Cui, X. wei, Zhang, J., Fu, Z. yi, Guo, X. rong, Sun, L. Z., & Ji, C. bo. (2013). Peptidome analysis of human skim milk in term and preterm milk. *Biochemical and Biophysical Research Communications*, *438*(1), 236–241. https://doi.org/10.1016/j.bbrc.2013.07.068

Weaver, L., & Walker, W. (1989). Uptake of macromolecules in the neonate. In *Lebenthal E (ed), Human Gastrointestinal Development, Raven Press: New York 1989: 731-748*.

Yokomizo, A., Takenaka, Y., & Takenaka, T. (2002). Antioxidative Activity of Peptides Prepared from Okara Protein. In *Food Sci. Technol. Res* (Vol. 8, Issue 4).

Zhang, Q., Cundiff, J. K., Maria, S. D., McMahon, R. J., Wickham, M. S. J., Faulks, R. M., & Van Tol, E. A. F. (2014). Differential digestion of human milk proteins in a simulated stomach model. *Journal of Proteome Research*, *13*(2), 1055–1064. https://doi.org/10.1021/pr401051u

Zhu, J., & Dingess, K. A. (2019). The functional power of the human milk proteome. In *Nutrients* (Vol. 11, Issue 8). MDPI AG. https://doi.org/10.3390/nu11081834


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Publicación V

Study of the phenolic compound profile and antioxidant activity of human milk from Spanish women at different stages of lactation: A comparison with infant formulas

Silvia Sánchez-Hernández, Adelaida Esteban-Muñoz, Cristina Samaniego-Sánchez, Rafael Giménez-Martínez, Beatriz-Miralles, Manuel Olalla-Herrera

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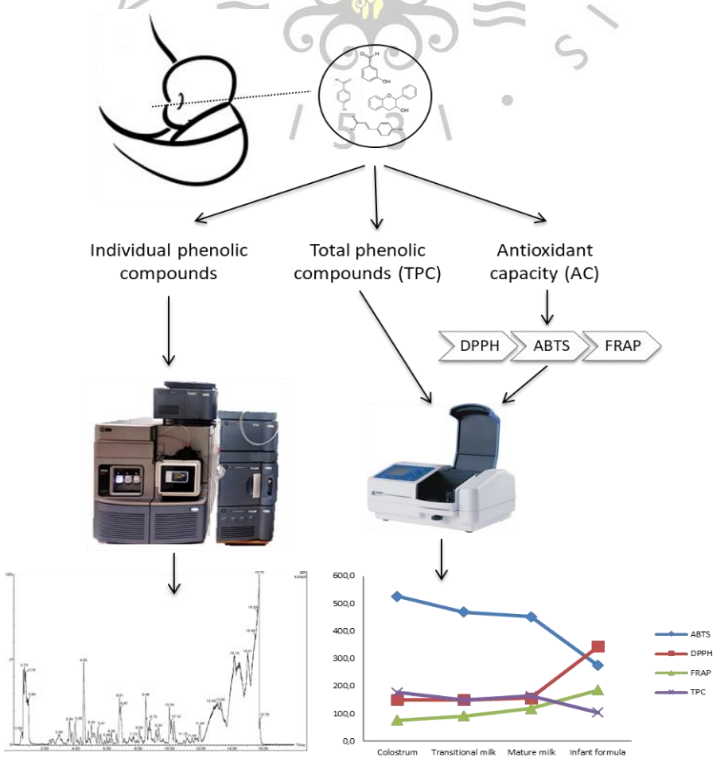


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Study of the phenolic compound profile and antioxidant activity of human milk from Spanish women at different stages of lactation: A comparison with infant formulas

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Abstract

Human milk (HM) has been proven to have important and essential antioxidant properties to counteract infant susceptibility to oxidative stress. Phenolic compounds are secondary metabolites which come from plants and are potent natural antioxidants. The ultra-performance liquid chromatography-tandem mass spectrometry (UPLCMS/MS) method used in the present study allowed the quantification of 26 phenolic compounds (ten hydroxybenzoic acids, seven hydroxycinnamic acids, four flavonoids, three hydroxybenzaldehydes and two other polyphenols) in HM samples at different stages of lactation (colostrum, transitional milk and mature milk) and infant formulas (IF). Many of the phenolic compounds identified have been reported to be present in HM for the first time. The total phenolic compound content (TPC) was quantified using the Folin assay and the antioxidant activity (AC) was evaluated with the DPPH, ABTS and FRAP assays. Significant differences were evidenced between HM and IF. HM from mothers with an adherence to a Mediterranean diet contained twice as many individual phenolic compounds as infant formulas, with a higher proportion of hydroxybenzoic acids. Conversely, IF showed a higher proportion of

hydroxycinnamic acids. Overall, the antioxidant activity of HM showed small variations during lactation.

Keywords: Phenolic compounds, phenolic acids, hydroxybenzoic acids, hydroxycinnamic acids, human milk, infant formulas, antioxidant activity

1. Introduction

Human milk (HM) is the only food properly adapted to the nutritional needs of infants during the first six months of life, although the World Health Organization (WHO), recommends breastfeeding up to 2 years of life (WHO & Pan American Health Organitation (PAHO), 2009). It provides all the necessary nutrients for the proper growth and development of the child, whilst also providing immune protection (Andreas et al., 2015; Poniedziałek et al., 2017). Nevertheless, the decisión to breastfeed is personal and is often influenced by many factors and, in various situations, breastfeeding may not be appropriate (Kozhimannil et al., 2014). The composition of milk is dynamic and changes over time, and adapts to the changing needs of the growing child. These variations depend on the stage of breastfeeding, maternal diet, maternal health and environmental exposure to toxins or heavy metals (Jackson et al., 2004; Lee & Kelleher, 2016).

Oxidative stress during the neonatal period has been associated with necrotizing enterocolitis (NEC), periventricular leukomalacia, and retinopathy (Inder & Volpe, 2000; Papp et al., 1999; Zhou et al., 2005). Nevertheless, it has been found that HM presents advantages in comparison to infant formulas (IF) (Aycicek et al., 2006; Zarban et al., 2009; Živković et al., 2015). In that sense, HM has been shown to have a preventive effect against frequent disorders linked to oxidative stress in the newborn (Păduraru et al., 2018; Yuksel et al., 2015).

Phenolic compounds are widely recognized for their antioxidant properties. They possess at least one phenol group: an aromatic ring joined to at least one hydroxyl functional group (Vázquez et al., 2015). These compounds are known to have a beneficial effect in the prevention of various disorders such as cardiovascular diseases, cancer or osteoporosis (Poniedziałek et al., 2018).

However, they cannot be synthesized in the human body, and must instead be provided through the intake of fruits, vegetables, and grains (Taamalli et al., 2019).

Phenolic compounds are usually classified as follows: phenolic acids, flavonoids, stilbenes, coumarins, lignans and tannins. Among flavanoids, there are flavonols, flavononols, flavanols, flavones, flavanones, anthocyanidins and isoflavonoids. Phenolic acids are divided into hydro xycinnamic and hydro xybenzoic acid derivatives, the first group including gallic, p-hydroxybenzoic, vanillic, syringic, protocatechuic, and ellagic acids, while the second group includes p-coumaric, caffeic, ferulic, sinapic, and chlorogenic acids (Shahidi & Ambigaipalan, 2015; Stój et al., 2020). Human milk has been shown to contain phenolic compounds and carotenoids that originate from the mothers' diet (Tsopmo, 2018). Some recent studies have focused on the study of flavonoids in HM (Khymenets et al., 2016; Romaszko et al., 2014; Song et al., 2013). Nevertheless, the presence of phenolic acids in HM has been scarcely investigated.

The aim of the present study was to provide more information regarding the phenolic compound profile, the total phenolic compound content (TPC) as well as the antioxidant capacity (AC) of the milk of women from the Spanish region of Granada. A second objective was to evaluate milk samples taken at different lactation time-points (colostrum, transitional milk and mature milk) and to compare them with different commercially available infant formulas (IF) in Spain.

2. Materials and methods

2.1. Chemicals and reagents

Reagents used for sample preparation and extraction of phenolic compounds were purchased from Panreac Quimica SL (Barcelona, Spain).

2,5-dihydroxybenzoic, vanillic acid, syringic acid, protocatechuic acid, 2,4,6-trihydroxybenzoic acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, protocatechuic aldehyde, gallic acid, 2,6-dihydroxybenzoic acid, ferulic acid, sinapic acid, chlorogenic acid, caffeic acid, 4- methoxycinnamic acid, dimethyl caffeic acid, p-coumaric acid, rutin, kaempferol-3-O-glucoside, quercetin-3-O-glucopuranoside, catechin, o-vanillin, p-hydroxyphenylacetic, p-vanillin,

pyrogallol and p-hydroxybenzaldehyde were purchased from Sigma-Aldrich SL (Madrid, Spain).

Reagents used to measure antioXidant capacity; DPPH (2,2-diphenil-1-picryl hydrazyl), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) for the Ferric reducing antioxidant power (FRAP) assay, ABTS (2,2-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid) and Trolox standard ((±)-6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were supplied by Sigma-Aldrich SL (Madrid, Spain) and Panreac Quimica SL (Barcelona, Spain). Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Hesse, Germany) and used to determine the TPC.

Doubly distilled deionized water was obtained from a Milli-Q purification system from Millipore (Milford, MA, USA). All reagents were of analytical grade.

2.2. Subjects

The present research was carried out in the obstetrics department of a regional hospital of Andalusia, namely the "Hospital Universitario Virgen de las Nieves". Details of the study were explained to all mothers who voluntarily gave written consent to participate. HM samples were provided by eighteen lactating women, with each mother donating three samples of colostrum, transitional and mature milk. The samples were collected from January to March 2019.

Ethics were approved by the relevant scientific committee and the trial was registered at ClinicalTrials.gov (number NCT): NCT02811172.

The main characteristics of donor mothers are shown in **Table 1**. Mothers with similar lifestyles and dietary patterns were selected to participate. All reported medium-to-high adherence to a Mediterranean diet. This was evaluated using a 14-item questionnaire, considering daily consumption of fruits and vegetables, and the use of virgin olive oil for cooking.

Table 1. Characteristics of the sampled mothers and newborns.

	Mean	Median	Range
Mothers			
Age, years	31.4	31.0	23.0-39.0
Pre-pregnancy weight, kg	68.8	62.5	46.0-130.0
Height, cm	163.6	163.0	150.0-177.0
Pre-pregnancy BMI, kg/cm^2	25.6	24.1	17.5-45.0
Post-pregnancy weight, kg	79.7	76.0	56.0-139.0
Post-pregnancy BMI, kg/cm^2	29.8	28.6	22.9-48.1
NW/OW, %			55/45
Weight gain (during pregnancy), kg	11.9	11.0	(-6.0)-25.0
Type of delivery (Vaginal/Cesarean), %			82/18
Parity	1.7	2.0	1.0-3.0
Newborns			
Gender (Male/Female), %			55/45
Gestational age, weeks	39.1	40.0	32.0-41.0
Birth weight, kg	3.2	3.3	1.8-4.1
Smoking habit			
Smoker/Nonsmoker, %			26/74

BMI: body mass index; NW: normal weight; OW: overweight

2.3. Samples

The study included HM samples which were categorized according to the length of time post-partum. AC and TPC were analyzed in fifty-four samples. Individual phenolic compounds were analyzed in twenty-one samples, randomly chosen (seven mothers out of eighteen).

Samples obtained between the 1st and 5th day post-delivery were assigned to the colostrum group (n=18, 4.8 ± 1.03 days). These samples were collected using the Marmet manual extraction technique (Ministry of Health, 2017). Samples obtained between the 6th and 15th day post-delivery were assigned to the transitional group (n=18, 10.0 ± 1.43 days). Finally, samples obtained after the 15th day post-delivery were assigned to the mature milk group (n=18, 20.5 ± 3.3 days).

For both of the latter groups, milk extraction was achieved by means of a mechanical breast pump Medela® (Baar, Switzerland), following the manufacturer's instructions. The milk from each breast was obtained at both the beginning and the end of each feed. All samples collected from participants were aliquoted and immediately stored at -70 ◦C until analysis.

In addition, seven different initiation formulas (0–6 months) for full- term infants were also analyzed. These are the most commonly consumed IF and include brands such as Nestlé, Bio HiPP, Ordesa, Nutribén, and Alter. They were purchased from different commercial and agricultural areas of the market.

2.4. Sample treatment

IF were reconstituted in water following the manufacturer's instructions. HM samples were thawed and thoroughly homogenized in a vortex.

Sample preparation and extraction of individual phenolic compounds was carried out following the method reported by (Li et al., 2009) with slight modifications with regard to the sample volume (5 mL of sample). The extract was then redissolved in 0.2 mL of 50% methanol and stored in the dark at 20 ◦C. It was subsequently analyzed via UPLC-MS/MS to obtain the final phenolic acid composition.

Sample preparation to determine TPC and AC was carried out following the method by Poniedziałek et al., 2018. Two phases were obtained: a solid one containing proteins and lipids and a liquid one that corresponded to the supernatant. This supernatant was separated out and used for further analysis.

2.5. Identification of individual phenolic compounds

A UPLC-MS/MS method was used to quantify the different phenolic compounds (Esteban Muñoz et al., 2018). Briefly, after gradient sepa- ration, detection on a Waters SYNAPT G2 HDMS Q-TOF high resolution spectrometer was performed in negative mode. Comparison with standard phenolic compounds with measurements in Multiple Reaction Monitoring (MRM) was used due to the large number of target analytes and the complexity of the matrix.

2.5.1. Chromatographic operating conditions

UPLC analysis was performed using a Waters ACQUITY I CLASS model chromatograph instrument (Waters, Mississauga, ON, Canada) equipped with a Waters XEVO TQ-XS, with ionization performed by UniSpray (US).

Phenolic compounds were separated via an Acquity UPLC HSS T3 1.8 µm column, with a gradient of solvent A (water) and solvent B (methanol containing 0.1% [v/v] acetic acid) for 25 min at a flow rate of 0.4 mL/min. The solvent gradient was programmed as follows: at 0 min 5% B, 15–15.10 min 95% B and 15.10–25 min 5% B (re-equilibration step). Identification of the phenolic compounds was accomplished by comparing the retention times of peaks and fragmentation data in samples, with those of the phenolic compound standards shown in **Fig. S1** and **Table S1**. UPLC-MS/MS analyses were carried out in triplicate.

2.5.2. Analytical validation

Validation was conducted by studying quality parameters for the chromatographic determination of phenolic compounds, pertaining to selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ) and precision. These quality parameters are shown in **Table S2** and follow guidelines for the validation of analytical methods laid out by the Association of Official Analytical Chemists (AOAC, 2012).

2.6. Quantification of the total phenolic compounds (TPC)

The overall amount of phenols was determined in triplicate using the classical Folin–Ciocalteu colorimetric method described by Singleton & Rossi. 1965, with some modifications (Poniedziałek et al., 2018). Absorbance was measured at 765 nm, using a Boeco S-22 ultraviolet–visible (UV–VIS) spectrophotometer (Hamburg, Germany). A standard solution of gallic acid (1 mg mL^{-1}) dissolved in methanol (1:1) was used to prepare a calibration curve. Results were expressed as gallic acid equivalents (mg GAE/L). The calibration curve range was 0.0–0.01 mg/mL (r = 0.998).

2.7. Evaluation of the antioxidant activity (AC)

The AC was measured via three different methods. The DPPH scavenging assay was used as proposed by Brand-Williams et al., 1995. The second method

was derived from the FRAP assay according to the pro- cedure described by Benzie and colleagues (Benzie et al., 1997). The third and final method estimated the antioxidant equivalent capacity as radical scavenging activity, based on reduction of the radical cation ABTS assay following the procedure of Re et al., 1999. These assays were slightly modified in our laboratory (Ramirez et al., 2019). Absorbance of the samples was measured at 515, 734 and 593 nm, respectively, using a Boeco S-22 UV–VIS spectrophotometer (Hamburg, Germany).

The absorbance signal was translated into antioxidant activity using Trolox as the external standard. Different calibration curve ranges were used depending on the method employed. Results of the assays are expressed as micromoles of Trolox equivalents per liter of sample (µmol of TE/L). All experiments were conducted in triplicate and values were expressed as averages ± standard deviation.

2.8. Statistical analyses

Statistical analyses were mainly performed using SPSS 20.0 (IBM, Chicago, IL, USA). Non-normal distribution of the data was verified using the Kolmogorov test. Therefore, non-parametric statistical tests were used: Wilcoxon t-test for comparisons of medians between two samples and Kruskal-Wallis test for comparisons of more than two groups (one-way ANOVA). A significance level of 5% was considered. Non-parametric correlation Spearman coefficients were calculated to define the nature of relationships between AC and phenol content using R 3.5.1. Software version (R Core Team, 2018, Austria). Correlation plots (correlograms) were obtained to show the results. Principal component analysis (PCA) was applied as a multivariate exploratory analysis to look for clustering variables. The variables were the sample groups (type of milk), the AC methods, TPC and individual phenolic compounds quantified by UPLC/MS-MS. PCA was performed on the covariance matrix. Linear Discriminant Analysis (LDA) was performed as an alternative method.

3. Results and discussion

3.1. Identification of phenolic compounds present in HM and IF

Few studies that consider the phenolic acid composition of HM are available. In the present work, phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) constituted the main group of phenolic compounds found in HM. Flavonoids, hydroxybenzaldehydes and other polyphenols were also found, although to a lesser extent.

The UPLC-MS/MS method used in the present study allowed the detection and quantification of 26 phenolic compounds (ten hydroxybenzoic acids, seven hydroxycinnamic acids, four flavonoids, three hydroxybenzaldehydes and two other polyphenols: pyrogallol and p-hydroxyphenylacetic acid) in samples of HM and IF, as shown **Table 2**.

According to the presented results, statistically significant differences exist between the different groups of HM with regard to the lactating stage and IF. In general, HM contained as much as twice of individual phenolic compounds than IF (660.61, 772.71, 651.72 and 375.18 µg/100 mL for colostrum, transitional, mature milk and IF, respectively).

Table 2. Identification and quantification of phenolic compounds (μg/100mL sample) in milk fracti

n	Compounds	Rt±SD (min)	Colostrum (n=7)		Transitional milk (n=7)		Mature mil	
			Mean	Median (IQR)	Mean	Median (IQR)	Mean	Med
	Phenolic acids / Hydroxybenzoic acids							
1	2,5-Dihydroxybenzoic acid	4.13 ± 0.037	5.61	4.54 (3.67-7.62)	7.41	4.62 (3.09-14.87)	6.43	7.40 (
2	Vanillic acid	4.13 ± 0.031	5.96	6.02 (5.91-6.68)	7.05	6.88 (6.04-8.08)	6.56	4.88 (
3	Syringic acid	4.17 ± 0.033	N.D.	N.D.	N.D.	N.D.	N.D.	
4	Protocatechuic acid	3.03 ± 0.037	15.16	6.48 (4.60-31.10)	22.55	15.70 (8.52-27.30)	13.16	13.60
5	2,4,6-trihydroxybenzoic acid	2.30 ± 0.229	2.71	1,53 (0.95-5.65)	2.68	2.34 (1.36-4.17)	3.08	2.87
6	p-Hydroxybenzoic acid	4.88 ± 0.037	0.54	0.47[a] (0.24-0.90)	0.67	0.69[b] (0.44-0.94)	0.72	0.85[c]
7	m-Hydroxybenzoic acid	3.74 ± 0.036	7.66	4.79[a] (2.64-13.75)	9.94	10.59[b] (5.51-12.39)	6.07	6.85[c]
8	Protocatehuicaldehide	3.73 ± 0.034	9.63	5.03[a] (2.83-16.87)	12.56	12.88[b] (6.59-15.81)	7.46	6.17[c]
9	Gallic acid	2.15 ± 0.025	582.26	482.96[a] (375.07-720.94)	661.06	569.55[b] (434.09-815.68)	575.45	287.96 (2
10	2,6-Dimethoxybenzoic acid	5.48 ± 0.089	N.D.	N.D.	N.D.	N.D.	N.D.	
	Phenolic acids / Hydroxycinnamic acid							
11	Ferulic acid	5.25 ± 0.074	0.62	0.52[a] (0.06-1.23)	0.71	0.26[b] (0.12-1.53)	0.34	0.31[c]
12	Sinapic acid	5.15 ± 0.081	N.D.	N.D.	N.D.	N.D.	N.D.	
13	Chlorogenic acid	3.43 ± 0.025	9.43	0.92[a] (0.57-26.82)	17.72	10.76 (2.39-40.02)	1.03	0.47[c]
14	Caffeic acid	4.42 ± 0.207	14.15	12.45[a] (4.31-16.75)	17.28	11.49[b] (3.80-29.26)	12.32	11.14[c]
15	4-Methoxycinnamic acid	7.14 ± 0.039	0.24	0.04* (0.02-0.67)	0.30	0.28 (0.14-0.48)	0.91	0.24
16	Dimethylcaffeic acid	6.36 ± 0.114	0.27	0.14[a] (0.13-0.28)	0.56	0.15 (0.15-0.25)	0.18	0.15[c]

17	p-coumaric acid	5.02 ± 0.141	0.44	0.29[a] (0.24-0.59)	1.20	0.94 (0.27-2.31)	0.29	0.21[c]
	Flavonoids / Flavonols							
18	Rutin	4.60 ± 0.024	N.D.	N.D.	N.D.	N.D.	N.D.	
19	Kaempferol-3-O-glucoside	5.26 ± 0.032	2.36	0.00* (7.08)	3.02	0.01* (5.24)	N.D.	
20	Isoquercetin	4.86 ± 0.022	0.11	0.13 (0.06-0.15)	0.19	0.16 (0.08-0.21)	0.16	0.11
21	Catechin	3.60 ± 0.147	0.04	0.04 (0.02-0.05)	0.02	0.02* (0.01-0.03)	0.29	0.37
	Other polyphenols / Hydroxybenzaldehydes							
22	o-Vanillin	7.44 ± 0.071	1.61	1.53[d] (1.41-1.62)	6.06	6.59 (5.74-6.69)	12.22	5.85 (
23	p-Hydroxybenzaldehyde	5.97 ± 0.203	2.57	1.09 (0.92-6.40)	3.54	4.13 (1.19-5.16)	1.62	1.10
24	p-Vanillin	5.03 ± 0.036	5.84	6.05[a] (3.32-8.58)	7.83	7.42[b] (6.52-10.41)	5.56	4.63
	Other polyphenols / Other polyphenols							
25	Pyrogallol	2.47 ± 0.035	1.07	0.75 (0.34-1.73)	1.11	0.95 (0.28-2.13)	2.33	1.66
26	p-Hydroxyphenylacetic acid	4.69 ± 0.038	0.27	0.24 (0.05-0.53)	0.41	0.35 (0.35-0.52)	0.40	0.20

N.D.: not detected. Superscript letters indicate significant differences ($p < 0.05$) evidenced by non-parametric testing. Significant di
(b) transitional milk and IF, (c) mature milk and IF, (d) colostrum and transitional milk". * < LOD".

3.1.1. Hydroxybenzoic acids

Most of the phenolic compounds identified in the milk fractions pertained to the hydroxybenzoic acids group, with percentages of 95%, 93.6%, 94.6% and 78.8% for colostrum, transitional milk, mature milk and IF, respectively, with respect to the total the individual phenolic compounds quantified by UPLC-MS/MS. As shown in **Fig. 1A**, HM contained more hydroxybenzoic acids than IF with an average of 94% versus 78.9% for IF. Significant differences were observed between colostrum and IF ($p < 0.05$), and between transitional milk and IF ($p < 0.001$). This was due to the large contribution of gallic acid, which was the main phenolic compound identified in all milk fractions **(Table 2)**. P-hydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid were exclusively found in HM, while syringic acid and 2,6-dimethoxybenzoic acid were only detected in IF.

The beneficial properties of hydroxybenzoic acids are widely described. They are involved in the sensory quality, taste and aroma of food (Muñoz et al., 2020). Those derived from gallic acid have been particularly studied, with recently published reviews covering their antimicrobial, anti-inflammatory, cardiovascular, gastrointestinal, metabolic and neuropsychological activity (Kahkeshani et al., 2019). It is worth noting the interfering role of gallic acid in various intracellular inflammatory pathways that induce ulcerative colitis in mice. The significantly higher proportion of gallic acid in colostrum and transi- tional milk with regard to mature milk might be of special relevance in the protective role of HM against newborn enterocolitis with regard to IF (Aycicek et al., 2006).

Protocatechuic acid, the second main hydroxybenzoic acid quantified in our samples **(Table 2)**, has been reported with an anti-inflammatory activity and an ability to improve antioxidant defenses (Juurlink et al., 2014). Protocatechuic aldehyde, the 3rd main hydroxybenzoic acid identified in HM **(Table 2)**, has been reported to have suppressive effects on adipogenesis (Byun et al., 2016).

Regarding the acids from the hydroxybenzoic acid group, only p-hydroxybenzoic acid had been previously found in breast milk (Li, et al. 2009). Regarding the hydroxybenzoic acids mentioned previously, the present study reports for the first time their presence in colostrum, transitional and mature milk,

although very recently, a liquid–liquid microextraction procedure has reported the identification of 11 phenolic compounds including gallic acid and caffeic acid in HM (Nalewajko-Sieliwoniuk et al., 2020).

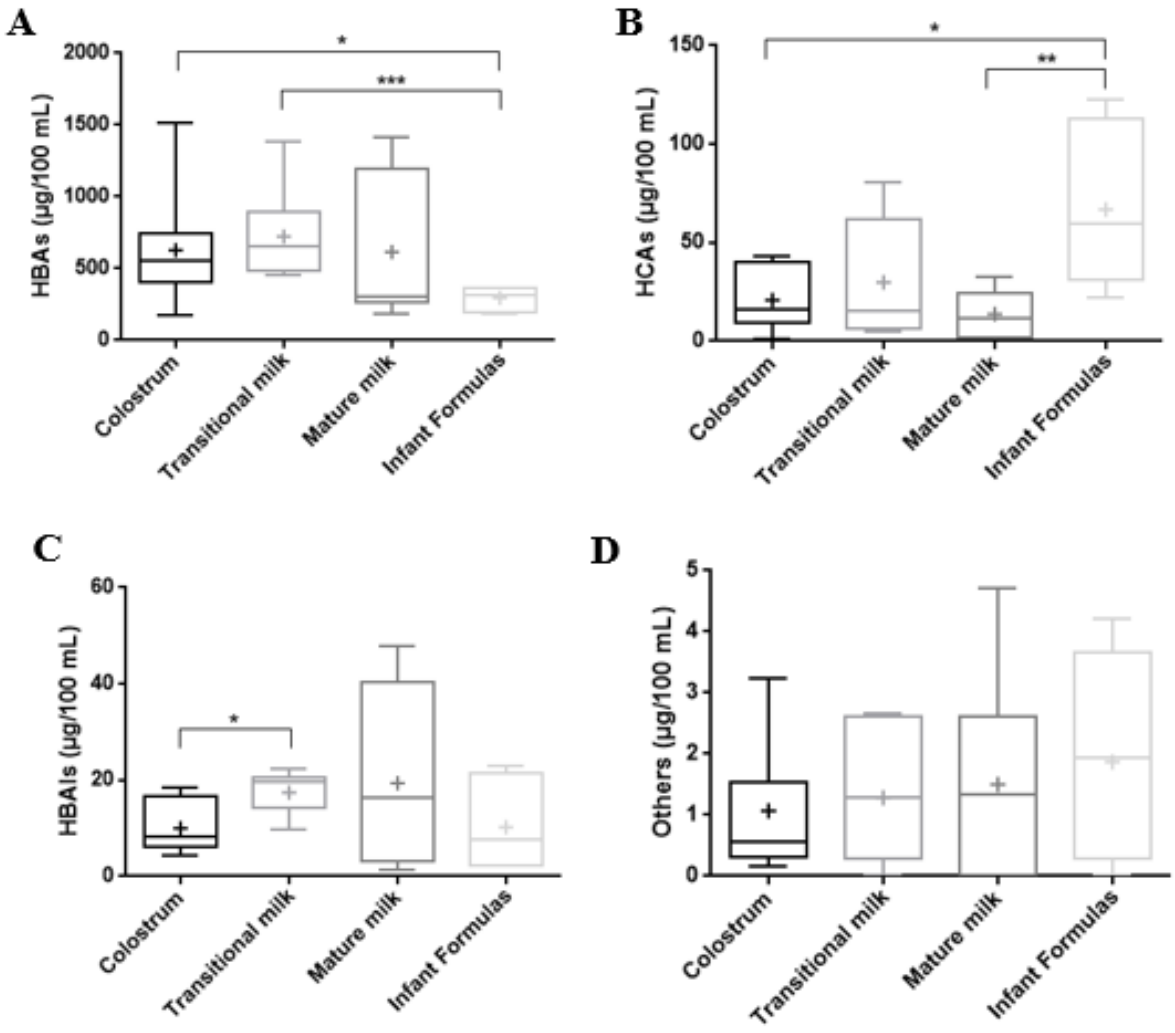


Fig. 1. Quantification of the amount of phenolic compounds present in human milk and infant formulas. (A) Hydroxybenzoic acid derivatives (HBAs), (B) Hydroxycinnamic acid derivatives (HCAs), (C) Hydroxybenzaldehyde derivatives (HBAIs) and (D) Other polyphenols. Significant differences are indicated by as follows: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

3.1.2. Hydroxycinnamic acids

Fig. 1B shows that IF contain a higher percentage of hydroxycinnamic acid with respect to the total of the individual phenolic com- pounds quantified by UPLC-MS/MS than HM (3.1%, 3.7% and 2.1% in colostrum, transitional and mature group, respectively, versus 17.3% in IF). This marked difference can be attributed to the high content of caffeic acid in IF samples as we evidenced significant differences ($p < 0,05$) between all HM milk fractions and IF with the following amounts of 14.15, 17.28, 12.32 and 50.92 µg/100 mL in colostrum, transitional milk, mature milk and IF; respectively **(Table 2)**.

Caffeic acid has been reported to prevent the oxidation of PUFAs (Socrier et al., 2019), which can explain the nutritional benefits of IF for newborns reported by Saphier and co-workers. (Saphier et al., 2019). Chlorogenic acid contributed importantly to this group **(Table 2)** and was evidenced to have anti-obesity, antioxidant, anti-inflammatory, anti-hypertension and anti-microbial effects (Naveed et al., 2018). Within the group of hydroxycinmic acids, ferulic and coumaric acid have been reported to be present in HM and IF (Li et al., 2009). Chlorogenic and caffeic acids constitute the main hydroxycinnamic acids that we identified in our milk fractions **(Table 2)**. To our knowledge, they have not been previously found in HM, nor have others such as 4-methoxycinnamic acid and dimethylcaffeic.

3.1.3. Flavonoids

Low concentrations of flavonoids were found in HM and in IF (data not shown) and no significant differences were found between the milk samples. The low amounts of flavonoids found could be due to the extraction method, which was more appropriate for phenolic acids. However, in accordance with our results, catechins, kaempferol and quercetin have been reported to be present in a study evaluating the phytochemical content of HM during different stages of lactation (Song et al., 2013).

3.1.4. Hydroxybenzaldehydes

Three phenolic compounds (o-vanillin and its p-vanillin isomer and p-hydroxybenzaldehyde) were quantified in the hydroxybenzaldehyde group, with significant differences emerging between colostrum and transitional milk ($p < 0.05$) **(Fig. 1C)**. To our knowledge, the presence of hydroxybenzaldehyde acids in HM has not been reported before.

P-vanillin is a well-known food and cosmetic additive with antioxidant and antimutagenic properties. It has also been suggested to have antifungal activity against human pathogenic fungi (Kim et al., 2014). P- hydroxybenzaldehyde has been found to have an antioxidant activity in foods such as oastand vinegar (Alonso et al., 2004; Molteberg et al., 1996; Natera et al., 2003).

3.1.5. Other polyphenols

Lastly, two other phenolic compounds were identified: pyrogallol and p-hydroxyphenylacetic acid. **Fig. 1D** shows that no statistically significant differences were found between samples.

Recently conducted studies, such as that of Peron et al., (2017), have highlighted the properties of a hydroxyphenylacetic acid derivative in relation to antiadhesion properties against uropathogenic bacteria. With regards to pyrogallol, recent studies confirm positive effects on a breast cancer ductal carcinoma in situ proliferation cell line (Nemec et al., 2016). Nevertheless, no previous reports in relation to these phenolic compounds in HM have been found.

3.2. Quantification of the amount of phenolic compounds in HM and IF

The highest TPC value evaluated with the Folin-Ciocalteu method was found in colostrum (184.4 mg GAE/L), and tended to decrease with the progress of breastfeeding (152.2 and 143.6 mg GAE/L for transitional and mature milk, respectively). IF showed a significantly ($p < 0.05$) lower total phenolic content than colostrum, transitional and mature milk (94.5 mg GAE/L), as shown in **Table 3**. Our results are in accordance with the work of Vázquez et al., who reported a

range of 30.89–122.75 mg GAE/L (Vázquez et al., 2015), whilst higher values (247.07 and 339.35 mg GAE/L) were recorded in a study with an intended high phenolic compounds diet (Al-Harbi, 2018).

Table 3. Statistical analysis related to the total phenolic compound content evaluated with the FOLIN assay and the antioxidant activity measured by DPPH, ABTS and FRAP assays in milk fractions.

Method	Equation	Sample	n	Mean (SD)	Median (IQR)
ABTS (µmol TE/L)	y = 196.84x + 4.7194 r^2 = 0.998	Colostrum	18	525.6 (364.1)	362.3 (249.6-816.7)
		Transitional milk	18	469.0 (326.7)	321.6 (220.4-669.7)
		Mature milk	18	452.4 (316.6)	325.9 (252.0-637.0)
		Infant formula	7	274.4 (147.8)	302.5 (103.4-363.8)
DPPH (µmol TE/L)	y = 135.85x + 1.0296 r^2 = 0.997	Colostrum	18	150.4 (78.5)	161.5[a] (84.6-203.7)
		Transitional milk	18	149.6 (82.2)	137.3[b] (101.4-177.3)
		Mature milk	18	156.4 (66.1)	157.2[c] (109.0-190.2)
		Infant formula	7	343.1 (70.6)	351.2 (105.5-382.2)
FRAP (µmol TE/L)	y = 609.45x – 1.0936 r^2 = 0.998	Colostrum	18	76.1 (58.2)	60.4[a] (24.3-123.1)
		Transitional milk	18	91.3 (58.1)	86.6[b](45.0-139.9)
		Mature milk	18	117.9 (71.3)	106.5 (58.0-164.0)
		Infant formula	7	185.7 (70.6)	156.3 (84.0-232.9)
TPC (mg GAE/L)	y = 116.26x + 0.0286 r^2 = 0.999	Colostrum	18	176.7 (73.6)	184.4[a] (111.8-199.5)
		Transitional milk	18	149.4 (52.6)	152.2[b] (116.2-174.8)
		Mature milk	18	163.6 (89.8)	143.6[c] (121.8-184.0)
		Infant formula	7	104.6 (24.2)	94.5 (54.5-127.6)

SD: standard deviation; IQR: Interquartile quartile range; Superscript letters indicate significant differences ($p < 0.05$) evidenced by non-parametric testing. Significant differences between: (a) colostrum and IF, (b) transitional milk and IF, (c) mature milk and IF".

The studied breast milk samples correspond to a relatively homogeneous population in terms of vegetables intake. On the contrary, Poniedziałek et al. (2018) as well as Cortez & Soria, (2016), reported lower values than those found in the present study.

3.3. Evaluation of the antioxidant properties of HM and IF

The antioxidant capacity (AC) was measured via three methods (DPPH, ABTS and FRAP assay) as shown in **Table 3**. The antioxidant activity of HM showed small variations during the different stages of lactation, however, statistically significant differences were observed in relation to IF **(Fig. 2)**.

DPPH levels in colostrum (161.5 µmol TE/L) tended to be higher, although not significantly, than those in transitional and mature milk (137.3 and 157.2 µmol TE/L, respectively). Zarban et al. and Živković et al. showed a trend towards decreasing levels of antioxidant activity by DPPH assay as lactation advanced to latter stages (Zarban et al., 2009; Živković et al., 2015), although slightly lower DPPH levels accompanied this pattern. This trend was similar with the application of the ABTS assay (362.3, 321.6 and 325.9 µmol TE/L for colostrum, transitional and mature milk, respectively). AC levels, determined by the DPPH method, were significantly lower than those reported using the ABTS method (Martysiak-Zurowska & Wenta, 2012). Although it has been determined that both methods are valid for measuring AC in HM, the ABTS test has higher repeatability and sensitivity (Martysiak-Zurowska & Wenta, 2012).

AC values evaluated via the ABTS assay in the present study were similar to those reported by other studies (Matos et al., 2009; Vander- Jagt et al., 2001), although some papers have registered higher values than those reported in this work (Akdag et al., 2014; Turoli et al., 2004). The storage temperature has been identified as one of the important parameters to have an impact in the results. A lower temperature might avoid the formation of lipid peroxides caused by an increase in free fatty acids due to lipoprotein lipase activity during storage. However, our storage conditions (-70 ◦C) were similar to the used by the cited authors except those used by Turoli and colleagues (20ºC), but the last observed that the increase in lipid peroxides did not correspond to a decreased total AC. On the other hand, the lactation period can influence this parameter. Data reported by Mehta and Petrova (2014) showed a significant decrease in AC during the first month of lactation, whilst Quiles et al. registered higher values of AC, in mothers of both preterm and full-term infants, in colostrum compared with mature and

transitional milk (Quiles et al., 2006) but such variations were not significant in the present study, even though a decreasing trend could be observed.

These values were contrary to those found using the FRAP assay, with these instead increasing with later breastfeeding (60.4, 86.6 and 106.5 µmol TE/L for colostrum, transitional milk and mature milk, respectively). These differences were, however, not statistically significantly. The variability in the AC of HM during lactation may be partly explained by the presence of bioactive proteins, such as leptin, lactoferrin and lysozyme (Mehta & Petrova, 2014).

IF showed a significantly higher AC than colostrum, transitional and mature milk when using the DPPH ($p < 0.001$) assay (351.2 µmol TE/L). AC was also higher in IF than in colostrum and transitional milk when using the FRAP ($p < 0.01$) assay (156.3 µmol TE/L). Although, lower values were found for the ABTS method (302.5 µmol TE/L), differences were not statistically significant.

Ascorbic acid, α-tocopherol, iron, uric acid, bilirubin and phenolic compounds were found to display ferric-reducing activity. Many of these are found in high quantities in IF, at levels that potentially provide a source of free iron capable of initiating free radical reactions (Friel et al., 2002). This may explain the higher values of AC found in IF when measured using the FRAP method (Zarban et al., 2009).

Higher antioxidant activity values using the ABTS method for all studied conditions were also found in HM in comparison to IF in a study carried out by Hanna et al. (2004).

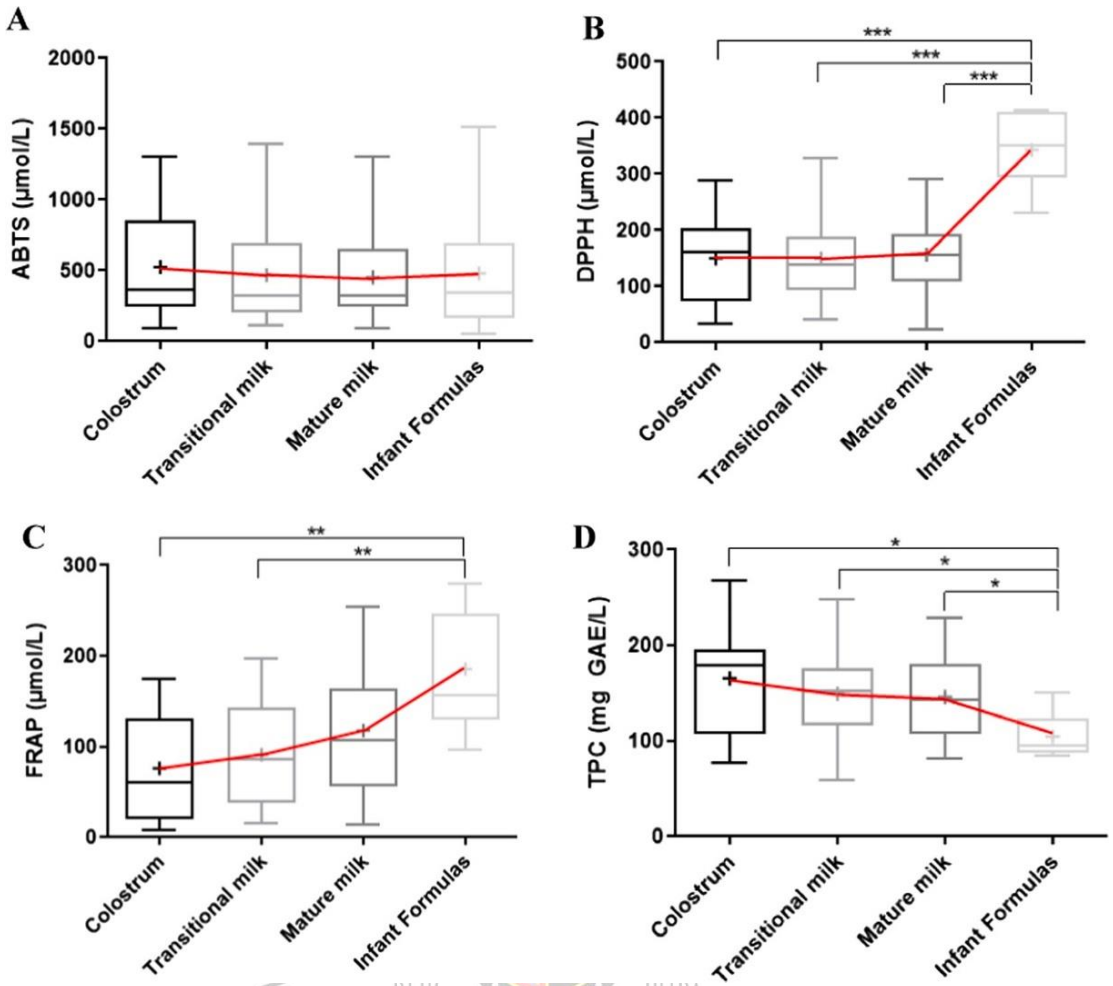


Fig. 2. Evolution the phenolic compound content and AC as a function of the lactation stage and comparison with infant formulas. (A) ABTS assay, (B) DPPH assay, (C) FRAP assay, (D) FOLIN assay (Median (IQR)). Significant differences are indicated by as follows: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Hydroxycinnamic acids (such as ferulic, caffeic, sinapic and p-coumaric acids) displayed a higher antioxidant activity than hydroxybenzoic acids, especially with the DPPH assay (Fukumoto & Mazza, 2000; Kikuzaki et al., 2002). In particular, caffeic acid and p-coumaric acids showed a high ability to neutralize DPPH but less efficiency was measured against ABTS (Gülçin, 2006; Masek, Chrzescijanska, & Latos, 2016). This could explain the higher AC values seen in IF when measured by the DPPH method (Doltra et al., 2013). The number of OH

moieties has also been proved to be an influential factor with the FRAP assay (Masek, Chrzescijanska, Latos, et al., 2016).

The antioxidant properties of HM are related to the combination of different compounds such as polyphenols, carotenoids and vitamins. The antioxidant levels observed in IF do not always match those reported in HM, in some instances being much higher (Gila-Diaz et al., 2019). This could be explained by the fact that IF usually contains a higher amount of vitamins A, E and C, when compared to HM (Cloetens et al., 2016).

HM, in particular colostrum, may be more susceptible to lipid oxidation than IF because of its higher content of LC-PUFAs (Li et al., 2009; Sánchez-Hernández et al., 2019; Turoli et al., 2004). However, it should be noted that other studies found no association effect of total PUFAs and LC-PUFA on AC values (Tijerina-Sáenz et al., 2009).

3.4. Correlation, principal component analysis (PCA) and linear discriminant analysis (LDA) of milk samples

Correlation matrix plots (correlograms) of AC and TPC of HM (colostrum, transitional milk, mature milk) and IF are shown in **Fig. 3**.

Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Correlations with p-values > 0.05 were considered non-significant and omitted from the graph. Strong correlations (rho > 0.7) were observed between the different AC assays for all samples (colostrum, transitional, and mature milk, and IF). With the FRAP assay, the ability of the sample to reduce the ferric ion is the criterion used to evaluate the antioxidant activity. The ABTS and DPPH assays measure, by the use of different free radicals, the electron donating ability and the scavenging activity related to the structure of the compounds. The fact that assays based on different principles correlate supports the confidence in the observed results. Such correlations have also been reported by other authors (Zarban et al., 2009; Živković et al., 2015).

Additionally, Principal Component Analysis (PCA) was performed to investigate associations between variables and to detect groups among samples. While we decided to retain the first three principal components (components with eigenvalues higher than one), we did not observe any clear clusters between samples of human milk (colostrum, transitional and mature milk) and infant formulas according to AC method (DPPH, ABTS and FRAP), total phenolic compounds (TPC) and individual phenolic compounds quantified by UPLC-MS/MS (**Figs. S2 and S3**, respectively).

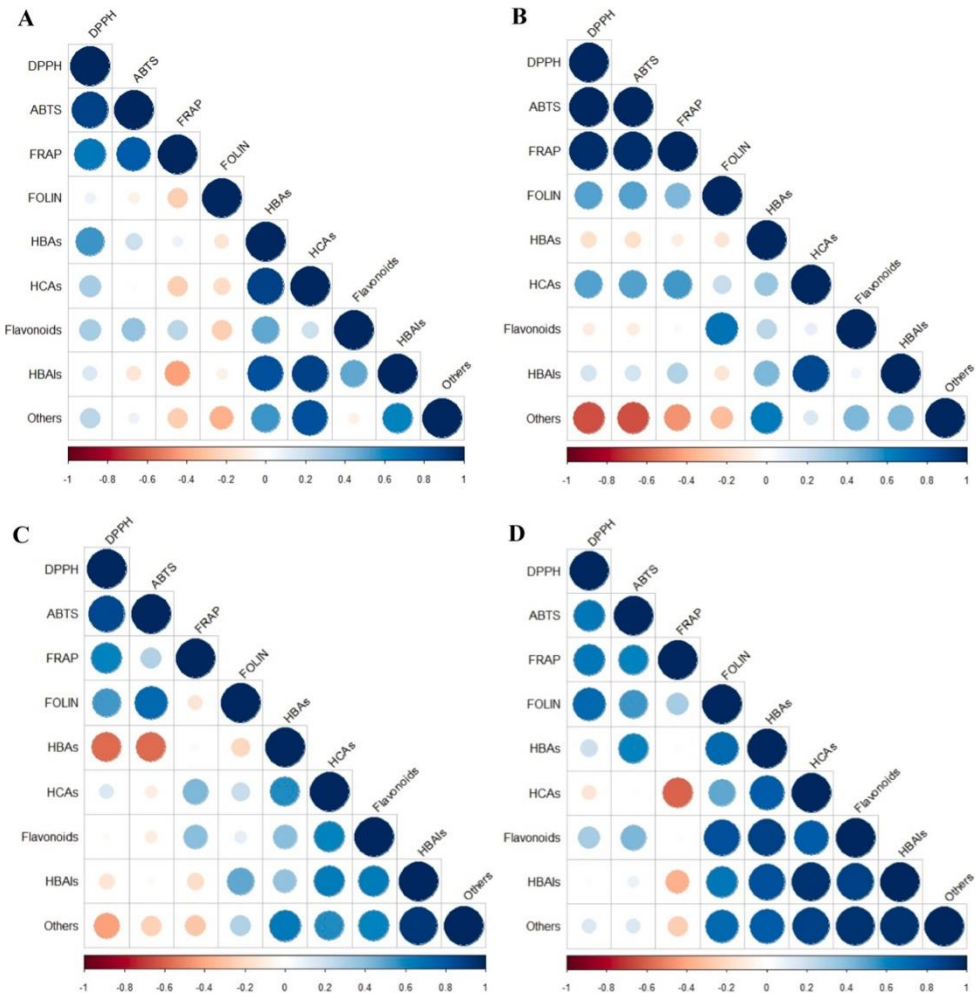
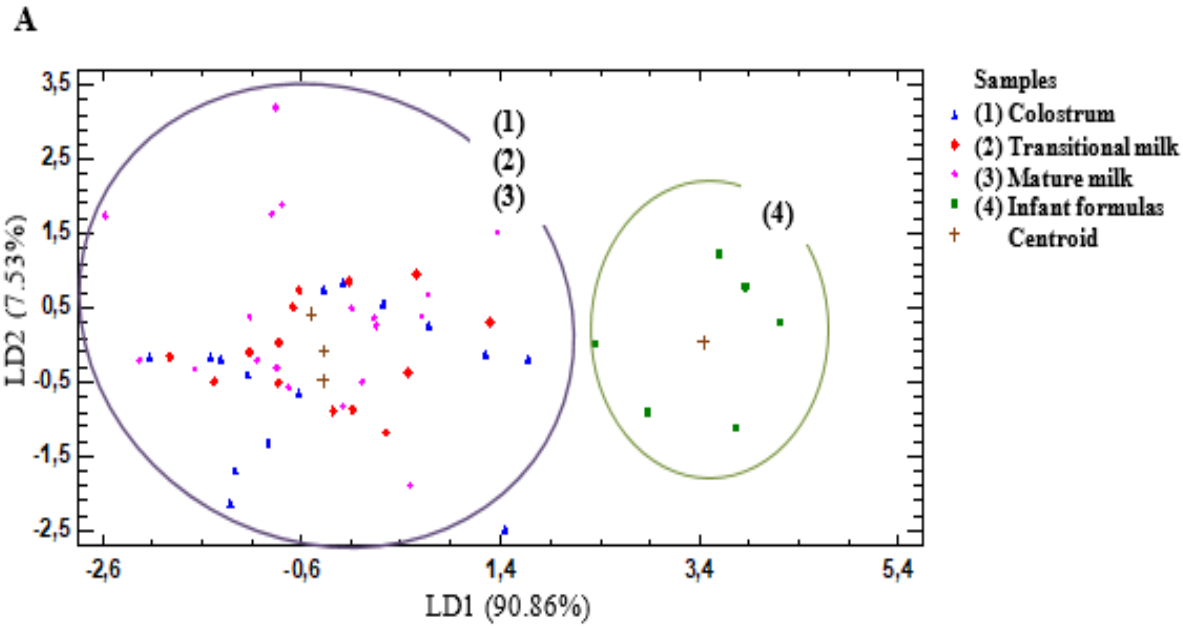


Fig. 3. Correlogram of the relationship between antioxidant capacity and phenol compound content of milk samples. (A) Colostrum, (B) Transitional milk, (C) Mature milk and (D) Infant Formulas.

Linear Discriminant Analysis (LDA) was performed as an alternative method, as it allows a better separation between groups in data sets of small sample size. In fact, as it can be seen in **Fig. 4**, LDA achieved a much clearer discrimination between samples, in comparison with PCA. When DPPH, ABTS, FRAP assays and TPC were considered **(Fig. 4A)**, this analysis was able to group the samples according to the class. IF and HM samples were clearly divided along the x axis, one group including only IF and another group including all HM samples, with no differences between colostrum, transitional and mature milk. However, according to the single phenolic profile (quantified by UPLC-MS/MS), the different stages of lactation can be distinguished; as reported in **Fig. 4B** clear differences among the colostrum, transitional and mature milk, and IF were noticed. Briefly, taking into account these results, milk categories are well defined and the type of milk sample is clearly clustered.



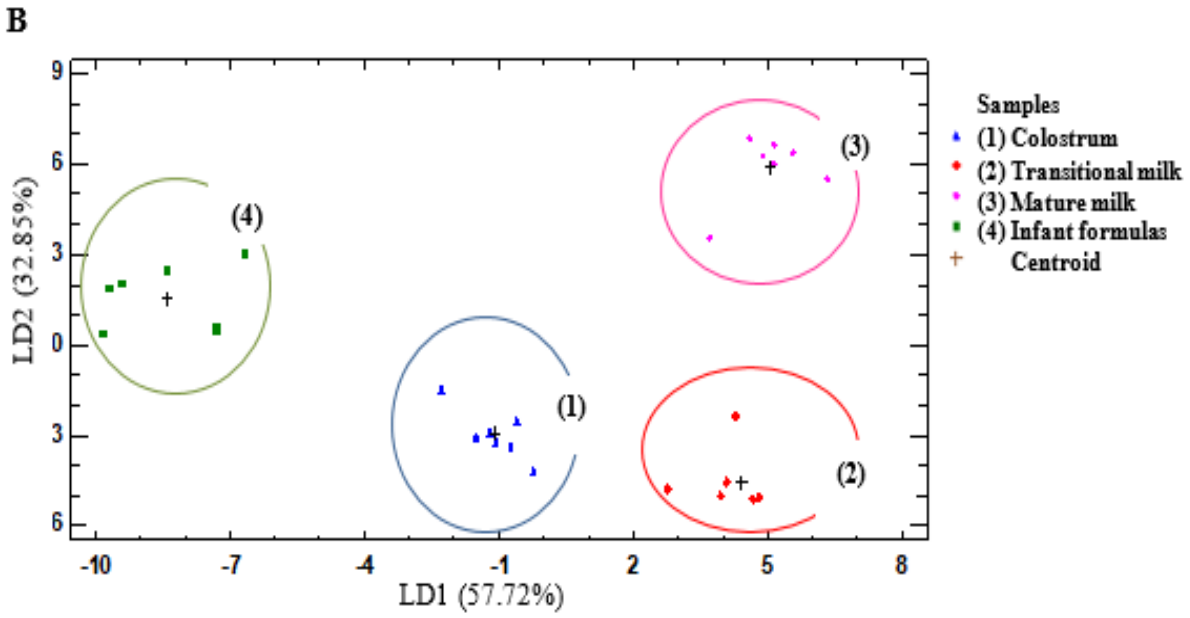


Fig. 4. Representation of the milk samples studied as a function of their phenolic compound content. Centroid: average value of the discriminant function for each one of the samples: (A) Total phenolic compounds and AC, (B) Individual phenolic compounds via UPLC.

4. Conclusions

The present study provides new data on the individual phenolic compounds present in HM of mothers with medium-to-high adherence to a Mediterranean diet. We quantified 26 compounds, in particular ten hydroxybenzoic acids, seven hydroxycinnamic acids, four flavonoids, three hydroxybenzoic aldehydes and two additional polyphenols.

Our data revealed that HM, despite being a non-static fluid that changes with time, does not differ in TPC or AC throughout the three stages of lactation. By contrast, clear differences were found in the antioxidant properties between HM and IF samples. Although the uptake of phenolic compounds by the newborn remains to be demonstrated, the analyzed HM samples presented a higher content of phenolic com- pounds than IF, which might be positive for preventing infant diseases. It has been found that HM has many advantages over formula, being able

to consider the phenolic compounds reported in the present work among these beneficial agents from now on.

This work provides data in favor of the implementation of breastfeeding, whilst also offering greater knowledge about the presence of phenolic compounds for the development of IF when this may not be possible. Information about the human milk antioxidant activity is still insufficient and future research is necessary.

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CRediT authorship contribution statement

Silvia Sánchez-Hernández: Methodology, Formal analysis, Investigation, Software, Data curation, Writing-original draft. Adelaida Esteban-Muñoz: Methodology, Formal analysis, Investigation, Software, Data curation, Writing-original draft. Cristina Samaniego-Sánchez: Methodology, Writing-review & editing. Rafael Giménez-Martínez: Data curation, Writing-review & editing. Beatriz-Miralles: Supervision, Visualization, Writing-review & editing. Manuel Olalla-Herrera: Resources, Conceptualization, Supervision, Project administration, Writing-review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110149.

References

Akdag, A., Nur Sari, F., Dizdar, E. A., Uras, N., Isikoglu, S., Erel, O., et al. (2014). Storage at -80◦C preserves the antioxidant capacity of preterm human milk. Journal of Clinical Laboratory Analysis, 28(5), 415–418. https://doi.org/10.1002/jcla.21703.

Al-Harbi, F. J. (2018). The impact of dietary polyphenols on the quality of expressed.

Alonso, ´A. M., Castro, R., Rodríguez, M. C., Guillén, D. A., & Barroso, C. G. (2004). Study of the antioxidant power of brandies and vinegars derived from Sherry wines and correlation with their content in polyphenols. Food Research International, 37(7), 715–721. https://doi.org/10.1016/j.foodres.2004.03.007.

Andreas, N. J., Kampmann, B., & Mehring Le-Doare, K. (2015). Human breast milk: A review on its composition and bioactivity. Early Human Development, 91(11), 629–635. https://doi.org/10.1016/J.EARLHUMDEV.2015.08.013.

AOAC, Association of Official Analytical Chemists (2012). Official methods of analysis of AOAC International.

Aycicek, A., Erel, O., Kocyigit, A., Selek, S., & Demirkol, M. R. (2006). Breast milk provides better antioxidant power than does formula. Nutrition, 22(6), 616–619. https://doi.org/10.1016/j.nut.2005.12.011.

Benzie, I. F. F., Strain, J. J., & Straint, J. J. (1997). Simultaneous automated measurement of total 'antioxidant' (reducing) capacity and ascorbic acid concentration. Redox Report, 3(4), 233–238.

https://doi.org/10.1080/13510002.1997.11747115.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology, 28(1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5.

Byun, J. W., Hwang, S., Kang, C. W., Kim, J. H., Chae, M. K., Yoon, J. S., et al. (2016). Therapeutic effect of protocatechuic aldehyde in an in vitro model of graves' orbitopathy. Investigative Ophthalmology and Visual Science, 57(10), 4055–4062. https://doi.org/10.1167/iovs.15-19037.

Cloetens et al. (2016). The antioxidant capacity of milk-the application of different methods in vitro and in vivo. April. https://doi.org/10.1170/T946.

Cortez, M. V., & Soria, E. A. (2016). The effect of freeze-drying on the nutrient, polyphenol, and oxidant levels of breast milk. Breastfeeding Medicine, 11(10), 551–554. https://doi.org/10.1089/bfm.2016.0102.

Doltra, A., Dietrich, T., Schneeweis, C., Kelle, S., Doltra, A., Stawowy, P., et al. (2013). Magnetic resonance imaging of cardiovascular fibrosis and inflammation: From clinical practice to animal studies and back. BioMed Research International, 676489 (10), 1–2. https://doi.org/10.1155/2013.

Esteban Muñoz, A., Barea Álvarez, M., Oliveras-López, M.-J., Giménez Martínez, R., Henares, J.´A. R., & Olalla Herrera, M. (2018). Determination of polyphenolic compounds by ultra-performance liquid chromatography coupled to tandem mass spectrometry and antioxidant capacity of Spanish subtropical fruits. Agricultural Sciences, 09(02), 180–199. https://doi.org/10.4236/as.2018.92014.

Friel, J. K., Martin, S. M., Langdon, M., Herzberg, G. R., & Buettner, G. R. (2002). Milk from mothers of both premature and full-term infants provides better antioxidant protection than does infant formula. Pediatric Research, 51(5), 612–618. https://doi.org/10.1203/00006450-200205000-00012.

Fukumoto, L. R., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. Journal of Agricultural and Food Chemistry, 48(8), 3597–3604. https://doi.org/10.1021/jf000220w.

Gila-Diaz, A., Arribas, S. M., Algara, A., Martín-Cabrejas, M. A., Pablo, ´A. L. L.

de, Pipaón, M. S. de, & Ramiro-Cortijo, D. (2019). A review of bioactive factors in human breastmilk: A focus on prematurity. In Nutrients (Vol. 11, Issue 6). MDPI AG. https://doi.org/10.3390/nu11061307.

Gulcin, I. (n.d.). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). In Toxicology (Vol. 217, Issues 2–3, pp. 213–220).

Hanna, N., Ahmed, K., Anwar, M., Petrova, A., Hiatt, M., & Hegyi, T. (2004). Effect of storage on breast milk antioxidant activity. Archives of Disease in Childhood: Fetal and Neonatal Edition, 89(6), F518–F520. https://doi.org/10.1136/adc.2004.049247.

Inder, T. E., & Volpe, J. J. (2000). Mechanisms of perinatal brain injury. Seminars in Neonatology, 5(1), 3–16. https://doi.org/10.1053/SINY.1999.0112.

Jackson, J. G., Janszen, D. B., Lonnerdal, B., Lien, E. L., Pramuk, K. P., & Kuhlman, C. F. (2004). A multinational study of α-lactalbumin concentrations in human milk. The Journal of Nutritional Biochemistry, 15(9), 517–521. https://doi.org/10.1016/J.JNUTBIO.2003.10.009.

Juurlink, B. H. J., Azouz, H. J., Aldalati, A. M. Z., Altinawi, B. M. H., & Ganguly, P. (2014). Hydroxybenzoic acid isomers and the cardiovascular system. In Nutrition Journal (Vol. 13, Issue 1, p. 63). BioMed Central Ltd. https://doi.org/10.1186/1475-2891-13-63.

Kahkeshani, N., Farzaei, F., Fotouhi, M., Alavi, S. S., Bahramsoltani, R., Naseri, R., et al. (2019). Pharmacological effects of gallic acid in health and disease: A mechanistic review. Iranian Journal of Basic Medical Sciences, 22(3), 225–237. https://doi.org/10.22038/ijbms.2019.32806.7897.

Khymenets, O., Rabassa, M., Rodríguez-Palmero, M., Rivero-Urgell, M., Urpi-Sarda, M., Tulipani, S., et al. (2016). Dietary epicatechin is available to breastfed infants through human breast milk in the form of host and microbial metabolites. Journal of Agricultural and Food Chemistry, 64(26), 5354–5360. https://doi.org/10.1021/acs.jafc.6b01947.

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., & Taniguchi, H. (2002). Antioxidant properties of ferulic acid and its related compounds. Journal of Agricultural and Food Chemistry, 50(7), 2161–2168.

https://doi.org/10.1021/jf011348w.

Kim, J. H., Lee, H. O., Cho, Y. J., Kim, J., Chun, J., Choi, J., et al. (2014). A vanillin derivative causes mitochondrial dysfunction and triggers oxidative stress in Cryptococcus neoformans. Plos One, 9(2). https://doi.org/10.1371/journal.pone.0089122.

Kozhimannil, K. B., Jou, J., Attanasio, L. B., Joarnt, L. K., & McGovern, P. (2014). Medically complex pregnancies and early breastfeeding behaviors: A retrospective analysis. Plos One, 9(8), Article e104820. https://doi.org/10.1371/journal.pone.0104820.

Lee, S., & Kelleher, S. L. (2016). Biological underpinnings of breastfeeding challenges: The role of genetics, diet, and environment on lactation physiology. In American Journal of Physiology - Endocrinology and Metabolism (Vol. 311, Issue 2, pp. E405–E422). American Physiological Society. https://doi.org/10.1152/ajpendo.00495.2015.

Li, W., Hosseinian, F. S., Tsopmo, A., Friel, J. K., & Beta, T. (2009). Evaluation of antioxidant capacity and aroma quality of breast milk. Nutrition, 25(1), 105–114. https://doi.org/10.1016/j.nut.2008.07.017.

Martysiak-Zurowska, D., & Wenta, W. (2012). A comparison of abts and dpph methods for assessing the total antioxidant capacity of human milk. Acta Scientiarum Polonorum, Technologia Alimentaria, 11(1), 83–89.

Masek, A., Chrzescijanska, E., & Latos, M. (2016). Determination of antioxidant activity of caffeic acid and p-coumaric acid by using electrochemical and spectrophotometric assays. International Journal of Electrochemical Science, 11, 10644–10658. https://doi.org/10.20964/2016.12.73.

Masek, A., Chrzescijanska, E., Latos, M., & Zaborski, M. (2016). Antioxidant potential of hydroxycinnamic acids in advanced oxidation processes. International Journal of Electrochemical Science, 11, 8848–8860. https://doi.org/10.20964/2016.10.12.

Matos, C., Moutinho, C., Balcão, V., Almeida, C., Ribeiro, M., Marques, A. F., et al. (2009). Total antioxidant activity and trace elements in human milk: The first 4 months of breast-feeding. European Food Research and Technology,

230(2), 201–208. https://doi.org/10.1007/s00217-009-1157-2.

Mehta, R., & Petrova, A. (2014). Is variation in total antioxidant capacity of human milk associated with levels of bio-active proteins? Journal of Perinatology, 34(3), 220–222. https://doi.org/10.1038/jp.2013.151.

Ministry of Health. (2017). Guía de Pr´actica Clínica sobre lactancia materna. https://portal.guiasalud.es/wp-content/uploads/2018/12/GPC_560_ Lactancia_Osteba_ compl.pdf.

Molteberg, E. L., Solheim, R., Dimberg, L. H., & Frølich, W. (1996). Variation in oat groats due to variety, storage and heat treatment. Ii: Sensory quality. Journal of Cereal Science, 24(3), 273–282. https://doi.org/10.1006/jcrs.1996.0059.

Muñoz, A. E., Hernández, S. S., Tolosa, A. R., Burillo, S. P., & Olalla-Herrera, M. (2020). Evaluation of differences in the antioxidant capacity and phenolic compounds of green and roasted coffee and their relationship with sensory properties. LWT-Food Science and Technology, 109457. https://doi.org/10.1016/j.lwt.2020.109457.

Nalewajko-Sieliwoniuk, E., Hryniewicka, M., Jankowska, D., Kojło, A., Kamianowska, M., & Szczepański, M. (2020). Dispersive liquid–liquid microextraction coupled to liquid chromatography tandem mass spectrometry for the determination of phenolic compounds in human milk. Food Chemistry, 327 (April). https://doi.org/10.1016/j.foodchem.2020.126996.

Natera, R., Castro, R., De Valme García-Moreno, M., Hern´andez, M. J., & García-Barroso, C. (2003). Chemometric studies of vinegars from different raw materials and processes of production. Journal of Agricultural and Food Chemistry, 51(11), 3345–3351. https://doi.org/10.1021/jf021180u.

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A. A., Khan, G. J., Shumzaid, M., XiaoHui, Z. (2018). Chlorogenic acid (CGA): A pharmacological review and call for further research. Biomedicine and Pharmacotherapy, 97(August 2017), 67–74. https://doi.org/10.1016/j.biopha.2017.10.064.

Nemec, M. J., Kim, H., Marciante, A. B., Barnes, R. C., Talcott, S. T., & Mertens-Talcott, S. U. (2016). Pyrogallol, an absorbable microbial gallotannins-

metabolite and mango polyphenols (Mangifera Indica L.) suppress breast cancer ductal carcinoma in situ proliferation in vitro. Food and Function, 7(9), 3825–3833. https://doi.org/10.1039/c6fo00636a.

Păduraru, L., Dimitriu, D. C., Avasiloaiei, A. L., Moscalu, M., Zonda, G. I., & Stamatin, M. (2018). Total antioxidant status in fresh and stored human milk from mothers of term and preterm neonates. Pediatrics and Neonatology, 59(6), 600–605. https://doi.org/10.1016/j.pedneo.2018.02.004.

Papp, A., Németh, I., Karg, E., & Papp, E. (1999). Glutathione status in retinopathy of prematurity. Free Radical Biology and Medicine, 27(7–8), 738–743. https://doi.org/10.1016/S0891-5849(99)00041-6.

Peron, G., Pellizzaro, A., Brun, P., Schievano, E., Mammi, S., Sut, S., et al. (2017). Antiadhesive activity and metabolomics analysis of rat urine after cranberry (Vaccinium macrocarpon Aiton) administration. Journal of Agricultural and Food Chemistry, 65(28), 5657–5667. https://doi.org/10.1021/acs.jafc.7b01856.

Poniedziałek, B., Rzymski, P. P., Pięt, M., Niedzielski, P., Mleczek, M., Wilczak, M., et al. (2017). Rare-earth elements in human colostrum milk. Environmental Science and Pollution Research, 24(33), 26148–26154. https://doi.org/10.1007/s11356-017-0359-6.

Poniedziałek, B., Rzymski, P., Pięt, M., Gąsecka, M., Stroi´nska, A., Niedzielski, P., et al. (2018). Relation between polyphenols, malondialdehyde, antioxidant capacity, lactate dehydrogenase and toxic elements in human colostrum milk. Chemosphere, 191, 548–554. https://doi.org/10.1016/j.chemosphere.2017.10.098.

Quiles, J. L., Ochoa, J. J., Ramirez-Tortosa, M. C., Linde, J., Bompadre, S., Battino, M., et al. (2006). Coenzyme Q concentration and total antioxidant capacity of human milk at different stages of lactation in mothers of preterm and full-term infants. Free Radical Research, 40(2), 199–206. https://doi.org/10.1080/10715760500404805.

Ramirez, J. P., Castañeda, M. C., Olalla, M., Villalón, M., López, H., & Samaniego, C. (2019). Changes in the antioxidant properties of extra virgin

olive oil after cooking typical mediterranean vegetables. Antioxidants, 8, 246. https://doi.org/10.20944/PREPRINTS201907.0226.V1

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3.

Romaszko, E., Wiczkowski, W., Romaszko, J., Honke, J., & Piskula, M. K. (2014). Exposure of breastfed infants to quercetin after consumption of a single meal rich in quercetin by their mothers. Molecular Nutrition and Food Research, 58(2), 221–228. https://doi.org/10.1002/mnfr.201200773.

Sánchez-Hernández, S., Esteban-Muñoz, A., Giménez-Martínez, R., Aguilar-Cordero, M. J., Miralles-Buraglia, B., & Olalla-Herrera, M. (2019). A comparison of changes in the fatty acid profile of human milk of Spanish lactating women during the first month of lactation using gas chromatography-mass spectrometry. A comparison with infant formulas. Nutrients, 11(12). https://doi.org/10.3390/nu11123055.

Saphier, O., Barak, T., Hamou, B., & Silberstein, T. (2019). Caffeic and gallic acids protect commercial infant milk formula from oxidative damage. Advances in Dairy Research, 07(02), 1–4. https://doi.org/10.35248/2329-888x.19.7.224.

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. Journal of Functional Foods, 18, 820–897. https://doi.org/10.1016/j.jff.2015.06.018.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. American Journal of Enology and Viticulture, 16(3).

Socrier, L., Quéro, A., Verdu, M., Song, Y., Molinié, R., Mathiron, D., Pilard, S., Mesnard, F., & Morandat, S. (2019). Flax phenolic compounds as inhibitors of lipid oxidation: Elucidation of their mechanisms of action. Food Chemistry, 274, 651–658. https://doi.org/10.1016/j.foodchem.2018.08.126

Song, B. J., Jouni, Z. E., & Ferruzzi, M. G. (2013). Assessment of phytochemical content in human milk during different stages of lactation. Nutrition, 29(1), 195–202. https://doi.org/10.1016/j.nut.2012.07.015

Stój, A., Kapusta, I., & Domagała, D. (2020). Classification of red wines produced from zweigelt and rondo grape varieties based on the analysis of phenolic compounds by UPLC-PDA-MS/MS. Molecules, 25(6). https://doi.org/10.3390/molecules25061342

Taamalli, A., Contreras, M. D. M., Abu-Reidah, I. M., Trabelsi, N., & Ben Youssef, N. (2019). Quality of phenolic compounds: Occurrence, health benefits, and applications in food industry. In Journal of Food Quality (Vol. 2019). https://doi.org/10.1155/2019/9594646

Tijerina-Sáenz, A., Innis, S. M., & Kitts, D. D. (2009). Antioxidant capacity of human milk and its association with vitamins A and e and fatty acid composition. Acta Paediatrica, International Journal of Paediatrics, 98(11), 1793–1798. https://doi.org/10.1111/j.1651-2227.2009.01437.x

Tsopmo, A. (2018). Phytochemicals in human milk and their potential antioxidative protection. In Antioxidants (Vol. 7, Issue 2). MDPI AG. https://doi.org/10.3390/antiox7020032

Turoli, D., Testolin, G., Zanini, R., & Bellù, R. (2004). Determination of oxidative status in breast and formula milk. Acta Paediatrica, International Journal of Paediatrics, 93(12), 1569–1574. https://doi.org/10.1080/08035250410022495

VanderJagt, D. J., Okolo, S. N., Costanza, A., Blackwell, W., & Glew, R. H. (2001). Antioxidant content of the milk of nigerian women and the sera of their exclusively breast-fed infants. Nutrition Research, 21(1–2), 121–128. https://doi.org/10.1016/S0271-5317(00)00261-X

Vázquez, C. V., Rojas, M. G. V., Ramírez, C. A., Chávez-Servín, J. L., García-Gasca, T., Ferriz Martínez, R. A., García, O. P., Rosado, J. L., López-Sabater, C. M., Castellote, A. I., Montemayor, H. M. A., & De La Torre Carbot, K. (2015). Total phenolic compounds in milk from different species. Design of an extraction technique for quantification using the Folin-Ciocalteu method. Food Chemistry, 176, 480–486.

https://doi.org/10.1016/j.foodchem.2014.12.050

World Health Organization (WHO) & Pan American Health Organitation (PAHO). (2009). La alimentación del lactante y del niño pequeño. In OMS (Vol. 1). https://eprints.ucm.es/22755/1/Manual-nutricion-dietetica-CARBAJAL.pdf

Yuksel, S., Yigit, A. A., Cinar, M., Atmaca, N., & Onaran, Y. (2015). Oxidant and antioxidant status of human breast milk during lactation period. Dairy Science and Technology, 95(3), 295–302. https://doi.org/10.1007/s13594-015-0211-z

Zarban, A., Taheri, F., Chahkandi, T., Sharifzadeh, G., & Khorashadizadeh, M. (2009). Antioxidant and radical scavenging activity of human colostrum, transitional and mature milk. Journal of Clinical Biochemistry and Nutrition, 45(2), 150–154. https://doi.org/10.3164/jcbn.08-233

Zhou, Y., Wang, Q., Evers, B. M., & Chung, D. H. (2005). Signal transduction pathways involved in oxidative stress-induced intestinal epithelial cell apoptosis. Pediatric Research, 58(6), 1192–1197. https://doi.org/10.1203/01.pdr.0000185133.65966.4e

Živković, J., Sunarić, S., Trutić, N., Denić, M., Kocić, G., Jovanović, T., Facultatis Medicae Naissensis, A., & M A Ry, S. M. (2015). Antioxidants and antioxidant capacity of human milk. Acta Facultatis Medicae Naissensis, 32(2), 115–125. https://doi.org/10.1515/afmnai-2015-0012


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2.5.1 Material Suplementario Publicación V

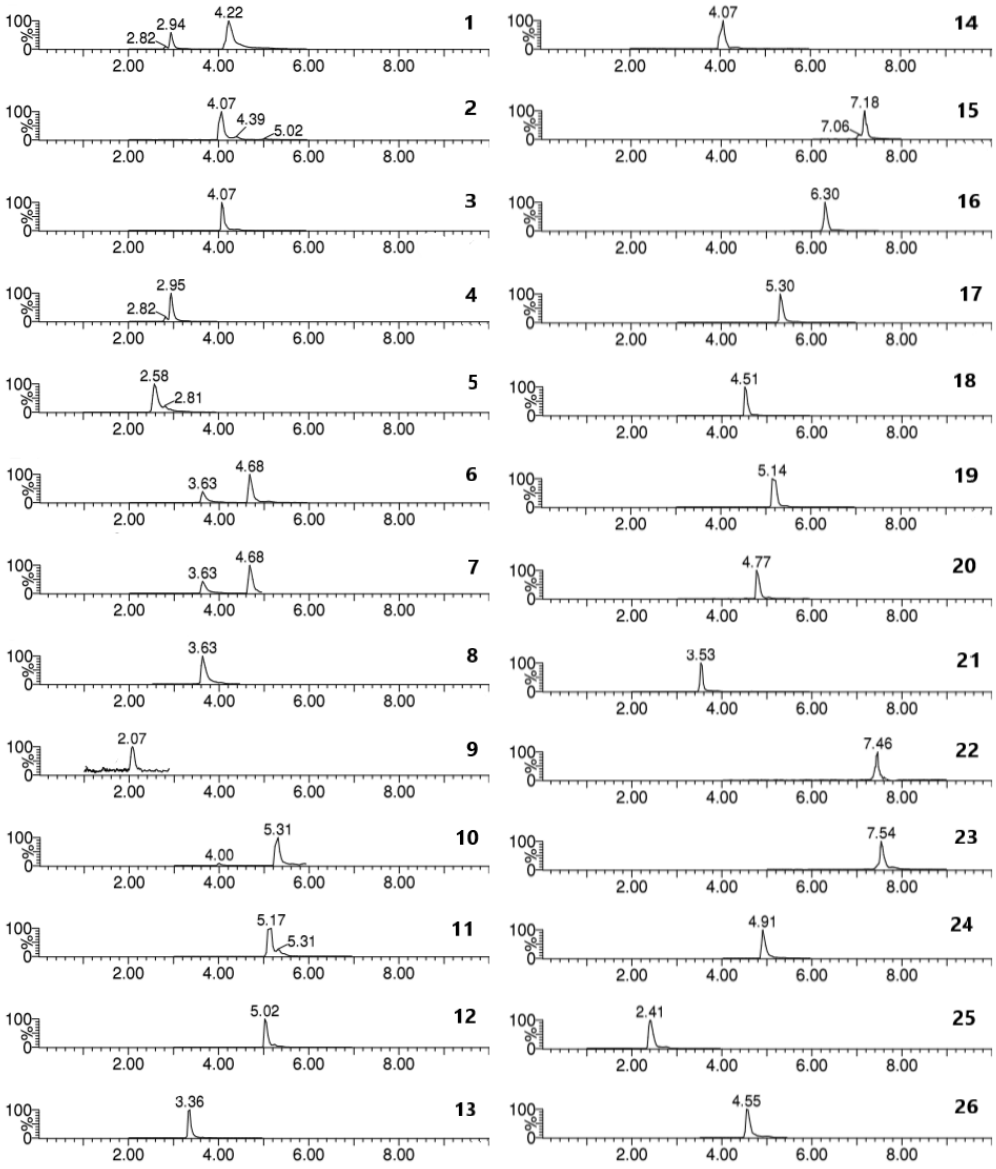

Fig. S1. Ultra-performance liquid chromatographic profile of 26 standard phenolic acids. 1: 2,5-dihydroxybenzoic; 2: vanillic acid; 3: syringic acid; 4: protocatechuic acid; 5: 2,4,6-trihydroxybenzoic; 6: p-hydroxybenzoic acid; 7: m-hydroxybenzoic acid; 8: protocatehuic aldehyde; 9: gallic acid; 10: 2,6-dihydroxybenzoic; 11: ferulic acid; 12: sinapic acid; 13: chlorogenic acid; 14: caffeic acid; 15: 4-methoxycinnamic; 16: dimethyl caffeic; 17: p-coumaric; 18: rutin; 19: kaempferol-3-O-glucoside; 20: isoquercetin; 21: catechin; 22: o-vanillin; 23: p-hydroxyphenylacetic; 24: p-vanillin; 25: pyrogallol; 26: p-hydroxybenzaldehide

Table S1. List of quantified phenolic acids and MRM parameters.

n	Phenolic compounds	Molecular Formula	Parent m/z	Cone voltage	Daughter m/z
	Phenolic acids / Hydroxybenzoic acids				
1	2,5-Dihydroxybenzoic	C7H6O4	152.99	8	108.81,64.24
2	Vanillic acid	C8H8O4	167.07	18	151.62,107.52
3	Syringic acid	C9H10O5	196.96	2	181.66,122.50
4	Protocatechuic acid	C7H6O4	153.06	42	80.30,108.81
5	2,4,6-Trihydroxybenzoic	C7H6O5	168.99	12	82.33,106.49
6	p-Hydroxybenzoic acid	C7H6O3	137.06	24	107.55
7	m-Hydroxybenzoic acid	C7H6O3	137.87	24	108.40
8	Protocatechualdehyde	C7H6O3	137.13	30	107.61,136.64
9	Gallic acid	C7H6O5	168.93	32	78.27,96.41
10	2,6-Dimethoxybenzoic acid	C9H10O4	181.09	12	106.5,136.6
	Phenolic acids / Hydroxycinnamic acids				
11	Ferulic acid	C10H10O4	192.96	2	133.51,177.64
12	Sinapic acid	C11H12O5	223.04	2	207.79,192.71
13	Chlorogenic acid	C16H18O9	353.19	34	190.74,84.40
14	Caffeic acid	C9H8O4	179.01	18	78.27

15	4-Methoxycinnamic	C10H10O3	177.03	18	116.56,102.49
16	Dimethylcaffeic acid	C11H12O4	207.11	12	102.48,130.48
17	p-Coumaric acid	C9H8O3	162.89	12	118.35,92.42
	Flavonoids / Flavonols				
18	Rutin	C27H30O16	609.31	2	300.12,270.92
19	Kaempferol-3-O-glucoside	C21H20O11	447.21	12	284.09,254.88
20	Isoquercetin	C21H20O12	463.19	8	270.89,300.10
21	Catechin	C15H14O6	289.05	14	202.78,108.44
	Other polyphenols / Hydroxybenzaldehydes				
22	o-Vanillin	C8H8O3	151.02	48	107.43,135.47
23	p-Hydroxyphenylacetic	C8H8O3	150.95	48	106.55,88.41
24	p- Vanillin	C8H8O3	151.02	22	135.53,91.42
	Other polyphenols / Other polyphenols				
25	Pyrogallol	C6H6O3	125.0	4	78.32,96.40
26	p-Hydroxybenzaldehyde	C7H6O2	121.13	12	91.44,120.61

*Rt: retention time of samples

Table S2. Quality parameters for the chromatographic determination of phenolic compounds.

n	Phenolic compounds*	Linear range (ppb)	Rt± SD (min)	Linear equation
	Phenolic acids / Hydroxybenzoic acids			
1	2,5-Dihydroxybenzoic	5 - 1000	4.22 ± 0.000	y = 122409x + 1000664
2	Vanillic acid	5 - 1000	4.04 ± 0.016	y = 5591.2x + 69561
3	Syringic acid	5 - 1000	4.08 ± 0.016	y = 4964.5x + 101920
4	Protocatechuic acid	5 - 1000	2.94 ± 0.009	y = 33773x + 397374
5	2,4,6-Trihydroxybenzoic	5 - 1000	2.58 ± 0.012	y = 19075x + 458517
6	p-Hydroxybenzoic acid	5 - 1000	4.70 ± 0.015	y = 13821x + 53472
7	m-Hydroxybenzoic acid	5 - 1000	3.66 ± 0.023	y = 3651.4x + 17260
8	Protocatechualdehyde	5 - 1000	3.65 ± 0.015	y = 50276x + 887358
9	Gallic acid	5 - 1000	2.08 ± 0.007	y= 601.3x + 7658.2
10	2,6-Dimethoxybenzoic acid	5 - 1000	5.30 ± 0.022	y = 7719x + 11116
	Phenolic acids / Hydroxycinnamic acids			
11	Ferulic acid	5 - 1000	5.10 ± 0.020	y = 10502x + 60674
12	Sinapic acid	5 - 1000	5.05 ± 0.016	y = 9414.3x + 20694
13	Chlorogenic acid	5 - 1000	3.36 ± 0.012	y = 5201.9x + 53904
14	Caffeic acid	5 - 1000	4.05 ± 0.032	y = 597.65x – 648.67
15	4-Methoxycinnamic	5 - 1000	7.14 ± 0.051	y = 574.14x + 7942.1
16	Dimethylcaffeic acid	5 - 1000	6.31 ± 0.016	y = 3786.3x + 8858.4
17	p-Coumaric acid	5 - 1000	5.35 ± 0.017	y = 1486.6x + 868.65
	Flavonoids / Flavonols			
18	Rutin	5 - 1000	4.54 ± 0.012	y = 6907.4x + 22289
19	Kaempferol-3-O-glucoside	5 - 1000	5.20 ± 0.023	y = 27579x + 20735
20	Isoquercetin	5 - 1000	4.80 ± 0.004	y = 22562x + 14034
21	Catechin	5 - 1000	3.57 ± 0.016	y = 1327.8x + 576.59
	Other polyphenols / Hydroxybenzaldehydes			

22	o-Vanillin	5 - 1000	7.42 ± 0.048	y = 11.202x + 196.11
23	p-Hydroxyphenylacetic	5 - 1000	7.53 ± 0.013	y = 954.48x + 25872
24	p- Vanillin	5 - 1000	4.93 ± 0.018	y = 48911x + 415071
	Other polyphenols / Other polyphenols			
25	Pyrogallol	5 - 1000	2.40 ± 0.010	y = 4300x + 70121
26	p-Hydroxybenzaldehyde	5 - 1000	4.59 ± 0.024	y = 27554x + 471064

Rtr: retention time; SD: standard deviation; R: correlation coefficient; LOD: Limit of detection; LOQ: Limit of quantific

based on the web http://phenol-explorer.eu/

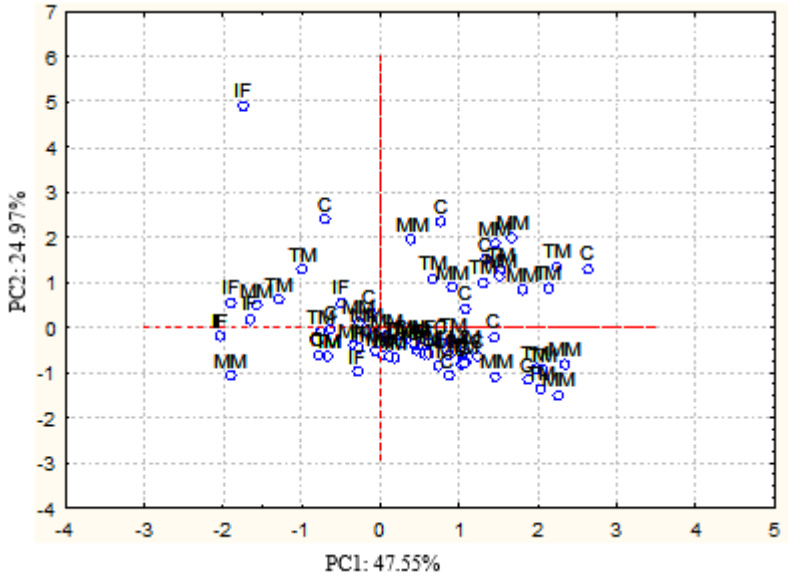

Figure S2. PCA representation according to method of antioxidant activity (DPPH, ABTS and FRAP) and total phenolic compounds (TPC).

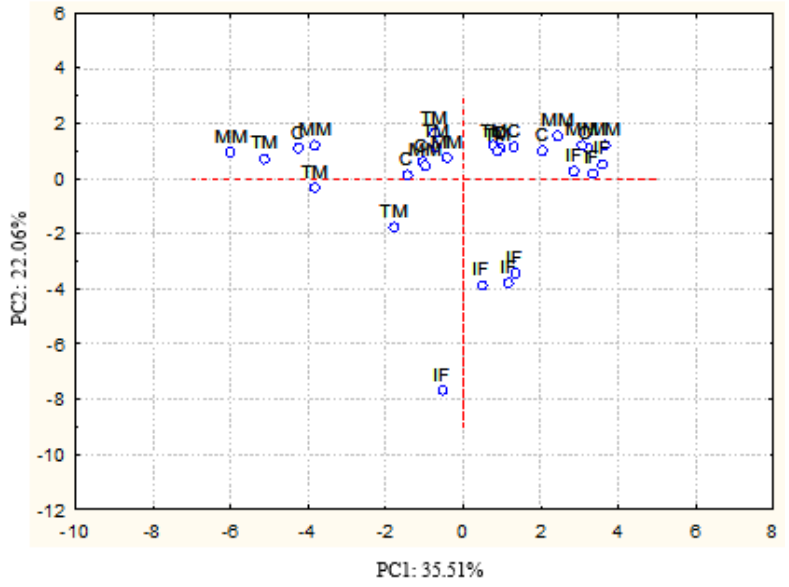

Figure S3. PCA representation according to individual phenolic compounds quantified by UPLC/MS-MS.

4. DISCUSIÓN


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El número de mujeres con sobrepeso u obesidad durante el embarazo y la lactancia ha aumentado notablemente durante las últimas décadas, y esta tendencia de aumento del peso corporal ha generado un creciente interés por las consecuencias que puede conllevar tanto para la madre como para el futuro bebé. Además, hay algunas sugerencias de que, además de aumentar el riesgo de embarazo y de complicaciones neonatales, el sobrepeso y la obesidad materna puedan estar asociados con cambios en la composición de macronutrientes de la HM. Son varios los estudios que han investigado las influencias de ciertas características maternas, como el tipo de parto o el BMI materno, en la composición de la HM. Sin embargo, todavía no hay evidencia y se necesitan estudios de mayor envergadura para comprender mejor las relaciones entre la adiposidad materna y la composición de la HM (Andreas et al., 2015; Leghi et al., 2020). Por otro lado, la composición de macronutrientes de la HM, a pesar de que varía dentro de las madres y durante el paso de la lactancia, se ha observado que se conserva notablemente entre las poblaciones a pesar de las variaciones en la nutrición materna (Prentice, 1985)

En 2015, la Organización de las Naciones Unidas aprobó la Agenda 2030 sobre el Desarrollo Sostenible, una oportunidad para que todos los países emprendan un nuevo camino con el que mejorar la vida de todos. La agenda cuenta con 17 Objetivos de Desarrollo Sostenible, y algunos de ellos son relevantes para la lactancia materna, aunque dos de ellos están directamente relacionados. El Objetivo de Desarrollo Sostenible 2 pretende poner fin al hambre y lograr la seguridad alimentaria y la mejora de la nutrición en todo el mundo y el Objetivo 3 pretende garantizar una vida sana y promover el bienestar para todos en todas las edades, centrándose así en la salud materno-infantil y en la reducción de las enfermedades no trasmisibles como el cáncer de mama, la diabetes, el sobrepeso o la obesidad y la reducción de las muertes infantiles evitables (Shingirai et al., 2016).

La lactancia materna exclusiva ha demostrado que mejora los efectos tanto a corto como a largo plazo en la supervivencia de los niños. Son muchas las

ventajas nutricionales de esta práctica, no sólo porque la HM sea un suministro nutricional perfectamente adaptado al bebé, sino también por su protección contra las infecciones, ya que a través de ésta se pueden transmitir elementos de su propio microbioma y respuestas inmunitarias, siendo también fuente de prebióticos específicos para apoyar el crecimiento de bacterias beneficiosas (Victora et al., 2016). La idoneidad de la HM como fuente de lípidos, proteínas, carbohidratos, y otros nutrientes ha sido ampliamente estudiada. No obstante, y pese a la importancia de esta práctica, el análisis de los datos disponibles muestra que, globalmente, menos de la mitad de los recién nacidos en el mundo son puestos al pecho dentro de la primera hora de vida (AEP, 2016).

La HM se considera el estándar de oro para el desarrollo de las IF y ha habido muchos intentos de diseñar IF similares a ésta, ya que las recomendaciones mundiales se basan principalmente en el análisis químico de la HM. En cambio, la composición de la HM es bastante variable, al igual que su ingesta por parte del lactante (Stam et al., 2013). Pese a todo, el hecho de que un seguimiento exhaustivo del embarazo y la lactancia materna no hayan sido prioritarios para del sistema sanitario, ha llevado a suponer que la HM puede ser sustituida por IF sin consecuencias perjudiciales (Victora et al., 2016), aunque son muchos los estudios que demuestran que el suministro de alimentos alternativos durante los primeros seis meses, o la adición de alimentos complementarios a la alimentación del bebé, les expone a posibles enfermedades como la diarrea, las infecciones respiratorias, diabetes o sobrepeso, entre otras (Shingirai et al., 2016).

La mayor variabilidad en la composición de la HM se encuentra en el contenido de grasa, cambiando su composición a lo largo del día y dependiendo de la fase de la lactancia materna, adaptándose de esta forma a las necesidades energéticas del bebé. Nuestros resultados muestran que el ácido palmítico es el principal SFA, el ácido oleico constituye la mayor parte de los MUFA y el ALA el de los PUFA, siendo los FA predominantes tanto en los grupos de calostro, leche de transición y leche madura, así como en las IF. También cabe destacar que el ALA, al igual que el ALA, son dos FA esenciales que pueden influir en los

procesos metabólicos y son importantes para la síntesis endógena de sus respectivos LC-PUFA (Siziba et al., 2019). Es en estos FA donde se encuentran las mayores diferencias en composición entre la HM y las IF, ya que continúan existiendo a pesar de que la diferencia se ha reducido con la mejora de sus cualidades nutricionales. Mientras que las IF presentan un porcentaje de PUFA n-3 significativamente mayor, debido a su alto contenido en ALA, que el que el observado en los distintos grupos de HM; su porcentaje en LC-PUFA es bastante más bajo, y puesto que la síntesis endógena de AA y DHA a partir de sus precursores es limitada en los recién nacidos, es necesaria una buena suplementación de las IF en estos FA para suplir las necesidades del lactante (Lorenzo et al., 2019). Por esta razón el estudio de la lipidómica de la HM sigue siendo esencial para proporcionar una comprensión más profunda de la salud infantil a corto y largo plazo, además de que los recientes avances en los métodos y en la instrumentación del estudio lipídico está dando lugar a investigaciones de la HM más completas (George et al., 2018).

El número de estudios sobre HM y su composición proteica ha aumentado drásticamente durante el último medio siglo. En nuestro estudio, centramos el interés en la composición en proteínas de la HM y cuáles de ellas son fuente importante de péptidos que puedan dar lugar a diferentes actividades biológicas. Para ello, estudiamos el perfil proteico individual caracterizando el perfil de péptidos grandes y proteínas mediante espectrometría de masas MALDI-TOF. Di Francesco et al., (2018) habían mostrado que el perfil de proteínas obtenido a través de MALDI-TOF de la leche en combinación con herramientas estadísticas demuestra ser un método de alto rendimiento y bajo costo con aplicaciones prometedoras como herramienta analítica. Este análisis ha mostrado una alta capacidad de clasificar y evaluar rápidamente las similitudes y diferencias en el perfil proteico de muestras de HM e incluso asociarlo con el BMI de la madre.

Por otro lado, el grado de degradación y digestibilidad de las proteínas de la HM se ha convertido en una cuestión fundamental tanto para el suministro de péptidos y aminoácidos como para las posibles bioactividades que puedan ejercer.

Puesto que los ensayos clínicos llevados a cabo para el estudio de la digestión de los alimentos son casi imposibles de llevar por razones éticas en poblaciones específicas sanas como los recién nacidos, tienen un gran interés los modelos *in vitro* que simulan las condiciones gastrointestinales del lactante (Ménard et al., 2018). Este estudio ha permitido detectar un comportamiento diferente en la pepsinólisis entre las proteínas del suero (principalmente la α-lactoalbúmina y la lactoferrina) que se muestran más resistentes a la digestión y las caseínas (principalmente la β-caseína) que se hidrolizan más extensamente, debido a la diferencia en su estructura. El modelo utilizado ha mostrado una buena correspondencia con lo reportado anteriormente tanto para las proteínas de la leche de vaca como la humana en estudios de digestión realizados *in vivo* (Bourlieu et al., 2015; De Oliveira et al., 2017).

No está claro en qué ubicación anatómica aparecen los péptidos funcionales durante la digestión infantil. Se ha sugerido que gran parte de los péptidos que se encuentran en las muestras de HM provienen de una proteólisis que comienza en el propio seno durante la lactancia o la bajada de la leche, ya que, dentro de la glándula mamaria, las proteasas como la plasmina, trombina, elastasa y calicreína inician la descomposición de las proteínas de la leche (Nielsen, Beverly, & Dallas, 2017). No obstante, existe un aumento muy abundante de péptidos funcionales en el estómago que se encuentran ausentes en la HM intacta, lo que da lugar a una proteólisis muy extensa en el estómago del bebé (Dallas et al., 2014). En nuestro estudio, la digestión *in vitro* de las proteínas de la HM da como resultado casi 700 péptidos, pero sólo algunos con demostrada actividad biológica, aunque para que estos péptidos bioactivos sean relevantes para el bebé, deben sobrevivir hasta que alcancen su sitio de acción. Para muchas de las bioactividades, este sitio es el tracto intestinal superior, donde pueden absorberse en el torrente sanguíneo para actuar sistémicamente, pero también pueden actuar localmente sobre bacterias, células inmunitarias y células epiteliales intestinales (Beverly et al., 2020). Los péptidos identificados procedieron, en su mayor parte, de la β-caseína, la α-lactoalbúmina y la lactoferrina, de acuerdo con su abundancia en la HM, aunque también se encontraron péptidos de otras proteínas minoritarias como la αs1-caseína o la κ-

caseína. Noventa secuencias de siete proteínas encontradas tras la digestión *in vitro* coincidieron con las que se han registrado en estudios *in vivo*. De todas estas secuencias, y tras la búsqueda previa en bases de datos de péptidos activos generales, como BIOPEP (Minkiewicz et al., 2019), o específicas de leche, como MBPDB (Nielsen, Beverly, Qu, et al., 2017), se encontraron doce péptidos que coincidieron completamente con las secuencias de péptidos bioactivos conocidos, además de múltiples péptidos identificados que fueron altamente homólogos (≥ 80% de coincidencia de secuencia) a péptidos bioactivos conocidos. Las bioactividades principales asociadas a los péptidos fueron antioxidantes, opioides, inmunomoduladoras, antimicrobianas y antibacterianas. Se ha descrito que los péptidos liberados por la digestión *in vivo* o *in vitro* de las proteínas de la HM afectan a los sistemas principales como el cardiovascular, nervioso, digestivo e inmunológico (Raikos & Dassios, 2014). Por tanto, los estudios de las aplicaciones potenciales de estos péptidos son necesarias para aplicar su posible inclusión en las IF, de manera que se consiga una mejor imitación de la funcionalidad de la HM (Beverly et al., 2021).

La HM contiene además muchos otros componentes considerados bioactivos, y uno de ellos son los denominados compuestos fenólicos. Muchos de los efectos protectores para la salud de los compuestos fenólicos están estrechamente relacionados con su actividad antioxidante, así como a sus propiedades antimutagénicas, anticancerígenas, antiinflamatorias, antimicrobianas y otras propiedades biológicas (Shahidi & Ambigaipalan, 2015; Taamalli et al., 2019). De todos los compuestos fenólicos, es el grupo de los flavonoides el que ha sido mayormente estudiado en la HM, demostrando tener actividades biológicas, como la antioxidante y la antiinflamatoria (Franke et al., 2006; Song et al., 2013). Sin embargo, pocos estudios han considerado la composición de ácidos fenólicos en la HM. La mayoría de los ácidos fenólicos, conocidos por su función de compuestos bioactivos polivalentes, forman parte de la dieta humana y se clasifican en derivados de los ácidos hidroxibenzoicos e hidroxicinámicos, siendo los ácidos hidroxicinámicos los más comunes y abundantes en los alimentos. En el presente trabajo se presentan nuevos datos sobre la composición fenólica y la

actividad antioxidante de la HM, en el que se cuantificaron diecisiete ácidos fenólicos diferentes y fueron los que constituyeron el principal grupo de compuestos fenólicos encontrados. Los ácidos hidroxicinámicos tienen gran importancia debido a sus efectos beneficiosos para la salud, ya que son reconocidos principalmente por su potente capacidad antioxidante, estando involucrados en prevención de varias enfermedades relacionadas con el estrés oxidativo, actividades antiinflamatorias y antimicrobianas (Sova & Saso, 2020). Por otro lado, los ácidos hidroxibenzoicos son compuestos que disminuyen el estrés oxidativo y la inflamación a través de la promoción de la expresión de enzimas antioxidantes (Juurlink et al., 2014). Es por esto que la presencia de ácidos fenólicos en la HM cobraría una especial importancia nutricional si se demostrase una alta biodisponibilidad una vez ingeridos. Los estudios *in vivo* sobre los mecanismos de absorción de los compuestos fenólicos están seriamente obstaculizados por la falta de acceso al epitelio intestinal humano, por lo que una alternativa es utilizar modelos experimentales de animales o cultivos celulares. Recientemente, Konishi y cols. (2006) llevaron a cabo un estudio en ratas Wistar macho, donde demostraron que el estómago y el intestino delgado constituyen un lugar de absorción activo de numerosos ácidos fenólicos, lo que significa que la concentración biodisponible del conjunto de ácidos fenólicos puede ser mayor que la encontrada para los flavonoides (Lafay & Gil-Izquierdo, 2008). Sin embargo, cuando se utilizan modelos animales, uno de los principales inconvenientes es encontrar una equivalencia con la fisiología y al metabolismo humano, y en particular, al del recién nacido, ya que no reflejan completamente las condiciones del tracto digestivo humano (Tarko et al., 2013).

Por otro lado, las diferencias en las propiedades antioxidantes que aparecen entre la HM y las IF pueden explicarse ya que, por ejemplo, la HM contiene más LC-PUFA (C20-22), que son muy susceptibles a la peroxidación lipídica, y por lo tanto están más asociados con una mayor lesión oxidativa en comparación con las IF. Del mismo modo, se ha observado que el calostro posee mayor contenido en antioxidantes en comparación con la leche madura, lo que demuestra que a lo largo del primer mes de lactancia, se produce una disminución gradual de la capacidad

antioxidante (Gila-Díaz et al., 2020). No obstante, la capacidad antioxidante de la HM se puede ver afectada por muchos factores, como el tabaquismo o el área geográfica (Li et al., 2009).

La HM constituye uno de los biofluidos más valiosos que existen, ya que es el único alimento que satisface completamente las necesidades nutricionales de los recién nacidos (Garwolińska et al., 2018). Además, a día de hoy, la estructura y la función biológica de los componentes de la HM, así como muchos de éstos que son capaces de alterase durante la digestión adquiriendo nuevas propiedades que pueden influir en la inmunidad y al crecimiento infantil, no se han estudiado lo suficiente (Andreas et al., 2015). Esto nos lleva a una necesidad de seguir investigando y profundizando aún más en el conocimiento de cada uno de los compuestos de la HM si se quiere mejorar la calidad de las IF, ya que se ha podido comprobar que estudiar la composición de ésta puede llegar a ser un desafío al tratarse de un fluido tan dinámico y cambiante.


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5. CONCLUSIONES


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1. Se observó una prevalencia de casi el 40% de mujeres que comenzaron su embarazo con sobrepeso u obesidad. El uso de medidas antropométricas consensuadas es una buena herramienta para estimar la composición corporal, y, por tanto, el estado nutricional materno durante las primeras semanas de embarazo. La muestra estudiada obtuvo una alta puntuación en el cuestionario de adherencia a la dieta mediterránea, pero en particular, la distribución de ingesta de macronutrientes no se ajustó del todo a las recomendaciones, encontrándose también déficit en algunos micronutrientes relevantes durante el embarazo.

2. Aunque las IF están enriquecidas en PUFA, necesarios para el crecimiento y desarrollo del feto durante el embarazo y los primeros años de vida, existen diferencias significativas con respecto a la HM. El contenido en AA y DHA fue superior en HM, mientras que el LA resultó similar, y el ALA fue superior en IF. En relación con la fase de lactancia, las diferencias en el perfil de FA son más acusadas entre el calostro y el resto de los tiempos de lactancia, justificando que se trata de un fluido dinámico y que se adapta a las necesidades nutricionales del lactante.

3. El análisis por espectrometría de masas MALDI muestra una alta capacidad para caracterizar el perfil proteico de la HM. Tanto el análisis discriminante como la aplicación de modelos predictivos, en concreto el denominado algoritmo genético, permiten clasificar las muestras según la etapa de lactancia y el estado metabólico de la madre.

4. Un modelo de digestión gastrointestinal in vitro que simula las condiciones del recién nacido muestra una alta resistencia a la digestión gástrica de las proteínas más abundantes de la HM, como la α-lactoalbúmina y lactoferrina, mientras que las caseínas, especialmente la β-caseína, se hidrolizan en cierta medida durante esta fase, debido a su estructura flexible que las hace más susceptibles a la proteólisis. Por el contrario, la digestión

intestinal da lugar a una rápida hidrólisis de las proteínas intactas que quedan tras la fase gástrica, en consonancia con lo observado en estudios del contenido digestivo en lactantes.

5. Noventa secuencias correspondientes a siete proteínas de la HM identificadas tras la digestión in vitro coincidieron con péptidos encontrados previamente en estudios in vivo de la digestión, indicando que se liberan secuencias similares de las caseínas y las principales proteínas del suero cuando se emplean condiciones digestivas neonatales apropiadas. Las bioactividades más relevantes de los péptidos encontrados para el recién nacido fueron antioxidantes, opioides, inmunomoduladoras, antimicrobianas y antibacterianas.

6. El método UPLC-MS/MS utilizado en el presente estudio permite la detección y cuantificación de veintiséis compuestos fenólicos. Los ácidos hidroxibenzoicos e hidroxicinámicos, pertenecientes al grupo de los ácidos fenólicos, constituyeron el grupo principal, algunos no descritos anteriormente en HM. En general, las muestras de HM presentaron un mayor contenido de compuestos fenólicos individuales, cuyo grupo mayoritario fue el de los ácidos hidroxibenzoicos, que las IF, cuyo grupo mayoritario fue el de los ácidos hidroxicinámicos.

7. El contenido en compuestos fenólicos totales tendió a disminuir con el avance de la lactancia materna, aunque el contenido fue mayor en todas las etapas de la lactancia que en las IF. La capacidad antioxidante medida por tres métodos diferentes (ABTS, DPPH y FRAP) mostró únicamente pequeñas variaciones entre las diferentes etapas de la lactancia. Por el contrario, las IF mostraron una capacidad antioxidante significativamente mayor que la HM en los métodos ABTS y DPPH.


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6. BIBLIOGRAFÍA


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AEP. (2016). Lactancia Materna en cifras: Tasas de inicio y duración de la lactancia en España y en otros países. 1–9. http://www.aeped.es/sites/default/files/documentos/201602-lactancia-materna-cifras.pdf

Altobelli, E., Angeletti, P. M., Verrotti, A., & Petrocelli, R. (2020). The Impact of Human Milk on Necrotizing Enterocolitis: A Systematic Review and Meta-Analysis. Nutrients 2020, Vol. 12, Page 1322, 12(5), 1322. https://doi.org/10.3390/NU12051322

Andreas, N. J., Kampmann, B., & Mehring Le-Doare, K. (2015). Human breast milk: A review on its composition and bioactivity. Early Human Development, 91(11), 629–635. https://doi.org/10.1016/J.EARLHUMDEV.2015.08.013

Ballard, O., & Morrow, A. L. (2013). Human Milk Composition. Nutrients and Bioactive Factors. In Pediatric Clinics of North America (Vol. 60, Issue 1, pp. 49–74). NIH Public Access. https://doi.org/10.1016/j.pcl.2012.10.002

Barreiro, R., Regal, P., López-Racamonde, O., Cepeda, A., & Fente, C. A. (2018). Comparison of the fatty acid profile of Spanish infant formulas and Galician women breast milk. Journal of Physiology and Biochemistry, 74(1), 127–138. https://doi.org/10.1007/s13105-017-0580-2

Beverly, R. L., Huston, R. K., Markell, A. M., McCulley, E. A., Martin, R. L., & Dallas, D. C. (2020). Milk Peptides Survive in Vivo Gastrointestinal Digestion and Are Excreted in the Stool of Infants. Journal of Nutrition, 150(4), 712–721. https://doi.org/10.1093/jn/nxz326

Beverly, R. L., Underwood, M. A., & Dallas, D. C. (2019). Peptidomics Analysis of Milk Protein-Derived Peptides Released over Time in the Preterm Infant Stomach. Journal of Proteome Research, 18(3), 912–922. https://doi.org/10.1021/acs.jproteome.8b00604

Beverly, R. L., Woonnimani, P., Scottoline, B. P., Lueangsakulthai, J., & Dallas,

D. C. (2021). Peptides from the Intestinal Tract of Breast Milk-Fed Infants Have Antimicrobial and Bifidogenic Activity. International Journal of Molecular Sciences, 22(5), 2377. https://doi.org/10.3390/ijms22052377

Binns, C., Lee, M., & Low, W. Y. (2016). The Long-Term Public Health Benefits of Breastfeeding. 28(1), 7–14. https://doi.org/10.1177/1010539515624964

Bohn, T., Carriere, F., Day, L., Deglaire, A., Egger, L., Freitas, D., Golding, M., Le Feunteun, S., Macierzanka, A., Menard, O., Miralles, B., Moscovici, A., Portmann, R., Recio, I., Rémond, D., Santé-Lhoutelier, V., Wooster, T. J., Lesmes, U., Mackie, A. R., & Dupont, D. (2018). Correlation between in vitro and in vivo data on food digestion. What can we predict with static in vitro digestion models? In Critical Reviews in Food Science and Nutrition (Vol. 58, Issue 13, pp. 2239–2261). Taylor and Francis Inc. https://doi.org/10.1080/10408398.2017.1315362

Boone, K. M., Geraghty, S. R., & Keim, S. A. (2016). Feeding at the Breast and Expressed Milk Feeding: Associations with Otitis Media and Diarrhea in Infants. Journal of Pediatrics, 174, 118–125. https://doi.org/10.1016/j.jpeds.2016.04.006

Bourlieu, C., Deglaire, A., de Oliveira, S. C., Ménard, O., Le Gouar, Y., Carrière, F., & Dupont, D. (2017). Towards infant formula biomimetic of human milk structure and digestive behaviour. OCL, 24(2), D206. https://doi.org/10.1051/ocl/2017010

Bourlieu, C., Ménard, O., Bouzerzour, K., Mandalari, G., Macierzanka, A., Mackie, A. R., & Dupont, D. (2014). Specificity of Infant Digestive Conditions: Some Clues for Developing Relevant In Vitro Models. Critical Reviews in Food Science and Nutrition, 54(11), 1427–1457. https://doi.org/10.1080/10408398.2011.640757

Bourlieu, C., Ménard, O., De La Chevasnerie, A., Sams, L., Rousseau, F., Madec, M. N., Robert, B., Deglaire, A., Pezennec, S., Bouhallab, S., Carrière, F., & Dupont, D. (2015). The structure of infant formulas impacts their lipolysis,

proteolysis and disintegration during in vitro gastric digestion. Food Chemistry, 182, 224–235. https://doi.org/10.1016/j.foodchem.2015.03.001

Brock, E. G., & Long, L. (2019). Breast feeding. In Obstetrics, Gynaecology and Reproductive Medicine (Vol. 29, Issue 5, pp. 136–140). Churchill Livingstone. https://doi.org/10.1016/j.ogrm.2019.02.003

Chamova, R., Pancheva, R., Dimitrova, T., & Bliznakova, D. (2018). Protective effect of breast milk on urinary tract infection in children aged 0-3 years. Journal of IMAB, 24(1). https://doi.org/10.5272/jimab.2018241.1918

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sørensen, E. S., & Petersen, T. E. (2004). In vitro digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. Trends in Food Science and Technology, 15(7–8), 373–383. https://doi.org/10.1016/j.tifs.2003.12.004

Chowdhury, R., Sinha, B., Sankar, M. J., Taneja, S., Bhandari, N., Rollins, N., Bahl, R., & Martines, J. (2015). Breastfeeding and maternal health outcomes: A systematic review and meta-analysis. In Acta Paediatrica, International Journal of Paediatrics (Vol. 104, Issue Suppl 467, pp. 96–113). Blackwell Publishing Ltd. https://doi.org/10.1111/apa.13102

Dallas, D. C., Guerrero, A., Khaldi, N., Borghese, R., Bhandari, A., Underwood, M. A., Lebrilla, C. B., German, J. B., & Barile, D. (2014). A Peptidomic Analysis of Human Milk Digestion in the Infant Stomach Reveals Protein-Specific Degradation Patterns. The Journal of Nutrition, 144(6), 815–820. https://doi.org/10.3945/jn.113.185793

Dallas, D. C., Guerrero, A., Khaldi, N., Castillo, P. A., Martin, W. F., Smilowitz, J. T., Bevins, C. L., Barile, D., German, J. B., & Lebrilla, C. B. (2013). Extensive in vivo human milk peptidomics reveals specific proteolysis yielding protective antimicrobial peptides. Journal of Proteome Research, 12(5), 2295–2304. https://doi.org/10.1021/pr400212z

Danielewicz, H., Myszczyszyn, G., Dębińska, A., Myszkal, A., Boznański, A., & Hirnle, L. (2017). Diet in pregnancy—more than food. In European Journal of Pediatrics (Vol. 176, Issue 12, pp. 1573–1579). Springer Verlag. https://doi.org/10.1007/s00431-017-3026-5

Dapcich, V., Salvador, G., Ribas, L., Pérez, C., Aranceta, J., & Serra, L. (2004). Guía de la alimentación saludable. Senc, 105.

De Oliveira, S. C., Bellanger, A., Menard, O., Pladys, P., Le Gouar, Y., Dirson, E., Kroell, F., Dupont, D., Deglaire, A., & Bourlieu, C. (2017). Impact of human milk pasteurization on gastric digestion in preterm infants: A randomized controlled trial. American Journal of Clinical Nutrition, 105(2), 379–390. https://doi.org/10.3945/ajcn.116.142539

Deglaire, A., De Oliveira, S. C., Jardin, J., Briard-Bion, V., Emily, M., Ménard, O., Bourlieu, C., & Dupont, D. (2016). Impact of human milk pasteurization on the kinetics of peptide release during in vitro dynamic term newborn digestion. Electrophoresis, 37(13), 1839–1850. https://doi.org/10.1002/elps.201500573

Deglaire, A., Oliveira, S. De, Jardin, J., Briard-Bion, V., Kroell, F., Emily, M., Ménard, O., Bourlieu, C., & Dupont, D. (2019). Impact of human milk pasteurization on the kinetics of peptide release during in vitro dynamic digestion at the preterm newborn stage. Food Chemistry, 281, 294–303. https://doi.org/10.1016/j.foodchem.2018.12.086

Demmelmair, H., & Koletzko, B. (2018). Lipids in human milk. In Best Practice and Research: Clinical Endocrinology and Metabolism (Vol. 32, Issue 1, pp. 57–68). Bailliere Tindall Ltd. https://doi.org/10.1016/j.beem.2017.11.002

Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., Dürr, D., Stoll, P., Vergères, G., & Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST in vitro digestion method to in vivo pig digestion. Food Research International, 102, 567–574. https://doi.org/10.1016/j.foodres.2017.09.047

Elango, R., & Ball, R. O. (2016). Protein and Amino Acid Requirements during Pregnancy. In Advances in nutrition (Bethesda, Md.) (Vol. 7, Issue 4, pp. 839S-844S). Oxford University Press. https://doi.org/10.3945/an.115.011817

Elwakiel, M., Boeren, S., Wang, W., Schols, H. A., & Hettinga, K. A. (2020). Degradation of Proteins From Colostrum and Mature Milk From Chinese Mothers Using an in vitro Infant Digestion Model. Frontiers in Nutrition, 7, 162. https://doi.org/10.3389/fnut.2020.00162

Eriksen, K. G., Christensen, S. H., Lind, M. V., & Michaelsen, K. F. (2018). Human milk composition and infant growth. Current Opinion in Clinical Nutrition and Metabolic Care, 21(3), 200–206. https://doi.org/10.1097/MCO.0000000000000466

FAO. (2001). Human energy requirements. Report os a Joint FAO/WHO/UNU Expert Consultation.

FAO. (2016). Standard for infant formula and formulas for special medical purposes intended for infants. Codex Alimentarius, 72–1981, 1–21. https://doi.org/10.1109/CVPR.2008.4587527

Franke, A. A., Halm, B. M., Custer, L. J., Tatsumura, Y., & Hebshi, S. (2006). Isoflavones in breastfed infants after mothers consume soy. American Journal of Clinical Nutrition, 84(2), 406–413. https://doi.org/10.1093/ajcn/84.1.406

Gan, J., Robinson, R. C., Wang, J., Krishnakumar, N., Manning, C. J., Lor, Y., Breck, M., Barile, D., & German, J. B. (2019). Peptidomic profiling of human milk with LC–MS/MS reveals pH-specific proteolysis of milk proteins. Food Chemistry, 274, 766–774. https://doi.org/10.1016/j.foodchem.2018.09.051

Gao, X., McMahon, R. J., Woo, J. G., Davidson, B. S., Morrow, A. L., & Zhang, Q. (2012). Temporal changes in milk proteomes reveal developing milk functions. Journal of Proteome Research, 11(7), 3897–3907. https://doi.org/10.1021/pr3004002

Garwolińska, D., Namieśnik, J., Kot-Wasik, A., & Hewelt-Belka, W. (2018). Chemistry of Human Breast Milk - A Comprehensive Review of the Composition and Role of Milk Metabolites in Child Development. In Journal of Agricultural and Food Chemistry (Vol. 66, Issue 45, pp. 11881–11896). American Chemical Society. https://doi.org/10.1021/acs.jafc.8b04031

George, A., Gay, M., Trengove, R., Geddes, D., George, A. D., Gay, M. C. L., Trengove, R. D., & Geddes, D. T. (2018). Human Milk Lipidomics: Current Techniques and Methodologies. Nutrients, 10(9), 1169. https://doi.org/10.3390/nu10091169

Gidrewicz, D. A., & Fenton, T. R. (2014). A systematic review and meta-analysis of the nutrient content of preterm and term breast milk. BMC Pediatrics, 14(1), 1–14. https://doi.org/10.1186/1471-2431-14-216

Gila-Díaz, A., Carrillo, G. H., Cañas, S., Pipaón, M. S. de, Martínez-Orgado, J. A., Rodríguez-Rodríguez, P., de Pablo, Á. L. L., Martin-Cabrejas, M. A., Ramiro-Cortijo, D., & Arribas, S. M. (2020). Influence of maternal age and gestational age on breast milk antioxidants during the first month of lactation. Nutrients, 12(9), 1–14. https://doi.org/10.3390/nu12092569

Goldberg, N., Rodriguez-Prado, Y., Tillery, R., & Chua, C. (2018). Sudden infant death syndrome: A review. In Pediatric Annals (Vol. 47, Issue 3, pp. e118–e123). Slack Incorporated. https://doi.org/10.3928/19382359-20180221-03

Grote, V., Verduci, E., Scaglioni, S., Vecchi, F., Contarini, G., Giovannini, M., Koletzko, B., & Agostoni, C. (2016). Breast milk composition and infant nutrient intakes during the first 12 months of life. European Journal of Clinical Nutrition, 70(2), 250–256. https://doi.org/10.1038/ejcn.2015.162

Guo, M. (2014). Human Milk Biochemistry and Infant Formula Manufacturing Technology. https://books.google.es/books?hl=es&lr=&id=SdPoDwAAQBAJ&oi=fnd&pg=PP1&ots=q2lS2CzwbZ&sig=kseXB4AB9Yuy40Q-ieitaXSaldw&redir_esc=y#v=onepage&q&f=false

Hall, B. (1979). Uniformity of human milk. The American Journal of Clinical Nutrition, 32(2), 304–312. https://doi.org/10.1093/ajcn/32.2.304

Hendricks, G. M., & Guo, M. (2014). Bioactive components in human milk. In Human Milk Biochemistry and Infant Formula Manufacturing Technology (pp. 33–54). Elsevier Ltd. https://doi.org/10.1533/9780857099150.1.33

Hernández-Ledesma, B., Quirós, A., Amigo, L., & Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. International Dairy Journal, 17(1), 42–49. https://doi.org/10.1016/j.idairyj.2005.12.012

Hoddinott, P., Tappin, D., & Wright, C. (2008). Breast feeding. In BMJ (Vol. 336, Issue 7649, pp. 881–887). BMJ Publishing Group. https://doi.org/10.1136/bmj.39521.566296.BE

Horta, B. L., Loret De Mola, C., & Victora, C. G. (2015). Long-term consequences of breastfeeding on cholesterol, obesity, systolic blood pressure and type 2 diabetes: A systematic review and meta-analysis. In Acta Paediatrica, International Journal of Paediatrics (Vol. 104, pp. 30–37). Blackwell Publishing Ltd. https://doi.org/10.1111/apa.13133

Horta, B. L., & Victora, C. G. (2013a). Long-term effects of breastfeeding. A Systematic review. www.who.int

Horta, B. L., & Victora, C. G. (2013b). Short-term effects of breastfeeding. A sistematic review on the beneficts of breastfeeding on diarrhoea and pneumonia mortality. www.who.int

Inatal. (2021). El embarazo semana a semana. https://inatal.org/el-embarazo/enciclopedia/71-gestacion.html

INE. (2021). Tipo de lactancia según sexo y comunidad autónoma. Población de 6 meses a 4 años. https://www.ine.es/jaxi/tabla.do?path=/t15/p419/a2006/p07/l0/&file=03111.

px&type=pcaxis&L=0

Ip, S., Chung, M., Raman, G., Chew, P., Magula, N., DeVine, D., Trikalinos, T., & Lau, J. (2007). Breastfeeding and maternal and infant health outcomes in developed countries. In Evidence report/technology assessment (Vol. 18, Issue 153, pp. 1–186). https://doi.org/10.1542/gr.18-2-15

Jakaitis, B. M., & Denning, P. W. (2014). Human Breast Milk and the Gastrointestinal Innate Immune System. In Clinics in Perinatology (Vol. 41, Issue 2, pp. 423–435). W.B. Saunders. https://doi.org/10.1016/j.clp.2014.02.011

Jardí, C., Aparicio, E., Bedmar, C., Aranda, N., Abajo, S., March, G., Basora, J., & Arija, V. (2019). Food consumption during pregnancy and post-partum. ECLIPSES study. Nutrients, 11(10), 2447. https://doi.org/10.3390/nu11102447

Juurlink, B. H. J., Azouz, H. J., Aldalati, A. M. Z., Altinawi, B. M. H., & Ganguly, P. (2014). Hydroxybenzoic acid isomers and the cardiovascular system. In Nutrition Journal (Vol. 13, Issue 1, p. 63). BioMed Central Ltd. https://doi.org/10.1186/1475-2891-13-63

Kahkeshani, N., Farzaei, F., Fotouhi, M., Alavi, S. S., Bahramsoltani, R., Naseri, R., Momtaz, S., Abbasabadi, Z., Rahimi, R., Farzaei, M. H., & Bishayee, A. (2019). Pharmacological effects of gallic acid in health and disease: A mechanistic review. Iranian Journal of Basic Medical Sciences, 22(3), 225–237. https://doi.org/10.22038/ijbms.2019.32806.7897

Kannieappan, L. M., Deussen, A. R., Grivell, R. M., Yelland, L., & Dodd, J. M. (2013). Developing a tool for obtaining maternal skinfold thickness measurements and assessing inter-observer variability among pregnant women who are overweight and obese. BMC Pregnancy and Childbirth, 13(1), 1–6. https://doi.org/10.1186/1471-2393-13-42

Keikha, M., Bahreynian, M., Saleki, M., & Kelishadi, R. (2017). Macro- and

Micronutrients of Human Milk Composition: Are They Related to Maternal Diet? A Comprehensive Systematic Review. Breastfeeding Medicine, 12(9), 517–527. https://doi.org/10.1089/bfm.2017.0048

Khan, S., Hepworth, A. R., Prime, D. K., Lai, C. T., Trengove, N. J., & Hartmann, P. E. (2013). Variation in fat, lactose, and protein composition in breast milk over 24 hours: Associations with infant feeding patterns. Journal of Human Lactation, 29(1), 81–89. https://doi.org/10.1177/0890334412448841

Koletzko, B. (2017). Human Milk Lipids. Annals of Nutrition & Metabolism, 69, 28–40. https://doi.org/10.1159/000452819

Kørvel-Hanquist, A., Djurhuus, B. D., & Homøe, P. (2017). The Effect of Breastfeeding on Childhood Otitis Media. In Current Allergy and Asthma Reports (Vol. 17, Issue 7, p. 45). Current Medicine Group LLC 1. https://doi.org/10.1007/s11882-017-0712-3

Lafay, S., & Gil-Izquierdo, A. (2008). Bioavailability of phenolic acids. In Phytochemistry Reviews (Vol. 7, Issue 2, pp. 301–311). https://doi.org/10.1007/s11101-007-9077-x

Lawrence, R. M. (2005). Host-resistance factors and immunologic significance of human milk. In Breastfeeding (pp. 171–214). Elsevier. https://doi.org/10.1016/b978-0-323-02823-3.50010-3

Leghi, G. E., Netting, M. J., Middleton, P. F., Wlodek, M. E., Geddes, D. T., & Muhlhausler, B. S. (2020). The impact of maternal obesity on human milk macronutrient composition: A systematic review and meta-analysis. Nutrients, 12(4). https://doi.org/10.3390/nu12040934

Li, W., Hosseinian, F. S., Tsopmo, A., Friel, J. K., & Beta, T. (2009). Evaluation of antioxidant capacity and aroma quality of breast milk. Nutrition, 25(1), 105–114. https://doi.org/10.1016/j.nut.2008.07.017

Liao, Y., Alvarado, R., Phinney, B., & Lönnerdal, B. (2011). Proteomic characterization of human milk whey proteins during a twelve-month

lactation period. Journal of Proteome Research, 10(4), 1746–1754. https://doi.org/10.1021/pr101028k

Liao, Y., Weber, D., Xu, W., Durbin-Johnson, B. P., Phinney, B. S., & Lö, B. (2017). Absolute Quantification of Human Milk Caseins and the Whey/Casein Ratio during the First Year of Lactation. https://doi.org/10.1021/acs.jproteome.7b00486

Lindquist, S., & Hernell, O. (2010). Lipid digestion and absorption in early life: an update. Current Opinion in Clinical Nutrition and Metabolic Care, 13(3), 314–320. https://doi.org/10.1097/MCO.0b013e328337bbf0

Llorente-Romero, Z. P., López-Marín, B. E., Deossa-Restrepo, G. C., & Arboleda-Montoya, L. M. (2021). Cross-sectional study to determine the relationship between anthropometric nutritional status of a group of lactating women of Medellin and the fatty acids profile of their mature breast milk. Revista Espanola de Nutricion Humana y Dietetica, 24(4), 292–310. https://doi.org/10.14306/RENHYD.24.4.797

Lodge, C. J., Bowatte, G., Matheson, M. C., & Dharmage, S. C. (2016). The Role of Breastfeeding in Childhood Otitis Media. In Current Allergy and Asthma Reports (Vol. 16, Issue 9, p. 68). Current Medicine Group LLC 1. https://doi.org/10.1007/s11882-016-0647-0

Lönnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. The American Journal of Clinical Nutrition, 77(6), 1537S-1543S. https://doi.org/10.1093/ajcn/77.6.1537S

Lönnerdal, B. (2014). Infant formula and infant nutrition: bioactive proteins of human milk and implications for composition of infant formulas. The American Journal of Clinical Nutrition, 99(3), 712S-717S. https://doi.org/10.3945/ajcn.113.071993

Lönnerdal, B. (2016). Human Milk: Bioactive Proteins/Peptides and Functional Properties. Nestle Nutrition Institute Workshop Series, 86, 97–107. https://doi.org/10.1159/000442729

Lopes, T. I. B., Cañedo, M. C., Oliveira, F. M. P., & Alcantara, G. B. (2018). Toward Precision Nutrition: Commercial Infant Formulas and Human Milk Compared for Stereospecific Distribution of Fatty Acids Using Metabolomics. OMICS: A Journal of Integrative Biology, 22(7), 484–492. https://doi.org/10.1089/omi.2018.0064

Lorenzo, I. S., Tonato, A. M. C., Puentes, A. de la G., Nieto, A., Herrmann, F., Dieguez, E., Castellote, A. I., López-Sabater, M. C., Rodríguez-Palmero, M., & Campoy, C. (2019). The effect of an infant formula supplemented with AA and DHA on fatty acid levels of infants with different FADS genotypes: The COGNIS study. Nutrients, 11(3). https://doi.org/10.3390/nu11030602

Mårild, S., Hansson, S., Jodal, U., Odén, A., & Svedberg, K. (2007). Protective effect of breastfeeding against urinary tract infection. Acta Paediatrica, 93(2), 164–167. https://doi.org/10.1111/j.1651-2227.2004.tb00699.x

Martín-Martínez, B. (2005). Estudio comparativo de la leche de mujer con las leches artificiales. Anales de Pediatria Monografias, 3(1), 43–53.

Martin, C. R., Ling, P. R., & Blackburn, G. L. (2016). Review of infant feeding: Key features of breast milk and infant formula. In Nutrients (Vol. 8, Issue 5). MDPI AG. https://doi.org/10.3390/nu8050279

Martínez-García, R. M., Jiménez-Ortega, A. I., Peral-Suárez, Á., Bermejo-López, L. M., & Rodríguez-Rodríguez, E. (2020). Importance of nutrition during pregnancy. Impact on the composition of breast milk. Nutricion Hospitalaria, 37(Ext2), 38–42. https://doi.org/10.20960/nh.03355

Mazurek, D., & Bronkowska, M. (2020). Maternal anthropometric factors and circulating adipokines as predictors of birth weight and length. International Journal of Environmental Research and Public Health, 17(13), 1–14. https://doi.org/10.3390/ijerph17134799

Mazzocchi, A., D'Oria, V., Cosmi, V., Bettocchi, S., Milani, G., Silano, M., Agostoni, C., Mazzocchi, A., D'Oria, V., Cosmi, V. De, Bettocchi, S., Milani,

G. P., Silano, M., & Agostoni, C. (2018). The Role of Lipids in Human Milk and Infant Formulae. Nutrients, 10(5), 567. https://doi.org/10.3390/nu10050567

Mehrotra, V., Kumar Sehgal, S., & Rajeev Bangale, N. (2019). Fat structure and composition in human milk and infant formulas: Implications in infant health Keywords: Functional constipation Infant feeding Stereospecific positioning of fatty acids Human breast milk Bowel habits in infants. https://doi.org/10.1016/j.cegh.2018.03.005

Ménard, O., Bourlieu, C., De Oliveira, S. C., Dellarosa, N., Laghi, L., Carrière, F., Capozzi, F., Dupont, D., & Deglaire, A. (2018). A first step towards a consensus static in vitro model for simulating full-term infant digestion. Food Chemistry, 240, 338–345. https://doi.org/10.1016/j.foodchem.2017.07.145

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. Food Chemistry, 145, 1039–1045. https://doi.org/10.1016/j.foodchem.2013.09.036

Miles, E. A., & Calder, P. C. (2017). The influence of the position of palmitate in infant formula triacylglycerols on health outcomes. In Nutrition Research (Vol. 44, pp. 1–8). Elsevier Inc. https://doi.org/10.1016/j.nutres.2017.05.009

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., MacIerzanka, A., MacKie, A., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. Food and Function, 5(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Minkiewicz, P., Iwaniak, A., & Darewicz, M. (2019). BIOPEP-UWM database of bioactive peptides: Current opportunities. International Journal of Molecular Sciences, 20(23). https://doi.org/10.3390/ijms20235978

Miralles, B., Sanchón, J., Sánchez-Rivera, L., Martínez-Maqueda, D., Le Gouar,

Y., Dupont, D., Amigo, L., & Recio, I. (2021). Digestion of micellar casein in duodenum cannulated pigs. Correlation between in vitro simulated gastric digestion and in vivo data. Food Chemistry, 343, 128424. https://doi.org/10.1016/j.foodchem.2020.128424

Mlenard, D., Monfils, S., & Tremblay, E. (1995). Ontogeny of Human Gastric Lipase and Pepsin Activities. In GASTROENTEROLOGY (Vol. 108).

Mohanty, D. P., Mohapatra, S., Misra, S., & Sahu, P. S. (2016). Milk derived bioactive peptides and their impact on human health – A review. In Saudi Journal of Biological Sciences (Vol. 23, Issue 5, pp. 577–583). Elsevier B.V. https://doi.org/10.1016/j.sjbs.2015.06.005

Mosca, F., & Giannì, M. L. (2017). Human milk: composition and health benefits. In La Pediatria medica e chirurgica : Medical and surgical pediatrics (Vol. 39, Issue 2, p. 155). https://doi.org/10.4081/pmc.2017.155

Motee, A., & Jeewon, R. (2014). Importance of exclusive breast feeding and complementary feeding among infants. Current Research in Nutrition and Food Science, 2(2), 56–72. https://doi.org/10.12944/CRNFSJ.2.2.02

Mousa, A., Naqash, A., & Lim, S. (2019). Macronutrient and micronutrient intake during pregnancy: An overview of recent evidence. In Nutrients (Vol. 11, Issue 2, p. 443). MDPI AG. https://doi.org/10.3390/nu11020443

Naveed, M., Hejazi, V., Abbas, M., Kamboh, A. A., Khan, G. J., Shumzaid, M., Ahmad, F., Babazadeh, D., FangFang, X., Modarresi-Ghazani, F., WenHua, L., & XiaoHui, Z. (2018). Chlorogenic acid (CGA): A pharmacological review and call for further research. Biomedicine and Pharmacotherapy, 97(August 2017), 67–74. https://doi.org/10.1016/j.biopha.2017.10.064

Nielsen, S. D., Beverly, R. L., & Dallas, D. C. (2017). Milk Proteins Are Predigested Within the Human Mammary Gland. Journal of Mammary Gland Biology and Neoplasia, 22(4), 251–261. https://doi.org/10.1007/s10911-018-9388-0

Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. Food Chemistry, 232, 673–682. https://doi.org/10.1016/j.foodchem.2017.04.056

Nolan, L. S., Parks, O. B., & Good, M. (2020). A review of the immunomodulating components of maternal breast milk and protection against necrotizing enterocolitis. In Nutrients (Vol. 12, Issue 1, p. 14). MDPI AG. https://doi.org/10.3390/nu12010014

Orozco-Muñoz, C., Sarasa-Muñoz, N. L., Hernández-Díaz, D., Cañizares-Luna, O., Álvarez-Guerra González, E., & Artiles-Santana, A. (2009). Anthropometric indicators for the characterization of body adiposity in healthy pregnant women at the beginning of pregnancy. Cuban Society of Cardiology, 10(4), 274–285.

Peres, K. G., Chaffee, B. W., Feldens, C. A., Flores-Mir, C., Moynihan, P., & Rugg-Gunn, A. (2018). Breastfeeding and Oral Health: Evidence and Methodological Challenges. Journal of Dental Research, 97(3), 251–258. https://doi.org/10.1177/0022034517738925

Pietrzak-Fiećko, R., & Kamelska-Sadowska, A. M. (2020). The comparison of nutritional value of human milk with other mammals' milk. Nutrients, 12(5). https://doi.org/10.3390/nu12051404

Prentice, A. (1985). Regional Variations in the Composition of Human Milk. In: Jensen RG, editor. Handbook of Milk Composition. Academic Press, Inc.; San Diego, CA (p. 919).

Raikos, V., & Dassios, T. (2014). Health-promoting properties of bioactive peptides derived from milk proteins in infant food: A review. In Dairy Science and Technology (Vol. 94, Issue 2, pp. 91–101). Springer. https://doi.org/10.1007/s13594-013-0152-3

Ramiro-Cortijo, D., Singh, P., Liu, Y., Medina-Morales, E., Yakah, W., Freedman, S. D., & Martin, C. R. (2020). Breast milk lipids and fatty acids in regulating neonatal intestinal development and protecting against intestinal injury. In Nutrients (Vol. 12, Issue 2, p. 534). MDPI AG. https://doi.org/10.3390/nu12020534

Saarela, T., Kokkonen, J., & Koivisto, M. (2007). Macronutrient and energy contents of human milk fractions during the first six months of lactation. Acta Paediatrica, 94(9), 1176–1181. https://doi.org/10.1111/j.1651-2227.2005.tb02070.x

Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. Food Chemistry, 239, 486–494. https://doi.org/10.1016/j.foodchem.2017.06.134

Schuindt da Silva, V., & Soares-Vieira, M. F. (2020). International society for the advancement of kinanthropometry (Isak) global: International accreditation scheme of the competent anthropometrist. Revista Brasileira de Cineantropometria e Desempenho Humano, 22, 1–6. https://doi.org/10.1590/1980-0037.2020v22e70517

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. Journal of Functional Foods, 18, 820–897. https://doi.org/10.1016/j.jff.2015.06.018

Shani-Levi, C., Alvito, P., Andrés, A., Assunção, R., Barberá, R., Blanquet-Diot, S., Bourlieu, C., Brodkorb, A., Cilla, A., Deglaire, A., Denis, S., Dupont, D., Heredia, A., Karakaya, S., Giosafatto, C. V. L., Mariniello, L., Martins, C., Ménard, O., El, S. N., … Lesmes, U. (2017). Extending in vitro digestion models to specific human populations: Perspectives, practical tools and bio-relevant information. In Trends in Food Science and Technology (Vol. 60, pp.

52–63). Elsevier Ltd. https://doi.org/10.1016/j.tifs.2016.10.017

Shingirai, M., Katsinde, S., & Chandrasekhar, S. (2016). Breast feeding and The Sustainable Development agenda. Indian Journal of Pharmacy Practice, 9(3). https://doi.org/10.5530/ijopp.9.3.2

Siziba, L. P., Lorenz, L., Brenner, H., Carr, P., Stahl, B., Mank, M., Marosvölgyi, T., Decsi, T., Szabo, E., Rothenbacher, D., & Genuneit, J. (2020). Changes in human milk fatty acid composition and maternal lifestyle-related factors over a decade: A comparison between the two Ulm Birth Cohort Studies. British Journal of Nutrition, 1–8. https://doi.org/10.1017/S0007114520004006

Siziba, L. P., Lorenz, L., Stahl, B., Mank, M., Marosvölgyi, T., Decsi, T., Rothenbacher, D., & Genuneit, J. (2019). Changes in human milk fatty acid composition during lactation: The ulm SPATZ health study. Nutrients, 11(12). https://doi.org/10.3390/nu11122842

Song, B. J., Jouni, Z. E., & Ferruzzi, M. G. (2013). Assessment of phytochemical content in human milk during different stages of lactation. Nutrition, 29(1), 195–202. https://doi.org/10.1016/j.nut.2012.07.015

Sova, M., & Saso, L. (2020). Natural sources, pharmacokinetics, biological activities and health benefits of hydroxycinnamic acids and their metabolites. Nutrients, 12(8), 1–30. https://doi.org/10.3390/nu12082190

Stam, J., Sauer, P. J., & Boehm, G. (2013). Can we define an infant's need from the composition of human milk? 1-3. The American Journal of Clinical Nutrition, 98(suppl), 521S-8S. https://doi.org/10.3945/ajcn.112.044370

Su, M. Y., Broadhurst, M., Liu, C. P., Gathercole, J., Cheng, W. L., Qi, X. Y., Clerens, S., Dyer, J. M., Day, L., & Haigh, B. (2017). Comparative analysis of human milk and infant formula derived peptides following in vitro digestion. Food Chemistry, 221, 1895–1903. https://doi.org/10.1016/j.foodchem.2016.10.041

Taamalli, A., Contreras, M. D. M., Abu-Reidah, I. M., Trabelsi, N., & Ben

Youssef, N. (2019). Quality of phenolic compounds: Occurrence, health benefits, and applications in food industry. In Journal of Food Quality (Vol. 2019). https://doi.org/10.1155/2019/9594646

Tarko, T., Duda-Chodak, A., & Zajac, N. (2013). Digestion and absorption of phenolic compounds assessed by in vitro simulation methods. A review. Roczniki Państwowego Zakładu Higieny, 64(2), 79–84.

Thompson, J. M. D., Tanabe, K., Moon, R. Y., Mitchell, E. A., Mcgarvey, C., Tappin, D., Blair, P. S., & Hauck, F. R. (2017). Duration of Breastfeeding and Risk of SIDS: An Individual Participant Data Meta-analysis. Pediatrics, 140(5). https://doi.org/10.1542/peds.2017-1324

Tsopmo, A. (2018). Phytochemicals in human milk and their potential antioxidative protection. In Antioxidants (Vol. 7, Issue 2). MDPI AG. https://doi.org/10.3390/antiox7020032

UNICEF. (2017). Breastfeeding. A mother's gift,for every child.

Vázquez, C. V., Rojas, M. G. V., Ramírez, C. A., Chávez-Servín, J. L., García-Gasca, T., Ferriz Martínez, R. A., García, O. P., Rosado, J. L., López-Sabater, C. M., Castellote, A. I., Montemayor, H. M. A., & De La Torre Carbot, K. (2015). Total phenolic compounds in milk from different species. Design of an extraction technique for quantification using the Folin-Ciocalteu method. Food Chemistry, 176, 480–486. https://doi.org/10.1016/j.foodchem.2014.12.050

Victora, C. G., Bahl, R., Barros, A. J. D., França, G. V. A., Horton, S., Krasevec, J., Murch, S., Sankar, M. J., Walker, N., Rollins, N. C., Allen, K., Dharmage, S., Lodge, C., Peres, K. G., Bhandari, N., Chowdhury, R., Sinha, B., Taneja, S., Giugliani, E., … Richter, L. (2016). Breastfeeding in the 21st century: Epidemiology, mechanisms, and lifelong effect. In The Lancet (Vol. 387, Issue 10017, pp. 475–490). Lancet Publishing Group. https://doi.org/10.1016/S0140-6736(15)01024-7

Vida-Candel, R., Sanchis-Valero, S., Mateu-Ciscar, C., Bellvis-Vázquez, E., Planells-López, E., Martínez-Ballester, A., Gómez-Sánchez, M. J., & Espuig-Sebastian, R. (2016). What is the best anthropometric indicator for the pregnancy control? Nutrición Clínica y Dietética Hospitalaria, 36(3), 87–96. https://doi.org/10.12873/363vilacandel

Wada, Y., & Lönnerdal, B. (2015). Bioactive peptides released from in vitro digestion of human milk with or without pasteurization. Pediatric Research, 77(4), 546–553. https://doi.org/10.1038/pr.2015.10

Wada, Y., & Lönnerdal, B. (2020). Bioactive peptides derived from human milk proteins: An update. Current Opinion in Clinical Nutrition and Metabolic Care, 23(3), 217–222. https://doi.org/10.1097/MCO.0000000000000642

Wada, Y., Phinney, B. S., Weber, D., & Lönnerdal, B. (2017). In vivo digestomics of milk proteins in human milk and infant formula using a suckling rat pup model. Peptides, 88, 18–31. https://doi.org/10.1016/j.peptides.2016.11.012

Walker, A. (2010). Breast Milk as the Gold Standard for Protective Nutrients. Journal of Pediatrics, 156(2). https://doi.org/10.1016/j.jpeds.2009.11.021

WHO. (2002). Infant and young child nutrition Global strategy on infant and young child feeding.

WHO. (2003). Estrategia Mundial para la Alimentación del Lactante y del Niño Pequeño Organización Mundial de la Salud Genebra.

WHO. (2017). Protecting, promoting and supporting breastfeeding in facilities providing maternity and newborn services. http://apps.who.int/bookorders.

Widen, E. M., & Gallagher, D. (2014). Body composition changes in pregnancy: Measurement, predictors and outcomes. In European Journal of Clinical Nutrition (Vol. 68, Issue 6, pp. 643–652). Nature Publishing Group. https://doi.org/10.1038/ejcn.2014.40

Zhu, J., & Dingess, K. A. (2019). The functional power of the human milk

proteome. In Nutrients (Vol. 11, Issue 8). MDPI AG. https://doi.org/10.3390/nu11081834

Živković, J., Sunarić, S., Trutić, N., Denić, M., Kocić, G., Jovanović, T., Facultatis Medicae Naissensis, A., & M A Ry, S. M. (2015). Antioxidants and antioxidant capacity of human milk. Acta Facultatis Medicae Naissensis, 32(2), 115–125. https://doi.org/10.1515/afmnai-2015-0012

Zou, L., Pande, G., & Akoh, C. C. (2016). Infant Formula Fat Analogs and Human Milk Fat: New Focus on Infant Developmental Needs. Annual Review of Food Science and Technology, 7(1), 139–165. https://doi.org/10.1146/annurev-food-041715-033120