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Escuela de Doctorado

TESIS DOCTORAL

Nuevos productos cárnicos saludables: carnes funcionales con propiedades antioxidantes e inmunomoduladoras

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Resumen

A lo largo de los años, estrechamente vinculada a la cada vez más elevada disponibilidad y variedad de alimentos de origen animal, procesados o naturales, el conocimiento sobre las repercusiones del consumo de carne ha ido en aumento. El consumo de carne en 2022 en España se sitúa en los 27.6Kg por persona al año. Respondiendo a esta tendencia, la presente tesis se enfoca en la reformulación de productos cárnicos con el fin de mejorar su perfil saludable. En particular, se investigan las propiedades antioxidantes y antiinflamatorias de los polifenoles extraídos de fuentes naturales y su impacto sobre la salud cuando se incorporan en productos cárnicos procesados. El consumo de carne procesada se ha asociado con un incremento del riesgo de enfermedades crónicas como el cáncer colorrectal y las enfermedades cardiovasculares debido a su contenido en grasas saturadas, sodio y compuestos formados durante su procesamiento. Para mitigar estos efectos negativos, la tesis propone la adición de compuestos fenólicos de origen vegetal, que se ha demostrado en estudios in vitro que tienen la capacidad de reducir la inflamación y el estrés oxidativo.

El proyecto incluye una revisión exhaustiva de las fuentes naturales de polifenoles como el romero, el té verde y la semilla de uva, destacando su eficacia en la prevención de la inflamación y el estrés oxidativo en líneas celulares de macrófagos (RAW 264.7) y colonocitos (Caco-2). Las etapas experimentales se llevaron a cabo evaluando primero el efecto de estos compuestos en cultivos celulares, y posteriormente en un modelo animal de obesidad inducida, donde se observó una mejora en la respuesta inflamatoria y una disminución del estrés oxidativo.

Los resultados del estudio sugieren que la reformulación de productos cárnicos mediante la adición de extractos naturales ricos en polifenoles puede ofrecer una estrategia viable para la mejora de la salud pública, al reducir el impacto negativo del consumo de carne procesada. El uso de polifenoles no solo contribuye a la reducción de biomarcadores inflamatorios y antioxidantes, sino que también mejora la calidad nutricional general del producto cárnico.

Adicionalmente, se destaca que los compuestos fenólicos no solo tienen un impacto directo sobre la salud antioxidante e inflamatoria, sino que también modulan diversas rutas metabólicas que están involucradas en enfermedades crónicas. Por ejemplo, los estudios en células y en animales revelaron que los extractos de plantas seleccionados pueden reducir la señalización de citoquinas proinflamatorias como el TNF-α, IL-6 y IL-1, que son clave en la patogénesis del cáncer y las enfermedades cardiovasculares.

El modelo experimental usado para evaluar los productos cárnicos reformulados incluyó un análisis detallado del tejido adiposo, hígado y bazo, donde se midieron cambios en los niveles de inflamación sistémica y local. Los resultados mostraron que la adición de compuestos fenólicos en la dieta contribuyó significativamente a la reducción del estrés oxidativo y a la mejora de los parámetros inflamatorios en estos tejidos. Estos hallazgos son alentadores para el desarrollo de nuevos alimentos funcionales que no solo satisfacen las necesidades nutricionales de la población, sino que también proporcionan beneficios para la salud a largo plazo.

Finalmente, la presente tesis discute el potencial de implementar estos cambios a nivel industrial, proponiendo estrategias para integrar estas reformulaciones en la producción en masa de alimentos cárnicos. Se exploran tanto los desafíos como las oportunidades que presenta la industria alimentaria para adoptar estas innovaciones, destacando la importancia de un enfoque multidisciplinario que combine la tecnología de alimentos con los avances en nutrición y salud pública.

OBJETIVOS

Objetivos Generales:

Evaluar la viabilidad de desarrollar productos cárnicos procesados que promuevan la salud mediante la incorporación de extractos naturales con propiedades antioxidantes y antiinflamatorias.

Evaluar los efectos de la reformulación de productos cárnicos sobre parámetros relacionados con la inflamación y el estrés oxidativo, a través de modelos celulares y animales.

Proponer alternativas viables y saludables a la industria alimentaria que reduzcan los riesgos asociados al consumo de carne procesada.

Objetivos Específicos:

Identificar fuentes naturales ricas en compuestos fenólicos, como el romero, el té verde y la semilla de uva, que posean capacidades antioxidantes y antiinflamatorias.

Desarrollar un proceso de reformulación de productos cárnicos que incorpore extractos fenólicos y evaluar su estabilidad durante el procesamiento y almacenamiento.

Evaluar in vitro los efectos antioxidantes e inmunomoduladores de los productos reformulados en macrófagos RAW 264.7 y colonocitos Caco-2, midiendo la producción de especies reactivas de oxígeno y citoquinas proinflamatorias.

Analizar en un modelo animal de obesidad inducida los efectos de los productos cárnicos reformulados en los parámetros de salud relacionados con la inflamación sistémica y el estrés oxidativo.

Proporcionar recomendaciones para la implementación de estos productos en la producción industrial de alimentos, asegurando que sean viables y efectivos desde el punto de vista nutricional y comercial.

INTRODUCCIÓN

Salud oxidativa e inflamatoria: conceptos generales

Concepto y formación de radicales libres y especies reactivas del oxígeno

Los organismos aerobios multicelulares complejos son capaces de oxidar biomoléculas ricas en carbono e hidrógeno gracias al oxígeno. Este proceso permite que la energía presente en los enlaces de estas biomoléculas pase a la unidad energética fisiológica conocida como ATP.

Los combustibles biológicos sufren una reducción escalonada permitiendo una liberación controlada de energía. No obstante, el oxígeno también puede ser reducido de forma incompleta: esto da lugar a compuestos con un desapareamiento en los espines electrónicos de la última capa molecular que son los llamados radicales libres (RL).

Estos radicales se agrupan función del átomo al que pertenece el electrón desapareado, pudiendo ser de oxígeno, carbono, azufre o nitrógeno, lo cual dependen del proceso por el que se hayan generado y esto condiciona que sean más o menos reactivos. Por ejemplo, se pueden generar mediante la adición de un electrón como ocurre con el hidroxilo formado a partir del peróxido de hidrógeno, mediante la pérdida de un protón como sucede en los ácidos grasos insaturados o los grupos tioles derivados de proteínas o con la rotura homolítica de un enlace covalente. La forma de destruir estos radicales consiste en reaccionar con otro radical o en reaccionar con moléculas no radicales que, aunque pasan a ser radicales son menos reactivas y acaban estabilizándose.

Por otro lado, tenemos las especies reactivas que pueden ser radicales libres o no, en este caso pueden dar lugar a ellos y son las denominadas especies reactivas del oxígeno (ROS).

Estos radicales libres y las especies reactivas del oxígeno se relacionan con la patogénesis de muchas enfermedades crónicas degenerativas siendo la mayoría patologías típicas del estado inflamatorio, del propio proceso de

envejecimiento, de diversos tipos de cáncer y de algunas enfermedades de tipo cardiovascular, Alzheimer, diabetes…

Para hacer frente a estos compuestos, el organismo dispone de sistemas de defensa antioxidante que intentan neutralizar los factores prooxidantes. Cuando estos sistemas no palian la totalidad de la agresión oxidativa es cuando se está en un estado de estrés oxidativo.

Sustratos biológicos de los radicales libres y especies reactivas

Los radicales libres y las especies reactivas pueden interactuar y modificar distintas moléculas biológicas siendo estas las bases de su patogenicidad. Ejemplos son las reacciones con:

- Hidratos de carbono: el hidroxilo es capaz de producir radicales de azúcar perdiendo estos un hidrógeno y derivando en el caso de la desoxirribosa de la rotura del ADN.
- Mucopolisacáridos: la presencia de radicales y ROS en el tejido sinovial degradan mucopolisacáridos como el ácido hialurónico propiciando la artritis.
- Aminoácidos alterando su papel fisiológico.
- Colesterol formando hidroperóxidos implicados en la aterosclerosis y en la enfermedad cardiovascular.
- Proteínas, alterando la función estructural de enzimas o la arquitectura celular de proteínas con dicha función. La reactividad de la proteína depende de la presencia de histidina, metionina, cisteína, triptófano y tirosina y se favorece por la presencia de iones metálicos derivando en pérdidas de la actividad enzimática, aumento de la susceptibilidad a las proteasas y cambios en la inmunogenicidad.
- Ácidos nucleicos: principalmente reaccionan tanto con las bases purínicas o pirimidínicas como las pentosas causando daños y roturas en el ADN.
- Ácidos grasos insaturados: son especialmente sensibles y la cadena de reacciones desencadenada por los radicales libres altera la membrana celular ya que estos se encuentra en los fosfolípidos. Dicho proceso

precede a numerosas enfermedades ya que la reacción sucede en cadena cuando un radical tiene suficiente energía para abstraer un átomo de hidrógeno del carbono metilénico alílico de un PUFA.

Antioxidantes como método de defensa de estos sustratos biológicos

Un antioxidante es cualquier sustancia que cuando está presente a bajas concentraciones en presencia de un sustrato oxidable, retrasa o previene la oxidación del mismo.

Los antioxidantes se pueden clasificar fisiológicamente en función de su acción:

- Antioxidantes de prevención que impiden la formación de radicales libres como la catalasa, la transferrina o la superóxidodismutasa.
- Antioxidantes eliminadores de radicales que inhiben el inicio de la cadena de oxidación evitando que se propague como los carotenoides y las vitaminas A y E.
- Enzimas de reparación y de novo o antioxidantes secundarios que reparan los daños oxidativos como las proteasas o las enzimas de reparación del ADN.

Relación entre los radicales libres y la inflamación

La inflamación representa una respuesta del organismo a una serie de estímulos mecánicos, químicos o infecciosos. Su misión es aislar, destruir o diluir conformando una protección local.

Dependiendo de la regulación humoral y la respuesta celular podemos estar frente a una inflamación crónica o aguda y las moléculas encargas de controlar estas respuestas son muy numerosas.

Durante esta inflamación los leucocitos polimorfonucleares, los monocitos y los macrófagos se activan y llevan a cabo el proceso de fagocitosis a través de un mecanismo que consume entre veinte y treinta veces más oxígeno que antes de la activación. Esto aumenta la producción

de NADPH a través de la ruta de las hexosas fosfato, incrementando la génesis de anión superóxido, radical hidroxilo, peróxido de hidrógeno y ácido hipocloroso, especies capaces de dañar la membrana celular y las biomoléculas asociadas a ella pero que cumple la misión e defensa del organismo destruyendo oxidativamente al agresor.

Si esta inflamación es de carácter crónico, el organismo se verá sometido de forma constante a estos factores y el balance de oxidación será superior encontrándonos ante una situación de estrés oxidativo.

Por ello las formas efectivas de intervenir en la patogénesis oxidativa son el aporte a través de la alimentación de antioxidantes naturales, de nutrientes precursores de defensas antioxidantes endógenas y sustancias con efecto antiinflamatorio.

Conceptos generales sobre la inflamación sistémica

La inflamación representa una respuesta biológica del organismo a una serie de factores mecánicos y químicos, o estímulos infecciosos. Su misión es aislar, destruir o diluir, como forma de protección localizada.

La inflamación puede ser crónica o aguda, dependiendo de las características de la respuesta humoral y de las moléculas involucradas. Cuando se altera el equilibrio inflamatorio con excesivas señales pro-inflamatorias (por ejemplo, en la vía de la ciclooxigenasa), pueden producirse daños fisiológicos (Wang et al. 2007).

La inflamación crónica es un estado derivado de situaciones fisiopatógenas como el síndrome metabólico derivado de la obesidad o enfermedades inflamatorias del intestino que implican una exposición prolongada a una serie de posibles sustancias patógenas.

Esas sustancias son principalmente mediadores inflamatorios como el factor de necrosis tumoral alfa (TNF-α), y están vinculados a la iniciación del cáncer (Balkwill 2006). La combinación de estos factores conduce a una desequilibrio del estado inflamatorio con un incremento de marcadores como las citoquinas inflamatorias, incluyendo TNF-α, interleucina (IL)-6, e IL-1, que también se asocian con enfermedades cardiometabólicas (Minihane et al., 2015).

Una forma potencial de prevenir la inflamación que puede derivar en la carcinogénesis o la enfermedad cardiovascular es a través del uso de extractos vegetales de especias, hierbas y alimentos que muestran tanto propiedades antioxidantes como antiinflamatorias. Por esta razón, los fitoquímicos antiinflamatorios podrían representar una ayuda exógena crucial para la prevención de enfermedades crónicas mediadas por procesos inflamatorios.

Nutrientes y salud oxidativa

Balance de factores oxidantes y la alimentación

La alimentación es de gran importancia para este balance oxidativo ya que de ella depende que la situación global del organismo se encuentre en un estado de estrés oxidativo o por el contrario que el organismo sea capaz de disponer de una eficaz defensa contra los procesos oxidativos.

En este balance oxidativo se encuentran factores oxidantes alimentarios, que pueden ser sustancias prooxidantes o que se oxidan fácilmente, como el Fe2+ y los ácidos grasos poliinsaturados, y factores oxidantes no nutricionales como los radicales libres o las especies reactivas de oxígeno anteriormente descritas. Mientras tanto, en la otra mano tenemos antioxidantes o componentes de difícil oxidación como son las vitaminas A, C y E, los B-carotenos, los ácidos grasos monoinsaturados y los compuestos fenólicos.

Sistemas antioxidantes relevantes

Vitamina E

Su principal función es inhibir la peroxidación lipídica por inactivación de los radicales peroxilo más velozmente de lo que éstos propagan los radicales a los ácidos grasos o proteínas adyacentes. En el proceso, el radical peroxilo se convierte en un peróxido lipídico y el α-tocoferol en un radical α-tocoferoxilo, el cual se regenera de nuevo a α-tocoferol por la acción del ubiquinol pero sobre todo de la vitamina C.

Como contrapartida, un exceso de vitamina E puede ser prooxidante por facilitar la trasferencia de la reacción de radicales en las lipoproteínas LDL.

Vitamina C

Aparte de ejercer diversos efectos fisiológicos, es el único antioxidante plasmático que puede proteger completamente del daño peroxidativo inducido por los radicales peroxilos acuosos y por los oxidantes liberados por los neutrófilos activados anteriormente citados.

Polifenoles

Se dividen en ácidos hidroxibenzoicos, ácidos hidroxicinámicos, estilbenos, lignanos y, el tipo más común en los vegetales, los flavonoides, responsables de parte del color y del sabor de los vegetales. Los polifenoles además se subdividen en antocianinas, flavonas, flavonoles, flavanonas, flavanoles e isoflavonas.

El principal problema de estos compuestos es la biodisponibilidad y aun así los estudios epidemiológicos destacan una asociación entre el consumo de polifenoles y la reducción del riesgo de padecer enfermedades cardiovasculares, tumores y alteraciones neurodegenerativas debido a la capacidad de estos de combatir el estrés oxidativo.

Capacidad antioxidante

Su poder antioxidante depende del número de anillos fenólicos y del número y posición de los grupos hidroxílicos y dobles enlaces. Estas mismas diferencias determinan su biodisponibilidad, tanto en la absorción a nivel gastrointestinal como en el metabolismo y la capacidad de distribución en tejidos y órganos.

Los polifenoles pueden actuar como antioxidantes primarios o secundarios, interrumpiendo las reacciones de los radicales libres gracias a la capacidad de ceder un electrón al radical peroxilo de los ácidos grasos. También retardan la reacción de iniciación de los radicales a través de la

quelación de los iones de los metales de transición (mayoritariamente hierro y cobre).

También ejercen una actividad indirecta ya que la mayoría son metabolizados in vivo dando lugar a sustancias que han perdido su poder antioxidante. La reducida cantidad que se puede encontrar en el organismo puede tener, no obstante, actividad farmacológica modulando ciertas funciones: regulan la expresión y actividad de enzimas tales como telomerasa, ciclooxigenasa, lipooxigenasa, xantina oxidasa, metaloproteinasa, protein-kinasa, inducen enzimas detoxificantes y regulan el ciclo celular.

Todos estos ámbitos en los que intervienen los flavonoides les confieren propiedades beneficiosas frente a enfermedades crónicas y degenerativas, entre las que cabe destacar su acción sobre la enfermedad cardiovascular debido a su impacto favorable sobre el endotelio vascular lo cual es esencial para la regulación del tono vasomotor y sobre el cáncer.

Acción de los polifenoles sobre el cáncer

Las especies reactivas del nitrógeno, así como el anión superóxido, el peróxido de hidrógeno, el radical hidroxilo y el óxido nitroso desempeñan un papel en la carcinogénesis al dañar el ADN. Los compuestos fenólicos intervienen en distintos mecanismos como inducción de enzimas detoxificantes de fase II previniendo la formación de los tumores, inhibición de las actividades AP-1 relacionada con la diferenciación, proliferación y apoptosis celular y activación de las quinasas MAPK. Además de estos mecanismos, los compuestos fenólicos actuando como antioxidantes previniendo el ataque de los radicales libres sobre el ADN protegiéndolo contra mutaciones que den lugar a procesos cancerígenos.

Los flavonoides y, particularmente, la quercetina, tienen una importante función de defensa de los coloncitos frente al ataque oxidativo, habiéndose visto que células de cáncer de colon HT29 cultivadas con antocianidinas se produce una reducción del nivel de ruptura de ADN.

Contexto nutricional de la carne y los productos cárnicos

La disponibilidad del consumo de carne por la domesticación animal y la capacidad de cocinarla ha supuesto a la humanidad un incremento en la toma de proteínas de alta calidad permitiendo su desarrollo biológico. Además, la carne es sin duda un alimento denso en nutrientes, no sólo de proteínas sino también Hierro (Fe), cinc (Zn), Selenio (Se), vitamina B12, niacina y grasa (Farran A. et al 2004)

No obstante, pese a estos aspectos positivos y que la carne supone un alimento más nutricionalmente denso que los de origen vegetal y que es consumido regularmente por la mayoría de población, cada vez hay más estudios observacionales que asocian el consumo de carne roja o procesada con enfermedades crónicas tales como diabetes, enfermedad cardiovascular y diversos tipos de cáncer. Esto ha dado lugar a recomendaciones basadas en la limitación o restricción del consumo de estos productos avaladas por el Superior Health Council (2013) y la World Cancer Research Fund (2012). Sin embargo, algunos autores recomiendan no aplicar restricciones severas a partir de cierta edad debido a los requerimientos de proteínas que tienen las personas mayores (Daly RM et al. 2014, Federal Commission for Nutrition 2011)

Estas limitaciones se basan en distintos metaanálisis dónde se aprecia el incremento de la mortalidad por enfermedad cardiovascular por un consumo superior a 84g de carne procesada por día (Pan A. et al 2012), en la incidencia de cáncer colorrectal por el consumo de 50g/día (Chan DSM et al. 2011) o aumento del riesgo de diabetes tipo 2 por 50g/día (Pan A. et al. 2013).

Una de las mayores asociaciones referentes al consumo de carne roja es con la incidencia de cáncer de colon. Se han tratado de describir los procesos mecanísticos que dan lugar a este, entre otros:

- Las grasas aumentan la liberación de ácidos biliares que actúan como surfactantes para la mucosa (Bruce 1987) y las grasas en exceso aumentan la resistencia a la insulina, dejando circular en

sangre factores que promueven el crecimiento tumoral (Calle & Kaaks, 2004). No obstante estudios posteriores con intervenciones con dietas bajas en grasa quitaron este factor como determinante (Beresford et al., 2006)

- El exceso de proteínas puede ser fermentado formando aminas y otros compuestos toxicos (Visek 1991) aunque estudios in vivo han determinado que no tiene efecto carcinogénico en roedores (Corpet et al., 1995)
- El hierro puede contribuir a la formación de radicales libres (Nelson 2001) aunque depende del grado de oxidación en el que se encuentra (Ilsley ey al., 2004).
- Y los compuestos nitrosados conformar nitrosaminas cancerígenas (Bingham et al. 1996)

En su revisión, Danis E. Corpet (2011) edita una tabla original de Bastide (2011) donde sintetiza las vías por las que la carne puede ser un factor de riesgo nutricional y lanza una propuesta para hacer la carne más saludable a través de suplementación con calcio, vitaminas, minerales y polifenoles entre otros.

Estrategias de mejora de salud en los productos cárnicos.

Una vez conocemos los mecanismos de la patogenicidad de la carne y las condiciones fisiológicas en las que se integra ésta, podemos deducir qué vías de intervención nos permiten obtener productos cárnicos más saludables. Las tendencias actuales de relación carne-enfermedad están focalizadas en asociaciones directas e indirectas con enfermedades cardiovasculares y distintos tipos de cáncer. Por ello, un objetivo de salud preferente debe ser intervenir en los mecanismos que dan lugar a estas enfermedades.

Una de las estrategias tradicionales es modificar los perfiles nutricionales reduciendo la cantidad de sustancias que puedan resultar nocivas. La Comisión Europea en su comunicado "Working document on the setting of Nutrient Profiles" de 2009 estableció unos límites teóricos de los

valores de sodio (700mg/100g) y grasas saturadas (5g/100g). Además, existen tendencias orientadas a reducir el valor calórico total o modificar aditivos artificiales por naturales ya que estos pueden ser parte de la causa de la patogénesis.

Junto con esta estrategia tradicional "sin", encontramos otra tendencia "con" a la adición de vitaminas C y E principalmente que no sólo tienen propiedades antioxidantes in vivo sino que intervienen en la nitrosilación previniendo la formación de nitrosotioles y nitrosilos que promueven mutaciones en ADN cortando una de las principales vías patogénicas.

Esta estrategia "con" está llevada a cabo a través de micronutrientes de reconocida eficacia, sin embargo, para obtener una respuesta más efectiva debemos recurrir a los compuestos fenólicos, de los que cada vez se saben más propiedades beneficiosas que pueden ser de gran utilidad para intervenir en estos procesos.

Fuentes relevantes de compuestos fenólicos

Algunas de las fuentes de compuestos fenólicos más relevantes presentes en la literatura con propiedades tanto antioxidantes como preventivas frente a la inflamación son los siguientes:

Olea europaea

El olivo y su fruto presentan un compuesto denominado hidroxitirosol, el cual reduce marcadores inflamatorios asociados a enfermedades crónicas y tiene un efecto directo antioxidante. Esto mitiga el daño oxidativo e inflamatorio (Bigagli E. 2017). Por otra parte, en estudios in vivo previene la inflamación aguda y crónica y la aterosclerosis (Lopez S 2017). También ayuda al tratamiento de colitis ulcerosa y prevención del cáncer de colon derivada de estas reduciendo citoquinas TNFa, IL-1B, IL-6 y INF (Vijay K. 2016).

Coffea canephora

Los ácidos clorogénicos presentes en la variedad de café robusta han demostrado que protegen del estrés oxidativo a las células hepáticas (HepG2)

suponiendo una ayuda a las defensas endógenas del organismo (Baeza G. 2014), son una fuente bioaccesible de compuestos con una actividad antioxidante multidireccional e inhibiendo la producción de LOX relacionada con algunos tipos de cáncer y enfermedad coronaria (Gawlik-Dziki U. 2014). También reducen la inflamación y fibrosis a través de la intervención en rutas de señalización (Shi H. 2013) y aumenta la resistencia de la peroxidación de LDL e inhibe COX-2 en RAW264 por lo que previene enfermedades cardiovasculares y cáncer de colon, hígado y lengua demostrado en modelos animales (Soo Shin H. 2015).

Camelia sinensis:

De esta planta se obtiene el té, una de las bebidas más populares del mundo debido a sus beneficios para la salud basados en la capacidad antioxidante y sus potentes bioactividades. Es rica en polifenoles derivados de la catequina, los cuales suponen hasta el 30% del peso de la hoja seca y contiene principalmente las siguientes epicatequinas: epicatequina (6%), epigalatocatequina (19%), galato de epicatequina (14%) y galato de epigalatocatequina (59%) (Hara, 2001), pero se ha demostrado que esto depende de la cosecha y los cultivos de la temporada (Fang 2016). También es rico en policosanol, una mezcla de alcoholes alifáticos bioactivos de cadena larga que promueven la salud (Choi, 2016).

La hoja de *Camellia sinensis* se ha utilizado para confeccionar algunos alimentos funcionales, como es el caso de productos de confitería, demostrándose conferirles un mayor potencial antioxidante (Gramza-Michałowska, 2016).

Rosmarinus officinalis:

El ácido rosmarínico presente en la planta del romero se reconoce como antiinflamatorio en modelos de inflamación crónica, reduce la angiogénesis interviniendo en la expresión de NF-kB p65 reduciendo por tanto factores de inflamación (citoquinas) y angiogénicos (Boonyarikpunchai W. 2014, Cao W. 2016, Ghasemzadeh M. 2017).

Vitis vinifera:

Los polifenoles presentes en la semilla de uva tienen una fuerte capacidad antioxidante y además actúan inhibiendo ciertos modelos de agregación plaquetaria dependientes del tromboxano que puedan propiciar enfermedad coronaria (Guerrero J.A. 2004) y también suponen una defensa antioxidante en medios lipídicos (Mohamed 2016).

Theobroma cacao:

Las catequinas presentes en el cacao tienen una probada capacidad antioxidante, previenen la disfunción endotelial y reducen la resistencia a la insulina (Corti 2009)

Allium nigrum:

Algunos autores han aislado compuestos bioactivos del ajo negro con el fin de evaluar su bioactividad. Cuando se compara el extracto acuoso de ajo negro con el extracto acuoso de ajo crudo en similares rango de dosis (entre 31,25 g/mL y 250 g/mL), se ha demostrado la inhibición del factor de necrosis tumoral alfa (TNFα) y la prostaglandina E2 (PGE2) (Kim et al. 2014, Kim et al. 2016) la cual aumenta con la fermentación y obtención del ajo negro.

Myciaria dubia:

Se ha demostrado que el jugo de camu camu, debido a su contenido de vitamina C, tiene un poder antioxidante fisiológico, que disminuye las especies de oxígeno reactivo total y la actividad antiinflamatoria y reduce la proteína reactiva C circulante en el ser humano a una dosis de 70 mL/día (Inoue et al. 2008) . Sin embargo, los polifenoles presentes en el camu camu, como las proantocianidinas, las elagitaninas y los derivados del ácido elágico, también tienen actividades antioxidantes y antiinflamatorias (Fraccasseti et al. 2013).

Ribes nigrum:

La grosella negra contiene importantes concentraciones de vitamina C y algunos polifenoles, principalmente antocianinas. Como se ha visto en otros extractos, las actividades sinérgicas son claves en los extractos naturales pero, en la grosella negra, los compuestos más notables son las prodelfinidinas (Tits et al. 1992). Estudios en animales (modelo ratas Wistar),

demuestran resultados antiinflamatorios debido a una reducción de los factores pro-inflamatorios tales como TNF-, IL-1, IL-6, e IL-10 con un pretratamiento intraperitoneal consistente en la administración de proantocianidinas de las hojas de *Ribes nigrum* en concentraciones de 10 mg/kg, 30 mg/kg, 60 mg/kg, y 100 mg/kg (Garbacki 2004). Además, los efectos quimiopreventivos de las antocianinas del *Ribes nigrum* se probaron contra la carcinogénesis hepática en ratas Sprague-Dawley utilizando una dosis entre 100 mg/kg y 500 mg/kg de extracto durante cuatro semanas, obteniendo como resultado efectos antihepatocarcinógenos (Bishayee et al. 2011).

Harpagophytum procumbens:

Comúnmente llamado "garra del diablo" por sus frutos, es una planta herbácea perenne cuyas raíces se utilizan tradicionalmente como agentes antiinflamatorios para el tratamiento sintomático de la artritis y el reumatismo. Crece principalmente en el desierto de Kalahari al sur de África. Se ha relacionado con propiedades saludables como la actividad antipalúdica, anticancerígena y uterotónica (Mncwangi et al. 2012).

Uncaria tomentosa:

Es una enredadera trepadora del Perú comúnmente conocida como uña de gato por sus espinas. Se ha utilizado a lo largo del tiempo en la medicina tradicional para tratar enfermedades como el reumatismo y el cáncer (Hetizman et al. 2005). Tiene hasta 32 compuestos fenólicos distintos, incluyendo ácidos hidroxibenzoicos, ácidos hidroxicinámicos, monómeros de flavan-3-ol, dímeros de procianidina, flavalignanos y dímeros de propelargonidina (Navarro Hoyos et al. 2015). Uno sus principales compuestos bioactivos es la mitrafilina, que fue aislada y evaluada por Rojas-Duran et al. 2012, usando una dosis de 30 mg/kg/día durante 3 días en ratones BALB, lo que resultó en modulación de las citoquinas reduciendo la señal inflamatoria.

La mayoría de los beneficios para la salud que suponen estos polifenoles son antagónicos a la patogenia de los productos cárnicos descrita actualmente en la literatura científica por lo que pueden suponer una forma efectiva de desarrollar nuevos productos cárnicos saludables.

Descripción de desarrollo del producto cárnico

Para el desarrollo del producto cárnico, nuestras hipótesis fueron (1) que la carne procesada juega un papel en la oxidación y procesos inflamatorios relacionados con la señalización de la inmunidad y (2) que dicha carne puede hacerse más saludable con la adición de extractos naturales con propiedades antioxidantes y antiinflamatorias, ya que un disminución del nivel de biomarcadores relacionados con la patogénesis, como las citoquinas o las especies de oxígeno reactivo (ROS) podría mejorar las propiedades beneficiosas para la salud de la carne.

Para probar esta hipótesis, el desarrollo del producto y la evaluación de sus capacidades se distinguen en varias etapas bien diferenciadas:

ETAPA 0	Durante la etapa previa al desarrollo y la verificación de las propiedades del producto se procede a realizar una exhaustiva revisión bibliográfica de todas las fuentes relevantes de compuestos fenólicos, de la que deriva la redacción de los apartados previos y la selección de los compuestos a evaluar para su adición al producto cárnico.
ETAPA 1	La primera etapa de la investigación consiste en evaluar el punto de partida: en ella se analizan numerosos extractos botánicos y de alimentos para contrastar su potencial eficacia de cara a su incorporación al producto cárnico. En paralelo, se evalúan numerosas matrices de productos cárnicos para conocer los niveles actuales de nutrientes, antioxidantes y compuestos potencialmente anti-inflamatorios presentes derivados de las materias primas con las que se elaboran (carnes, especias, aromas…). Esta etapa debe concluir con la elaboración del producto cárnico potencialmente saludable que será evaluado en fases posteriores del proyecto.
ETAPA 2	La segunda etapa consiste en la evaluación con un cribado masivo (pero más selectivo que la etapa 1) de los extractos naturales seleccionados, así como de los productos cárnicos más propensos a presentar propiedades saludables y de

	algunos ejemplos de combinaciones de ambas. El medio para realizar este cribado es el uso de cultivos celulares, tanto de macrófagos (RAW 264.7) como células del epitelio del colon (Caco-2) a los cuales se les inducía una respuesta inmune a través del uso de lipopolisacáridos y se comprobaba la modulación inmune y antioxidante que pudieran tener estos extractos y productos cárnicos. Además, se realizó para poder reducir al máximo el uso de animales de experimentación en la fase 3, la fase pre-clínica, acotando los tratamientos suficientemente.
ETAPA 3	La tercera y última etapa del proyecto consiste en la evaluación de la eficacia antioxidante, anti-inflamatoria y promotora de la salud en general del producto cárnico desarrollado, así como del extracto natural usado y del producto cárnico por separado. Esta evaluación se realiza con un ensayo pre-clínico en un modelo animal con ratones C57BL/6

Fundamentos de los ensayos celulares (Etapa 2)

El cultivo de tejidos es una técnica experimental desarrollada desde finales del siglo XIX como continuación de las técnicas de embriología siendo considerado a R.G. Harrison como el padre de esta ciencia en el 1907.

En la actualidad el cultivo celular in vitro supone una herramienta básica e imprescindible para estudiar en detalle algunos procesos como la reproducción y diferenciación celular, el cáncer y la medicina regenerativa a través de células troncales o células madre. Además, algunos cultivos celulares ya se reconocen como métodos de sustitución a los ensayos animales, como el uso de células epiteliales para testar la aptitud dermatológica de algunos compuestos o su toxicidad.

Para la consecución de la etapa 2 del proyecto, han sido empleados para tratar de demostrar los beneficios del producto desarrollado y los compuestos que lo acompañan en una primera fase de cribado.

Durante la inflamación se produce una activación fagocítica de los macrófagos, monocitos y leucocitos polimorfonucleares presentes en el lumen intestinal resultando por un lado en un importante vertido de radicales libres hacia los tejidos biológicos y en otro en una respuesta inflamatoria mediada mayormente por productos derivados de la ciclooxigenasa-2 (Riccioti & FitzGerald, 2011).

La alteración del equilibrio de estos procesos durante la inflamación aguda y de bajo grado que se produce tras la ingesta de alimentos (Arya et al. 2010) da lugar a un incremento en el número de moléculas proinflamatorias y citoquinas las cuales están relacionadas con la aparición del cáncer (Lin, 2007)

Estos procesos afectan a todo un ecosistema compuesto por las células del lumen intestinal junto con la microbiota, decidiéndose estudiar dos líneas celulares en representación de aquellas que median en mayor medida el proceso inflamatorio, los macrófagos (RAW264.7) y de las células que recubren el epitelio colónico, los colonocitos (Caco-2).

Macrófagos RAW264.7

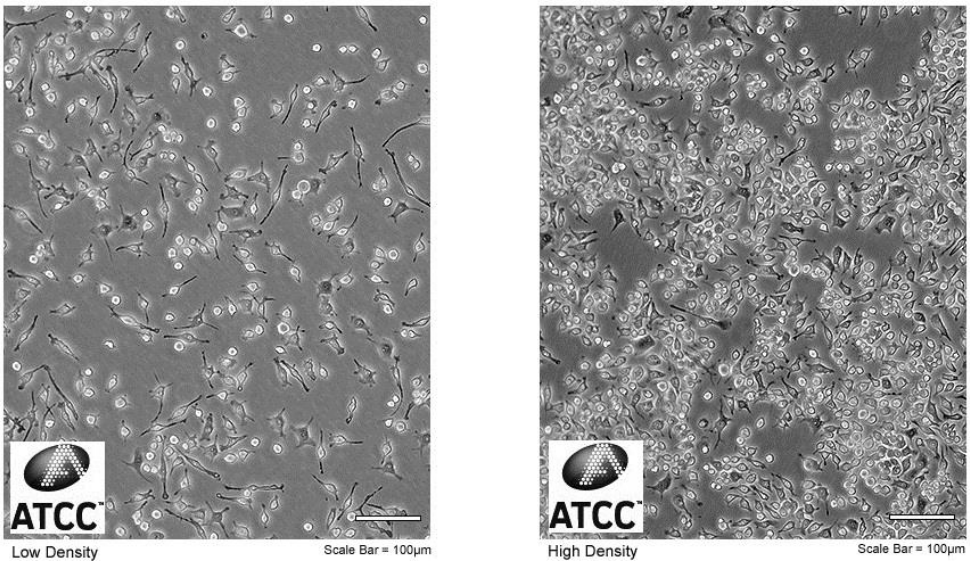


Imagen de la ATCC para la identificación morfológica de los macrófagos RAW264.7 a diferentes densidades celulares

Esta ha sido la línea celular empleada mayoritariamente para demostrar los potenciales efectos beneficiosos observados en la etapa anterior.

Son macrófagos inmortalizados a través de la inducción de la leucemia de Abelson por un virus promotor de tumores. Provienen de un ratón (Mus musculus) de la cepa BALB/c que vivió en el año 1978 cuyas células inmortales han sido replicadas hasta la actualidad por la American Type Culture Collection (ATCC).

Los macrófagos aparecen en el lumen intestinal como línea defensiva del organismo y mediadores de la inflamación y señalización celular tras diferenciarse de su tipo celular previo, el monocito.

La modulación que podamos conseguir en la producción de agentes pro-inflamatorios y del estatus oxidativo de los mismos será un reflejo de los posibles beneficios que tendrá el producto sobre la salud intestinal.

Colonocitos Caco-2

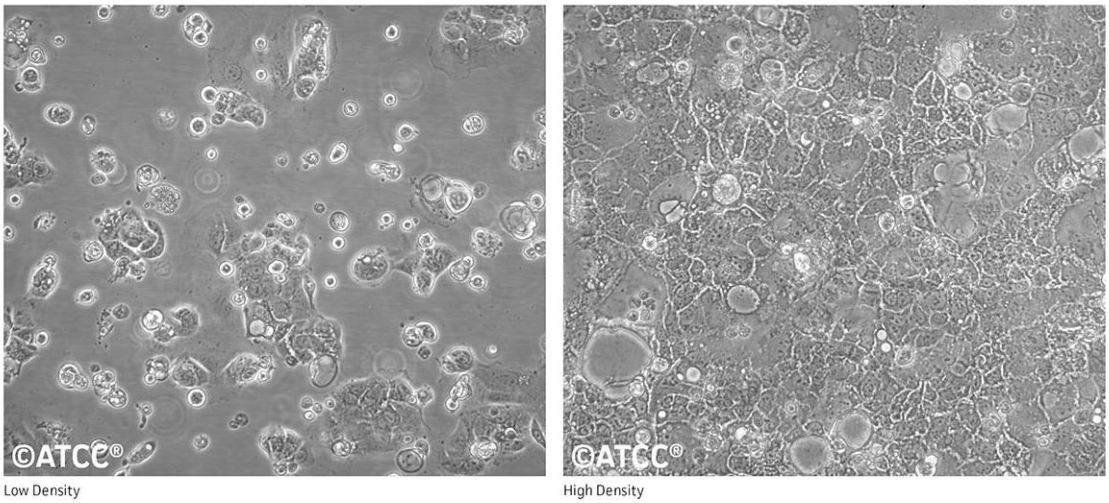


Imagen de la ATCC para la identificación morfológica de los colonocitos Caco-2 a diferentes densidades celulares.

La línea celular Caco-2 ha sido utilizada dentro del proyecto para evaluar la influencia de la exposición por un lado al producto cárnico y

extractos empleados y por otro a los macrófagos RAW264.7 durante un proceso inflamatorio.

Caco-2 ha sido obtenida a través de la inmortalización de colonocitos provenientes de un adenocarcinoma colónico humano presente en un varón caucásico de 72 años en el año 1974. Al igual que sucede con la línea RAW264.7, estas células son replicadas y distribuidas por la American Type Culture Collection.

Son células adherentes que crecen hasta la confluencia. Además estas células tienen la capacidad de diferenciarse cuando alcanzan la confluencia dando lugar a una capa de epitelio intestinal madura con su consiguiente diferenciación enterocítica.

Objetivo y fundamentos del ensayo preclínico

El objetivo de la tercera etapa es la validación de todos estos ensayos in vitro en un modelo pre-clínico. Para ello se tratará de observar cómo interacciona la mezcla de bioactivos desarrollada en un organismo vivo y cómo afecta a la salud modulando el estatus inflamatorio y oxidativo del mismo.

Selección del modelo animal

Se ha de seleccionar un modelo animal donde se pueda inducir un proceso inflamatorio y cuantificar la mejoría que pueda aportar añadir el bioactivo o el producto cárnico reformulado a la dieta.

El modelo animal seleccionado finalmente es el de ratón (Mus musculus) por diversas razones:

- Capacidad de extrapolar resultados de inflamación y oxidación a humanos.
- Especie animal más usada en la investigación por lo que cuenta con una amplia bibliografía de respaldo y comparativa.

- Experiencia previa del grupo de investigación para el manejo de esta especie.

Tras observar que tanto la cepa de *Mus musculus* BALB/c como la cepa C57BL/6J se adaptan a las exigencias del proyecto, la selección final se decanta por C57BL/6J dada la experiencia previa del grupo.

C57BL/6J es la cepa de ratón consanguínea más ampliamente utilizada y fue la primera en tener su genoma secuenciado. Entre sus características destacan por ser muy susceptibles al desarrollo de obesidad a través de la dieta y a padecer diabetes tipo 2 y aterosclerosis. (The Jackson Laboratory 2018).

Una vez seleccionada la cepa se escogió la obesidad como modelo de investigación ya que el síndrome metabólico derivado de ésta aumenta el estrés oxidativo y el estatus inflamatorio (van der Heijden et al. 2015) permitiendo observar con mayor facilidad la potencial modulación sobre estos parámetros que pueda tener una intervención.

Fundamento del modelo de obesidad

El hígado y el tejido adiposo son los principales encargados de regular el metabolismo. En el modelo de obesidad, la hipertrofia del tejido adiposo y la deposición de grasa en el hígado actúan como disruptores generando el síndrome metabólico (Suganami & Ogawa 2010).

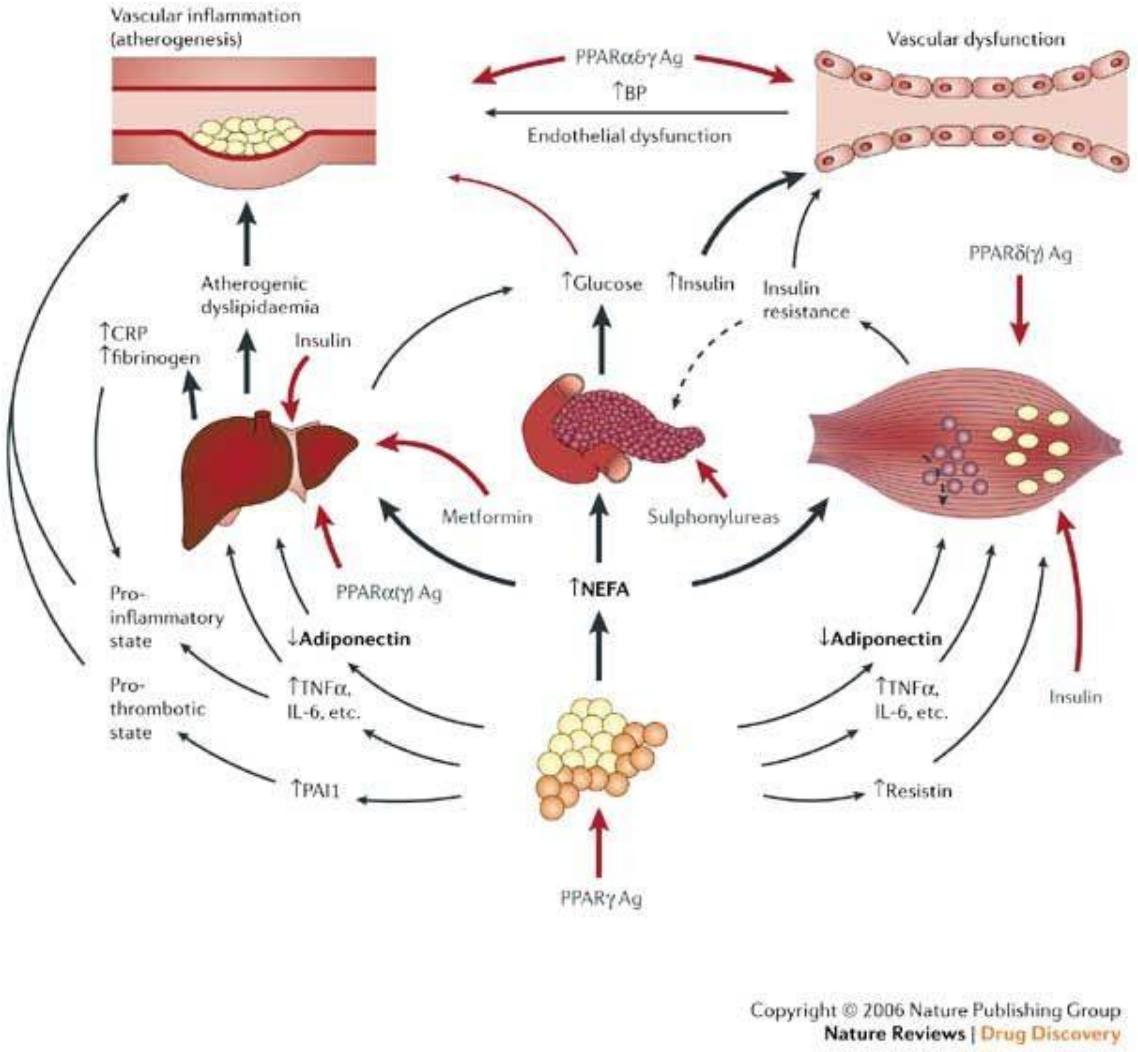
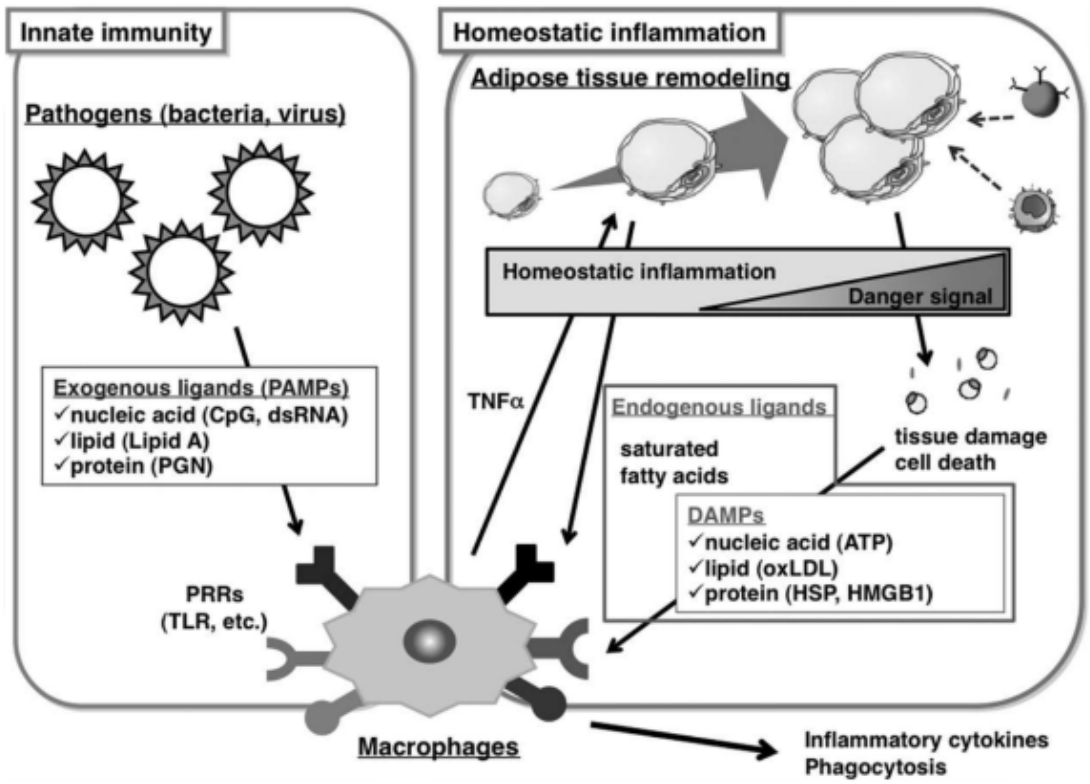


Ilustración por Grundy (2006) de las alteraciones relacionadas con el síndrome metabólico donde aparecen citoquinas como mediadores el estado inflamatorio sistémico.

El modelo de obesidad es corroborado a través de las curvas de crecimiento y los cambios morfológicos del individuo a estudio tras la incorporación del pienso obesogénico. También se puede complementar con una evaluación al detalle de los depósitos grasos a través de la tomografía SPEC-CT.

Este modelo nos va a permitir generar un estatus inflamatorio aumentado derivado de la actividad de los macrófagos en el tejido adiposo (Ferrante 2007) para poder medir la actividad sistémica e inflamación post-pandrial local en el tracto gastrointestinal (Klop et al. 2012), la cual también podría repercutir sobre el estatus inflamatorio sistémico.



Inflamación del tejido adiposo como inflamación homeostática. (Suganami & Ogawa 2010)

De igual forma, uno de los factores patogénicos más importantes derivados de la obesidad usada como modelo es el incremento del estrés oxidativo (Marseglia et al. 2015, Matsuda et al. 2013, Collins et al. 2016) que también se estudiará el efecto de la intervención tanto a nivel local como sistémico.

Una ventaja de usar el modelo de obesidad para el ensayo de inmunidad-oxidación es que, de forma transversal, se puede evaluar el estado de salud en otros órganos.

En el caso del bazo, existe una correlación entre la inflamación presente en la obesidad y el tamaño del mismo, pudiendo asociarse la esplenomegalia con un peor estado de salud derivado de la inflamación (Gotoh et al. 2012).

El modelo también afecta al hígado, promoviendo el depósito de grasa y generando la enfermedad del hígado graso no-alcohólico o NAFLD) por sus siglas en inglés) (Ragab et al. 2015).

Fase In-vitro

antioxidants

MDPI

Article

Regulation of Inflammatory Response and the Production of Reactive Oxygen Species by a Functional Cooked Ham Reformulated with Natural Antioxidants in a Macrophage Immunity Model

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Abstract: Nowadays, more consumers demand healthier products. A way to offer such products is to functionalize them using health-promoting bioactive compounds. Meat and meat products are high in essential nutrients; however, their excessive consumption implies a high intake of other substances that, at levels above recommended uptake limits, have been linked to certain non-communicable chronic diseases. An effective way to reduce this danger is to reformulate meat products. In this study, natural botanical extracts rich in anti-inflammatory and antioxidant compounds were used to improve the health properties of a cooked ham with an optimal nutritional profile (i.e., low in fat and salt). The RAW 264.7 mouse cell line was used as an inflammatory model and was stimulated with *Escherichia coli* lipopolysaccharide to evaluate changes in inflammatory biomarkers such as tumour necrosis factor alpha, the interleukins (ILs) IL-1β and IL-6, nitric oxide and intracellular reactive oxygen species (ROS). The results showed that the use of natural extracts in optimized cooked ham significantly downregulated inflammatory markers and reduced the levels of intracellular ROS. Thus, the present study proposed a new functional cooked ham with potential health properties via anti-inflammatory and antioxidant in vitro activity.

Keywords: functional meat; functional food; cooked ham; polyphenol; antioxidant; reactive oxygen species; anti-inflammatory; cytokines

1. Introduction

Meat is an important source of essential nutrients. It provides high-quality proteins, vitamins such as B12 and B6 and minerals such as iron and selenium. However, recent studies have linked meat—mostly red and processed meats—with the incidence of non-communicable diseases such as colorectal cancer [1,2].

Although epidemiological studies support this premise, the mechanistic associations are not well understood. Meat contains several molecules that could be involved in non-communicable diseases [3]. For example, the consumption of one component of meat, heme iron—an essential source of dietary iron and a nutrient—should be moderated, as, if it is consumed in excess, it increases the risk of type II diabetes by causing oxidative damage to pancreatic β cells [4] and also causes inflammatory disruption due to the catalysis of fat peroxidation, which can lead to cardiovascular diseases [5]. On the other hand, the high density of nutrients in meat, such as fats, compel its consumption to be moderated in order to prevent metabolic syndrome [6] or diseases related to the excessive intake of saturated fats and the content of sodium in processed meats. Studies have linked pathogenesis with substances such as N-nitrous compounds [7] and heterocyclic amines [8]; however, these works have limitations, as

their authors did not specify key elements such as the characteristics of thermal treatment and other processes applied to meat, which influence the development of N-nitrous compounds and heterocyclic amines [9].

During inflammatory processes, the phagocytic activation of macrophages, monocytes, and polymorphonuclear leukocytes results in the pouring of free radicals into biological tissues and an inflammatory response called respiratory burst [10]. The deregulation of those processes due to the low-grade inflammatory response implying postprandial processes [11] produces pro-inflammatory molecules and cytokines, which are linked with tumour progression [12] together with free radicals. Cytokines also have other physiological functions, such as mediating macrophage chemotaxis and angiogenesis by interleukin (IL)-1 [13]; they are also involved in the differentiation of immune cells via IL-6 [14] and have been widely used as an inflammatory biomarker [11,15–17].

In this study, in order to prevent oxidative and inflammatory deregulation, some natural compounds were selected based on previous studies of antioxidant and anti-inflammatory potential. For example, catechins have been shown to upregulate endogenous antioxidants such as superoxide dismutase and catalase [18] and to have anti-proliferative and anti-angiogenesis activities [19]. Additionally, rosmarinic acid has been demonstrated to have anti-inflammatory properties [20,21], and hydroxytyrosol, an emerging and potent antioxidant derived from *Olea europaea*, has been shown to protect against oxidative stress and inflammation [22]. Moreover, chlorogenic acids have been shown to modulate inflammation [23].

In this study, our hypotheses were (1) that processed meat plays a role in oxidative and inflammatory processes related to immunity signalling and (2) that such meat can be made healthier with the addition of natural extracts with antioxidant and anti-inflammatory properties, since a decrease in the level of pathogenesis-related biomarkers such as cytokines or reactive oxygen species (ROS) could improve the healthiness of cooked ham. To test these hypotheses, a macrophage model of inflammation was used to test both the anti-inflammatory and antioxidant potential of standard and enriched cooked ham. For this purpose, a cooked ham was selected as a base product due to its improved macronutrient profile (i.e., low sodium content and saturated fat). Then, the ham was enriched with natural extracts that can palliate the possible pathogenic effects of the meat due to the antioxidant and anti-inflammatory processes [24].

The aim of the present study was to evaluate the inflammatory role of processed meat and to develop a functional meat product enriched in natural extracts with antioxidant and anti-inflammatory properties.

2. Materials and Methods

2.1. Reagents

The enzymes pepsin (catalogue no. P7000), pancreatin (catalogue no. P1750) and α-amylase (catalogue no. A6380) for in vitro digestion, 2,4,6-tris(2-pyridil)-s-triazine (TPTZ) (catalogue no. 93285), iron (III) chloride hexahydrate (catalogue no. 31232), Folin–Ciocalteu phenol reagent (catalogue no. F9252), gallic acid (catalogue no. 91215), lipopolysaccharide from *Escherichia coli* serotype O127:B8 4.5 g/L (catalogue no. L4516), 2′,7′-dichlorofluorescin diacetate (catalogue no. D6883), Griess reagent (catalogue no. 03553) and thiazolyl blue tetrazolium bromide (catalogue no. M5655) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 2 mM glutamine, 1% non-essential amino acids, 1% penicillin–streptomycin) was purchased from Gibco BTL Life Technologies (Paisley, Scotland). Dimethyl sulfoxide (DMSO) and MCYTOMAG-70K plate for Luminex assay were supplied by Merck KGaA (Darmstadt, Germany).

2.2. Samples

BienStar® ham (produced by ElPozo Alimentación, S.A., Alhama de Murcia, Murcia, Spain) is a cooked ham with an improved nutritional profile (25% less fat and 35% less sodium than a

standard cooked ham). In this study, uncooked BienStar® ham dough (which contains 85% pork ham, water, potassium chloride, corn dextrose, sugar, corn syrup, flavouring, sodium citrate, and sodium erythorbate) was used as the base product to develop a new functional meat. The original BienStar® dough was used as the control sample (C), and two new functional meat foods were obtained by adding to one sample of dough a natural antioxidant aqueous solution containing 12.5 mg/mL of chlorogenic acid, 10 mg/mL of catechins and 1.5 mg/mL of rosmarinic acid (called CCR), and adding to another sample of dough a natural antioxidant aqueous solution containing 1.5 mg/mL of hydroxytyrosol (called CCH). Both aqueous solutions were mixed with a water standard to a concentration of 5% *w*/*w* with BienStar® ham dough. The three meat samples (C, CCR and CCH) were cooked at 80 °C for 40 min.

In order to simulate physiological digestion, the cooked ham samples were digested following the method described by Minekus et al. [25]. The resulting homogenates were filtered using 0.22 µm PVDF filters in order to sterilize them for cell culturing (see Section 2.5), thus obtaining the final samples.

2.3. Ferric-Reducing Antioxidant Power (FRAP) Assay

The antioxidant power of the digested samples was measured using the ferric-reducing antioxidant power (FRAP) method [26]. Subsequently, the samples were added to a cell culture in order to correlate the increased antioxidant content of the cooked ham with the modulation of oxidative status in macrophages.

To prepare the working solution, a 10 mM TPTZ solution was prepared in 40 mM HCl and then mixed with 20 mM $FeCl_3 \cdot 6H_2O$ in distilled water and 0.3 M sodium acetate anhydrous buffer solution at a ratio of 1:1:10 (*v*:*v*:*v*). Standard Trolox solution was prepared to determine the calibration curve at a concentration range of 10–200 µM. Samples and the standard were mixed with the FRAP working solution at 1:10 and then incubated at 37 °C for four minutes. Then, the absorbances of the samples and the standard were measured at a wavelength of 595 nm. The results were expressed as µm Trolox equivalents per 100 g of digested sample.

2.4. Determination of Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method [27]. A total of 100 µL of digestion liquid extracts were diluted in 3 mL distilled water and 0.5 mL of Folin–Ciocalteu reagent was added and incubated for 3 min. Then, a mixture of water and 20% sodium carbonate was added and mixed and the mixture was incubated for 20 min. Then, the absorbance of the mixture was measured at 750 nm. A standard gallic acid solution was prepared for the determination of the calibration curve.

2.5. Cell Culture

The RAW 264.7 macrophages were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK; number 86010202). Cells were maintained as indicated by the provider in Dulbecco's Modified Eagle's Medium (4.5 g/L glucose, 3.7 g/L sodium bicarbonate) supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37 °C with 5% CO_2, 95% air atmosphere, and 95% relative humidity. The medium was replaced every two days and subcultures were made at a ratio of 1:5 in a 75 cm^2 culture flask.

2.5.1. Cell Viability

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) [28]. Cells were seeded at 2×10^4 in 96 well plates at digestion extract concentrations of between 1 and 10% *v*/*v* with medium applied for 24 h. Then, after removing the medium, 100 µL of the MTT solution (5 mg/mL in phosphate-buffered saline) was added and the mixture was incubated for 4 h at 37 °C. A total of 100 µL of DMSO was added to each well to dissolve formazan and then the

absorbance was measured at 540 nm. Results lower than 90% of cell viability resulted in the elimination of the sample.

2.5.2. Lipopolysaccharide-Induced Inflammation Assay

Lipopolysaccharide (LPS) from *E. coli* was used to stimulate RAW 264.7 to produce free radicals, NO, and IL [29]. Cells were seeded at a density of 1×10^4 and incubated for 24 h to ensure total adherence and functionality. Standard media were enriched with 5% *v*/*v* of digested distilled water as a control without sample for measuring the basal status without inflammation and total stimulus with LPS. The same procedure was performed for measuring changes with control digested cooked ham and the CCH and CCR digested enriched cooked hams. Then, 200 µL of each enriched medium was added to the cell cultures and the mixtures were left for 24 h. After the incubation, the enriched medium was removed and 200 µL of medium with 1 µg/mL LPS was added to all wells except the basal control and the mixture was left for 24 h. After the last incubation, the medium was stored for NO and interleukin measurement and fresh medium was added in preparation for the ROS assay.

2.5.3. Measurement of Reactive Oxygen Species

The 2′-7′-dichlorofluorescein diacetate (DCFH-DA) was used to measure ROS [30]. Cell supernatant was enriched with 12.5 µM DCFH-DA and incubated for 30 min at 37 °C. After incubation, the supernatant was removed, plates were washed twice with phosphate buffered saline (pH = 7.4) and fluorescence was measured at λ_{ex} = 488 nm and λ_{em} = 530 nm.

2.5.4. Measurement of Nitric Oxide

Cell supernatant for NO determination was mixed 1:1 with Griess reagent and incubated at room temperature for 20 min. Then, absorbance was measured at a wavelength of 540 nm.

2.5.5. Measurement of Cytokines

The interleukins IL-1β and IL-6 and tumour necrosis factor alpha (TNFα) were measured using Luminex multiplex immunoassay technology. A Luminex MAGPIX system with a MCYTOMAG-70K plate was used. As per the manufacturer's instructions, a total of 25 µL of LPS-stimulated RAW 264.7 supernatant was used to determine cytokine concentrations using the Luminex xPONENT software. The results were obtained in pg/mL and expressed as a percentage of respective cytokine inhibition.

2.5.6. Statistics

Statistical analysis was performed using the GraphPad Prism 7.0 software for Windows (GraphPad Software, San Diego, CA, USA). Results were expressed as the mean plus or minus of the standard error of the mean (SEM). Multiple comparisons were performed using one-way (FRAP, TPC, ROS and NO results) or two-way ANOVA (cytokine results) followed by Tukey's multiple comparison test. The *p*-values less than 0.0001 were considered significant.

3. Results

3.1. Preliminary Antioxidant Assays

The results of the FRAP assay, which was performed in order to check for increases in antioxidant activity, showed that both of the enriched cooked hams presented statistically significant differences ($p < 0.0001$), with the antioxidant capacity of each ham being twice that of the control ham (Figure 1).

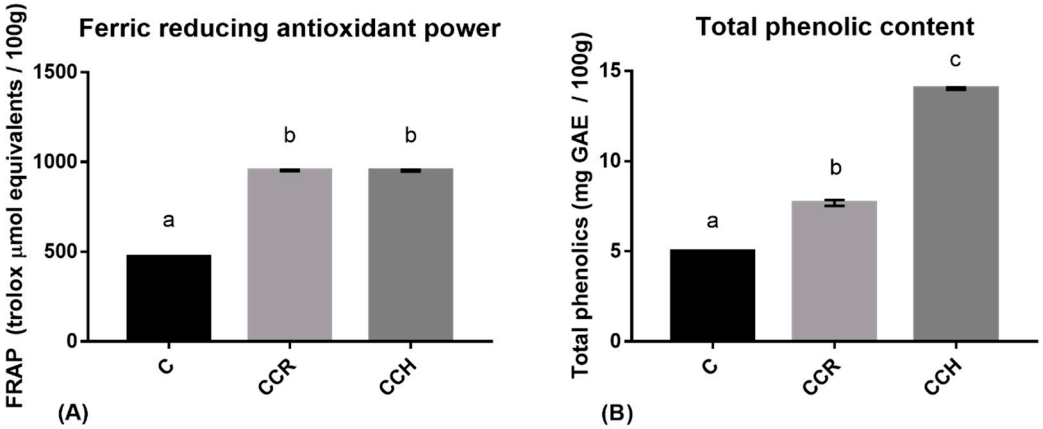

Figure 1. The results of the ferric-reducing antioxidant power (FRAP) assay (**A**) and total phenolic content (**B**) for the control cooked ham (C), the cooked ham reformulated with 5% *w*/*w* of an aqueous solution of chlorogenic acids, catechins, and rosmarinic acid (CCR), and the cooked ham reformulated with 5% *w*/*w* of an aqueous solution of chlorogenic acids, catechins, and hydroxytyrosol aqueous solution (CCH). Different letters represent differences that are statistically significant ($p < 0.0001$).

The phenolic content of the CCR ham was 54.21% higher than that of the control ham, while that of the CCH ham was 180% higher than that of the control ham.

These results verify the effectiveness of reformulating cooked ham in terms of antioxidant capacity and phenolic content. The differences in the phenolic content and antioxidant capacity of the CCH and CCR hams indicate that a lower phenolic content can be associated with the same reducing activity depending on the compound added (hydroxytyrosol or rosmarinic acid, respectively).

3.2. Intracellular Reactive Oxygen Species

The downregulation of ROS production compared to non-treated stimulated RAW 264.7 is expressed as % of ROS inhibition. This parameter decreased significantly when macrophages were treated with the digested extract from the control cooked ham, with an inhibition of 19.28 ± 0.31%. This shows that, even without reformulating, the cooked ham had intracellular antioxidant properties. Technological antioxidants for food preservation could have protective intracellular effects against ROS.

As shown in Figure 2A, when the cooked ham was reformulated with CCR or CCH prior to digesting and treating macrophages, its ability to decrease the level of intracellular ROS increased significantly, with higher ROS inhibition being observed with the CCH formula than the CCR formula. The differences among the two formulas can be attributed to the presence of hydroxytyrosol, which seems to be significantly more effective at inhibiting ROS than rosmarinic acid.

The fact that both the reformulated hams showed a higher inhibition of ROS and NO production ($p < 0.0001$) than the control ham indicates an improvement in the reduction of intracellular ROS and NO production.

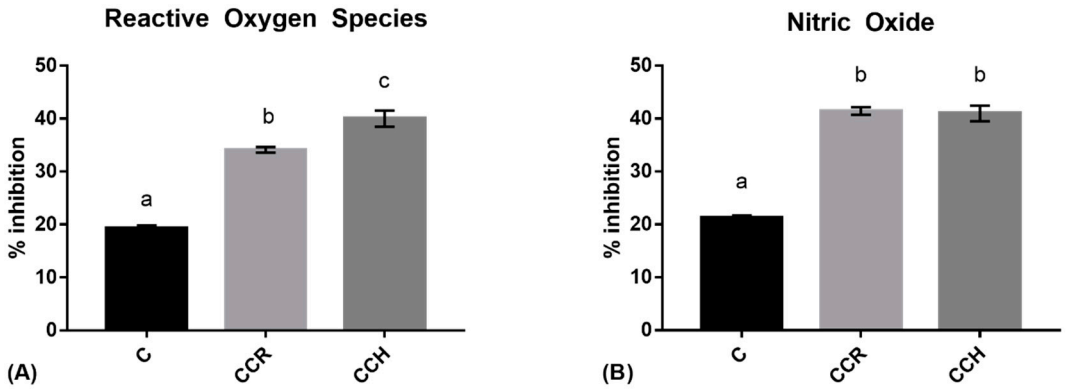

Figure 2. The ability of digested extracts of ham to inhibit reactive oxygen species (ROS) (**A**) and to inhibit nitric oxide production (**B**) from lipopolysaccharide-activated RAW 264.7 macrophages. Different letters represent statistically significant differences ($p < 0.0001$).

3.3. Nitric Oxide

As shown in Figure 2B, the amount of extracellular NO was slightly lower for the cells treated with the digested extract of the standard cooked ham compared to non-treated stimulated RAW 264.7. As was observed for ROS, reformulating with natural extracts significantly increased NO inhibition in stimulated macrophages ($p < 0.0001$).

3.4. Cytokine Measurement

The release of cytokines, which is used as a biomarker of inflammation, was not significantly different among polyphenol-enriched cooked hams, despite the fact that higher TNFα inhibition was observed in the CCH enriched ham. However, the hams treated with CCR and CCH, respectively, inhibited the extracellular signalling of IL-1β and IL-6. Additionally, the ham treated with CCH more effectively inhibited TNFα.

The results in Figure 3 show the effectiveness of polyphenol in modulating the inflammatory macrophage response, which could indicate a new strategy to produce healthier meat products.

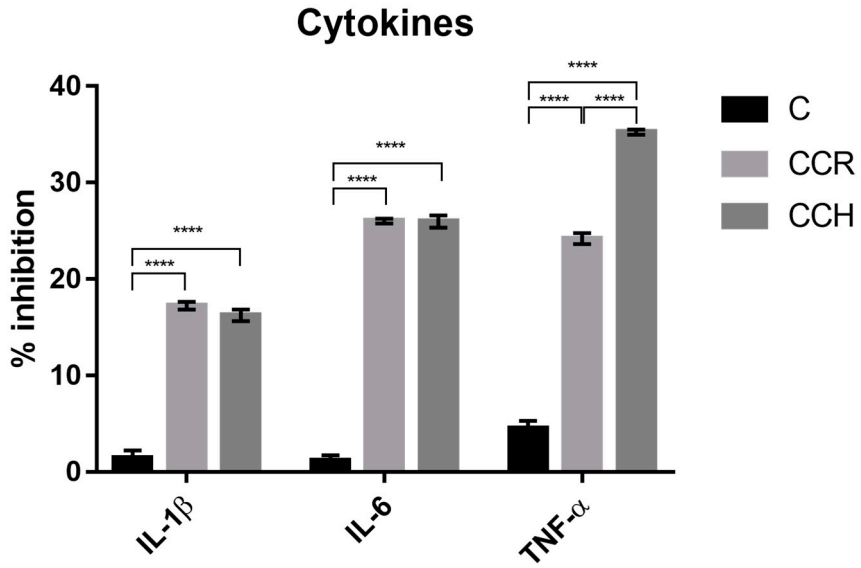

Figure 3. The effect of digested extracts of the three cooked hams on the inhibition of the cytokines interleukin (IL)-1β and IL-6 and tumour necrosis factor alpha (TNFα) in lipopolysaccharide-activated RAW 264.7 macrophages. **** $p < 0.0001$.

Although the control ham showed slight inhibition of IL-1β, IL-6, and TNFα, the inhibition of these cytokines was significantly higher for the hams reformulated with polyphenol blends ($p < 0.0001$).

4. Discussion

Due to the widespread consumption of meat, there has been widespread research into the production of healthier meat products. Some authors have focused on inhibiting potential carcinogenic substances generated during meat processing using natural antioxidants [31,32]—such as rosemary extract, which is rich in rosmarinic acid—which have been shown to decrease lipid oxidation [33–35] and improve the healthiness of processed meat.

Previous studies on the antioxidant capacity of meat have shown that, by itself, meat acts as a radical scavenger or reducing agent [36] with an activity similar to that measured in non-reformulated meat in this assay. Since pigs are monogastric animals, the antioxidant capacity of their meat can be influenced by their diet [37]. Such an influence may explain the polyphenol content of the control sample in this study; however, it does not affect the comparison of the results since the same meat was used to prepare the three samples. Previous studies showed that, in a standard diet, meat and meat products represent up to 10.51% of total antioxidant intake [38]. However, in this study, the total phenolic content of the meat product was found to increase after treatment with natural extracts, doubling the antioxidant capacity of the meat product.

These findings are consistent with the fact that the control cooked ham was demonstrated to have antioxidant properties. The denaturation of proteins through heat processing has been shown to release antioxidant peptides and some Maillard reaction products [36]. This can explain the antioxidant properties and the presence of technological antioxidants in processed meats such as the cooked ham used in the present study.

The level of intracellular ROS can be reduced both by enhancing endogenous intracellular antioxidants using polyphenols [39] and the antioxidant capacity of technological antioxidants evaluated by the FRAP method. The differences observed in this study between the CCH and CCR hams can be explained by the fact that higher values of TPC were observed in the CCH ham and that the hydroxytyrosol used to treat this ham was highly bioavailable [40] compared to other polyphenols [41], and was, therefore, less degraded by the digestion process used in the present study.

Antioxidants have been widely described as protective agents against degenerative diseases [42] produced by physiological mechanisms which involve the pouring of ROS [43]. The inhibition of these radicals by untreated and reformulated cooked ham may, therefore, lead to the prevention of oxidative damage. However, more detailed in vivo studies should be performed.

Nitric oxide is synthesized by macrophages as a signalling molecule of inflammation and a toxic defence against infectious organisms [44]. Its presence indicates the activation of NO synthase, which in this study was due to the simulated infectious stimulus by an *E. coli* lipopolysaccharide. Reducing postprandial physiological inflammation could prevent cardiovascular diseases caused by lipemia enteric processes [45] and some pathways of colon cancer promotion [44]. A previous study observed inhibitions of NO emissions (measured as nitrite) of around 50% by treating cell cultures with pure concentrations of flavonoids such as quercetin and kaempferol at a concentration of 50 μM [46]. Furthermore, another study related heme oxygenase-1 protein expression with the inhibitory effects of flavonoids on NO emission [47].

In our research, the control cooked ham showed a slight inhibition of NO production from macrophages. In macrophages, NO is synthesized by NO synthase, whereas superoxide is mainly produced by nicotinamide adenine dinucleotide phosphate oxidase. Peroxynitrite is produced in vivo when reactions occur between superoxide and NO. Considering that peroxynitrite generation has been attributed to inflammatory diseases such as chronic heart failure, myocardial infarction, stroke, diabetes, and cancer, the consumption of cooked ham (under a balanced diet) may control these inflammatory diseases.

Heat processes and digestion can release peptides that modulate nitric oxide in a similar way to that achieved by Song et al. [48], who used peptides from the sea cucumber. In the present study, reformulating ham with CCH and CCR was found to significantly increase the capacity to inhibit the production of ROS and NO compared to the control cooked ham, thus increasing the anti-inflammatory capacity of the ham.

Cytokines have been shown to be inflammatory mediators [49]. The results of the present study show that the control cooked ham slightly inhibited the production of TNFα by stimulated macrophages, while the CCH and CCR reformulated hams both significantly downregulated the secretion of IL-1β, IL-6, and TNFα. The cytokine IL-6 has been described as both inflammatory and anti-inflammatory; under specific conditions, including LPS-induced inflammation, it was considered to be inflammatory [50]. Reducing the level of inflammatory cytokines could allow the prevention of inflammatory diseases, as suggested by Arranz et al. [51], using rosemary extract, and by Wu et al. [52], using dioscin. Other foods have been tested to determine their influence on inflammatory status. For example, digested einkorn-based bread was tested with stimulated Caco-2 cells, achieving a significant reduction in IL-6 production [53], and similar levels of ROS and NO inhibition were observed using digested bread with HepG2 cells [54]. Cytokines were also shown to be downregulated by bile salt used in digestion [55]. In the present study, positive and negative inflammation control wells were treated with digested distilled water at the same concentration as in the sample wells.

5. Conclusions

The present study found that cooked ham with an optimal nutritional profile had antioxidant and anti-inflammatory properties related to the inhibition of substances which are involved in gut inflammation—namely, reactive oxygen species, nitric oxide, and cytokines—produced by macrophages. These health-promoting inhibitory properties were significantly increased when the cooked ham was enriched with bioactive phytochemicals. In conclusion, the incorporation of botanical extracts rich in phenolic compounds (i.e., chlorogenic acid, catechins, rosmarinic acid, and hydroxytyrosol) in cooked ham is an excellent strategy to produce a healthy functional meat product.

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Conflicts of Interest: The authors declare no conflict of interest.

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Anti-Inflammatory and Antioxidant Effects of Regular Consumption of Cooked Ham Enriched with Dietary Phenolics in Diet-Induced Obese Mice

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Abstract: Oxidative damage and chronic inflammation have been proven as one of the major factors associated with obesity, which increases the incidence of non-communicable chronic diseases. In this sense, the development of new functional products aiming at the palliation of oxidative stress and inflammatory disruption can be a determining factor for public health as seen in previous researches. In this study, a blend of potentially bioavailable dietary phenolics was added to low sodium and low-fat cooked ham. A diet-induced obesity model in C57/BL6J mice has been used for testing the effectiveness of the phenolic blend and the new functionalized product, which bioavailability was tested by UPLC-ESI-QTOF-MS. After obesity induction, different oxidative and inflammatory biomarkers were evaluated. Results in the murine induced obesity model, demonstrate a robust statistically significant improvement in key parameters related with obesity risk in the groups feed with a phenolic-enriched diets (P) + high-fat diet (HFD) and phenolic enriched cooked ham (PECH) + HFD. In both groups there was an improvement in body composition parameters, inflammatory biomarkers and antioxidant enzymes levels. Specifically in the group feed with the phenolic enriched cooked ham (PECH + HFD) there was an improvement of total fat volume (23.08% reduction), spleen index (22.04% of reduction), plasmatic MCP-1 (18% reduction), IL-6 (38.94% reduction), IL-10 (13.28% reduction), TNF-α (21.32% reduction), gut IL-1β (10.86% reduction), gut IL-6 (13.63% reduction) and GPx (60.15% increase) and catalase (91.37% increase) enzymes. Thus, the functionalized ham could be considered an appropriate dietary polyphenol source, which might improve the oxidative and inflammatory status and could finally result in the potential decrease of the risk of certain non-communicable chronic diseases.

Keywords: ham; oxidative stress; inflammatory biomarkers; polyphenols

1. Introduction

Obesity has become the most significant public health problem [1], and it is considered as the result of excessive energy intake compared to the energy expenditure [2]. It characterized by a body mass

index equal or higher than 30 kg/m^2, with an increase in adipocyte number and size [3]. Obesity leads to augmented cardiovascular and diabetes risk factors [4] with some disorders such as dyslipidaemia, non-alcoholic fatty liver disease, as well as oxidative and inflammatory disruptions [5–8]. Therefore, the reversal of these disorders and the prevention of obesity are essential challenges for society.

Nowadays, obesity is usually treated with changes in lifestyle [9] or drugs [10]. Recent studies have demonstrated that dietary polyphenols can contribute to improve obesity disorders due to their antioxidant and anti-inflammatory properties [11–13]. (Poly)phenolic compounds are the most common and ubiquitous groups of secondary metabolites, widely distributed in plant foods such as fruits, vegetables and beverages. They have been reported to exhibit a broad spectrum of potential biological activities, including their antioxidant and anti-inflammatory properties, related to the prevention of chronic diseases such as cancer, diabetes, cardiovascular, and neurodegenerative diseases [14,15]. For decades, their potential anti-inflammatory effects have been associated with their antioxidant activity reduce reactive oxygen species (ROS) and nitric oxide (NO) production, reduced lipid peroxidation), and by the modulation of a plethora of cell-signalling pathways and inflammation mediators (cytokines, eicosanoids, etc.) [16]. However, despite the numerous preclinical studies, the clinical evidence for these health benefits is still elusive, at least in part, as their health effects depend on both, their bioavailability and metabolism, and the interindividual response after their intake [17]. Even so, although only a small number of dietary phenolics are considered bioavailable, their potential benefits may be achieved by the consumption of a natural phenolic-enriched diet, as well as by their administration as food supplements or nutraceuticals. In this sense, in recent years, to respond to consumer's demands, functionalizing food through phenolic enrichment has been considered an advantage for dealing with chronic diseases, including obesity disorders.

Meat and meat products are widely consumed in most dietary patterns [18,19]. They are a source of nutrients such as high-quality proteins, vitamins (B6 and B12) and minerals (Fe, Zn or Se among others). Regarding lipid profile, due to animal genetic, nutrition and managements, there is a wide range of lean meats (less than 5% of fat, and a reduced saturated fatty acid profile), that are included in some weight-loss diets [20]. There is an industrial trend drive by consumers demand to improve the nutritional profile of cooked hams by lowering its lipid content (less than 1.5%), reduce saturated fatty acids and maintain its high protein content (18,5%) and quality. As previously mentioned, plant polyphenols provide clear health benefits. The use of polyphenols in the meat industry have been explore aiming to improve the preservation of meat [21,22] and transform meat products in a source of polyphenols to produce a beneficial effect on the health of consumers [23,24]. One of the most consumed pieces of lean meat is cooked ham, which consists of brined hind legs of swine, either smoked or not [25].

The present study aims to demonstrate that the regular consumption of a meat product enriched with plant polyphenols have a positive impact in a murine model, mainly on oxidative stress, chronic inflammation, adiposity and obesity. For this purpose, the effects of a ham with improved nutritional profile (low in salt, fat and saturated fatty acids) incorporating a mixture of polyphenols (catechins, chlorogenic acids and hydroxytyrosol—HXT—selected for their efficacy on obesity disorders [11,26–28]) was investigated in a murine model.

2. Materials and Methods

2.1. Ham Preparation and Diet Composition

Cooked ham used for this study was provided by a local meat industry (ElPozo Alimentación, S.A., Alhama de Murcia, Murcia, Spain). This type of cooked ham has an improved nutritional profile with a total fat content below 1.5%, and an amount of saturated fat and sodium below 0.5% (BienStar®, ElPozo Alimentación, Alhama de Murcia, Murcia, Spain). It was used as the base product for developing the new phenolic-enriched ham. Uncooked ham dough was mixed 5% *w/w* with water for the control sample and with a natural antioxidant aqueous solution of 12.5 mg/mL of chlorogenic acid, 10 mg/mL

of catechins and 1.5 mg/mL of HXT. Both hams were cooked at 80 °C for 40 min. After being cooked, hams were lyophilized (Telstar Lyoquest Lyophilizer, Tarrasa, Barcelona, Spain) to obtain a powder suitable for mixing with the mice diet.

Five diets were prepared for the present study: (1) Pelletized standard diet (SD) (Teklad Global 14% Protein Rodent Maintenance Diet. Envigo, Barcelona, Spain) and (2) high-fat diet (HFD) (Teklad Custom Diet TD.06414 60/fat. Envigo) were obtained from the manufacturer. Then, (3) phenolic-enriched high-fat diet (P + HFD) was obtained by adding the natural antioxidant mix previously described to a final concentration of 166 mg/kg to high-fat diet composition, (4) lyophilized cooked ham enriched high-fat diet (CH + HFD) was obtained by adding the lyophilized standard cooked ham to a final concentration of 50 g/kg, and (5) lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH + HFD) was obtained by adding the lyophilized phenolic-enriched cooked ham to a final concentration of 50 g/kg.

Casein, lard, and maltodextrin, which are the primary macronutrient sources of HFD, were used for standardizing macronutrient profile of every diet in the intervention phase after adding or not both lyophilized cooked hams (Table 1). All HFD diets were re-pelletized.

Table 1. Macronutrient profile of standard diet and high-fat diets used throughout the study.

Macronutrients Composition and Energy Density	Standard Diet (SD)	High-Fat Diets (HFD)
Protein (%)	14.3	23.5
Total carbohydrate (%)	48.0	27.3
Fiber (%)	4.1	6.5
Fat (%)	4.0	34.3
Energy density (Kcal/g)	2.9	5.1
• Calories from protein (%)	20	18.3
• Calories from carbohydrates (%)	67	21.4
• Calories from fat (%)	13	60.3

2.2. Animals, Treatments and Ethical Considerations

The experiment was approved by the Animal Experimentation Ethics Committee of the University of Murcia, Spain, following the directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

For the present study, 36 male C57/BL6J mice six weeks old were provided by Envigo. They were randomly housed in nine cages with four animals each. They were divided based on the previously described diet interventions. Mice were fed *ad libitum* with the SD during the adaptation period (weeks 6 to 11). SD was maintained for control mice, housed in cage 1, and obesity was induced through the high-fat diet along five weeks (weeks 11 to 16), cages 2 to 9. Once the diet-induced obesity (DIO) period was over, HFD was maintained for obesity control cages (2 and 3), and the following intervention diets were applied: P + HFD to cages 4–5, CH + HFD to cages 6–7 and PECH + HFD to cages 8–9. Consumed food was quantified to detect possible differences throughout the study.

2.3. Sampling

At the end of treatments, blood was collected from the submandibular vein into a heparin-coated tube. Blood samples were centrifuged at 4000 g for 10 min (4 °C), and plasma was collected and frozen at −80 °C for further analysis. Mice were housed by groups of 4 individuals from the same treatment in metabolic cages with a separation system for feces and urine, and both were collected.

For protein (cytokine) determination, gut samples were obtained using Tissue Ruptor and Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Urine samples were vortexed during a minute, centrifuged at 300× *g* for 10 min at 4 °C. Next, urine samples were diluted 1:5 with acidified water (0.1% formic acid), filtered through a 0.22 µm polyvinylidene fluoride (PVDF) filter and injected in the UPLC-ESI-QTOF-MS equipment.

Livers were collected in 10% phosphate-buffered formalin and stored less than 24 h at 4 °C. Sections of 5 µm were obtained using Leica Microtome RM2155 (Leica, Wetzlar, Germany) stained with hematoxylin and eosin. Images were obtained using a digital camera Leica DC500 with Leica DMRB optical microscope. Samples were analysed in triplicate for fat content according to AOAC methods [29]

2.4. Body and Spleen Weight and Body Composition

Mice were weighed weekly, between 4:00−5:00 p.m., using a precision weight scale (0.1 g) (Highland Adam 600 × 0.01 g, UK) and right after the sacrifice. The spleen was immediately removed after collecting blood samples and sacrificing and then weighted. The following formula was used for obtaining the spleen index: Spleen index (mg/g) = spleen weight (mg)/live body weight before sacrifice (g).

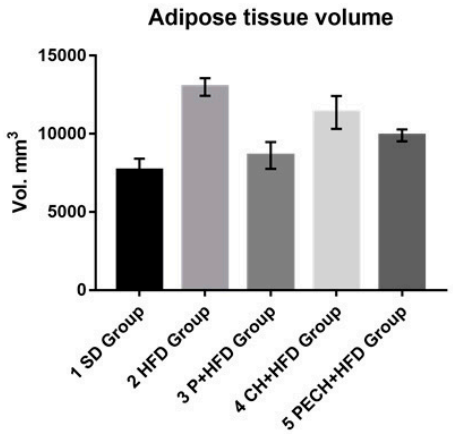
Total fat volume was determined, immediately after mice euthanasia, through the method described by Moreno [30] using the Albira CT system—a small animal positron emission tomography (PET)/single photon emission computed tomography (SPECT)/computed tomography (CT) imaging system—(Bruker Molecular Imaging, Woodbridge, CT, USA). Images were computerized (using the filtered back-projection algorithm via the Albira Suite 5.0 Reconstructor), 3D image obtained (Figure 1) had 125-µm isotropic voxels (each of an array of elements of volume that constitute a notional three-dimensional space, especially each of an array of discrete elements into which a representation of a three-dimensional object is divided) and was analysed through PMOD software (PMOD Technologies, Zurich, Switzerland). Finally, images were segmented according to adipose tissue density and voxels corresponding to adipose tissue were interpreted as total fat volume, expressed as mm^3.

2.5. Evaluation of Antioxidant Capacity

The evaluation of antioxidant capacity and antioxidant enzymes in plasma were performed following the methods described in [31] Glutathione peroxidase (GPx) assay was performed based on the method described by Paglia and Valentine [32] with a commercially available kit (Ransel test kit, Randox Laboratories Ltd., Crumlin, County Antrim, UK). Catalase activity was determined according to Johansson and Borg [33]. Ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain [34], and paraoxonase 1 (PON1) was measured following the method described by Tvarijonaviciute et al. [35].

2.6. Cytokine Measurement

The cytokines monocyte chemoattractant protein 1 (MCP-1), interleukin (IL)-1β, IL-6, IL-10, and tumoral necrosis factor-alpha (TNF-α) were measured using Luminex multiplex immunoassay technology. A Luminex MAGPIX system with a MCYTOMAG-70K plate (Merck KGaA, Darmstadt, Germany) was used. According to the manufacturer instructions, a total of 25 µL of plasma or gut lysate was used to determine cytokine concentrations in triplicate using the Luminex xPONENT ® software (Hertogenbosch, The Netherlands). The results were obtained in pg/mL.



A

Group comparisons	Significant differences
1 SD Group vs. 2 HFD Group	Yes****
1 SD Group vs. 3 P+HFD Group	No
1 SD Group vs. 4 CH+HFD Group	Yes***
1 SD Group vs. 5 PECH+HFD Group	Yes*
2 HFD Group vs. 3 P+HFD Group	Yes***
2 HFD Group vs. 4 CH+HFD Group	No
2 HFD Group vs. 5 PECH+HFD Group	Yes**
3 P+HFD Group vs. 4 CH+HFD Group	Yes**
3 P+HFD Group vs. 5 PECH+HFD Group	No
4 CH+HFD Group vs. 5 PECH+HFD Group	No

B

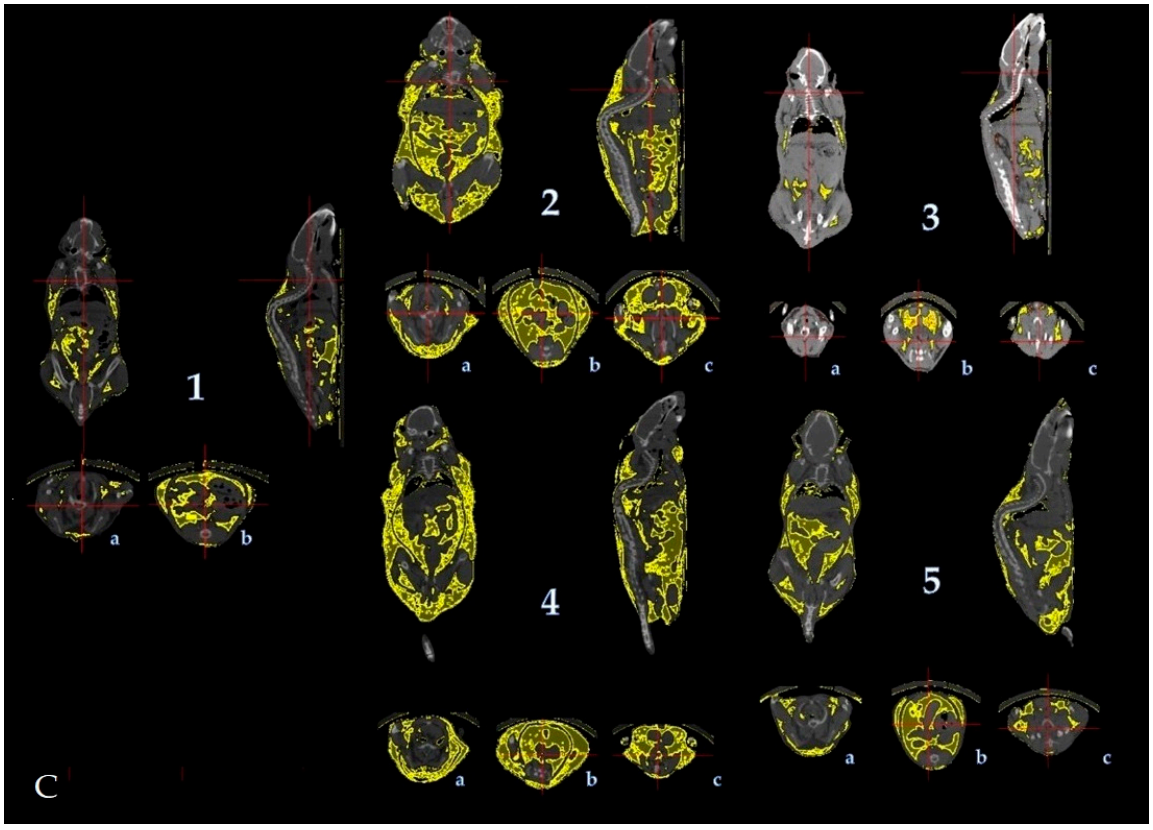


Figure 1. Effect of the different diets on the adipose tissue volume. (**A**) Adipose tissue volume expressed as mm^3 at the end of the study. Numbers and groups correspond as following: (1) Standard diet, (SD), (2) high-fat diet (HFD), (3) phenolic-enriched high-fat diet (P+HFD), (4) lyophilized cooked ham enriched high-fat diet (CH+HFD) and (5) lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH). (**B**) Statistical comparison Significant differences are expressed as * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$ (**C**) PET/SPECT/CT computerized images showing areas with a density attributed to adipose tissue coloured by yellow (voxels).

2.7. OxLDL Measurement

Plasma oxidized low-density lipoprotein (OxLDL) concentrations were determined using commercially available standard ELISA kits (Mouse Oxidized Low-Density Lipoprotein (OxLDL) ELISA Kit 96-strip-wells, mybiosource.com, San Diego, CA, USA) following the manufacturer's instructions. All samples were analysed at least in duplicate.

2.8. UPLC-ESI-QTOF-MS Analysis of Urine

Phenolic-derived metabolites were identified in urine by UPLC-ESI-QTOF-MS, as previously described [36]. The injection volume was 3 μL. A target screening strategy was applied for the qualitative screening of 60 possible metabolites that could be present after consumption of the polyphenol mix. Including both parent phenolic compounds, phase II derived metabolites (glucuronides, sulphates, etc.) and phenolic acids (Table S1). Organic solvents of analytical grade were obtained from Merck KGaA and Milli-Q ultrapure water from Millipore Corp. (Bedford, MA, USA).

2.9. Statistics

Statistical analysis and graphics were performed using the GraphPad Prism 7.0 software for Windows (GraphPad Software, San Diego, CA, USA) and Sigma Plot 13.0 (Systat Software, San Jose, CA, USA). Multiple comparisons were performed using one-way, or two-way ANOVA followed by Tukey multiple comparison test. Results are expressed as the mean plus or minus of the standard error of the mean (SEM). The *p*-values of less than 0.05 were considered significant.

3. Results

3.1. Changes in Body Weight and Body Composition

As shown in Figure 2, weight changes were small during the adaptation period with SD. Significant differences were found after the first week of diet-induced obesity period. Bodyweight changes comply with specification sheet from Teklad Custom Diet TD.06414 60/fat. It can be stated that enriched HFD diets have not an anorexigenic effect because average food intake among groups did not show significant differences (data not shown) and because body weight differences between HFD groups were not statistically significant.

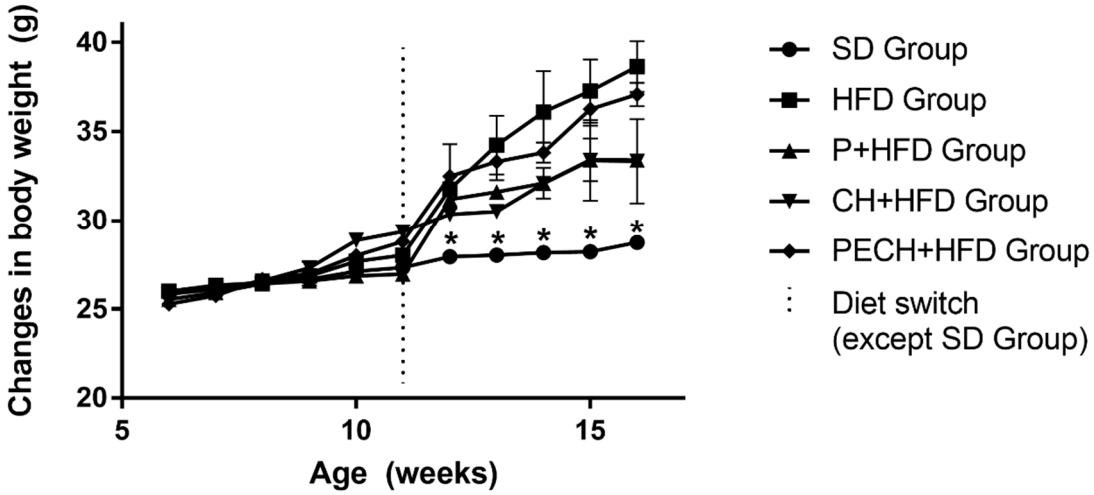
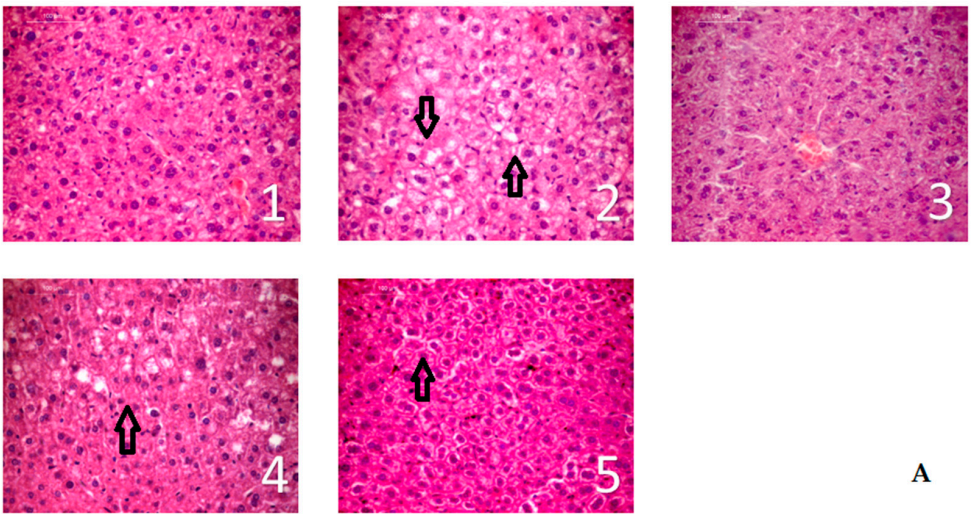


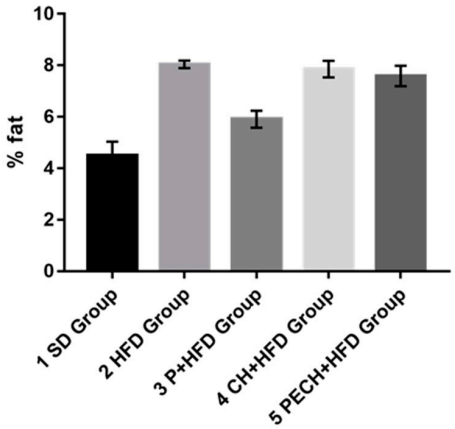
Figure 2. Changes in body weight during the study. Each group starts with SD (standard diet, $n = 4$) during the adaptation period. Diets were switched from SD to each high-fat diet intervention (high-fat diet (HFD, $n = 8$), phenolic-enriched high-fat diet (P + HFD, $n = 8$), lyophilized cooked ham enriched high-fat diet (CH + HFD, $n = 8$) and lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH+HFD, $n = 8$) at week 11. Significant differences between SD Group and each other are expressed as * $p < 0.05$.

Significant differences were observed between SD group and HFD group on total adipose volume, verifying the fattening effect of high-fat diet compared with standard diet. No differences were observed between SD and P + HFD, indicating that P+HFD diets could control adipogenesis. There were no differences between HFD and CH + HFD group. However, PECH + HFD group presented less adipose volume than HFD group.

Total fat content in liver was calculated to evaluate the effects of high fat diets over liver steatosis ans presented in Figure 3. A higher adiposity was observed in stained cuts from livers corresponding to HFD and CH + HFD diets (Figure 3A). Every obese group had higher liver fat concentration than SD group (Figure 3B). However, P + HFD group had lower concentrations of fat content than CH + HFD and PECH + HFD groups. A higher phenolic content was correlated with less liver fat content.



A



B

Group comparisons	Signifficant differences
1 SD Group vs. 2 HFD Group	Yes****
1 SD Group vs. 3 P+HFD Group	Yes**
1 SD Group vs. 4 CH+HFD Group	Yes****
1 SD Group vs. 5 PECH+HFD Group	Yes****
2 HFD Group vs. 3 P+HFD Group	Yes***
2 HFD Group vs. 4 CH+HFD Group	No
2 HFD Group vs. 5 PECH+HFD Group	No
3 P+HFD Group vs. 4 CH+HFD Group	Yes***
3 P+HFD Group vs. 5 PECH+HFD Group	Yes**
4 CH+HFD Group vs. 5 PECH+HFD Group	No

C

Figure 3. Effect of the different diets on the fat content in liver. (**A**) Liver cuts stained with hematoxylin-eosin. Non-stained areas correspond with lipid deposits between hepatocytes, pointed by black arrows. Numbers and groups correspond as following: (1) Standard diet, (SD), (2) high-fat diet (HFD), (3) phenolic-enriched high-fat diet (P + HFD), (4) lyophilized cooked ham enriched high-fat diet (CH + HFD) and (5) lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH). (**B**) The graph shows total fat content in humid samples as %. (**C**) Statistical comparison. Significant differences are expressed as ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$.

Changes in body weight and body composition have demonstrated the effectivity of every high fat diet inducing obesity in mice. Nevertheless, diets including phenolic compounds have shown lower obesity induction than those without phenolics.

3.2. *Inflammatory Markers*

The concentrations of MCP-1 and cytokines are expressed in pg/mL. As represented in Figure 4, the HFD diet increased inflammatory markers compared to the SD diet. While anti-inflammatory IL-10 modulation did not show significant differences between SD and HFD, the amount of pro-inflammatory cytokines IL-6 and TNF-α and chemokine MCP-1 did it ($p < 0.0001$).

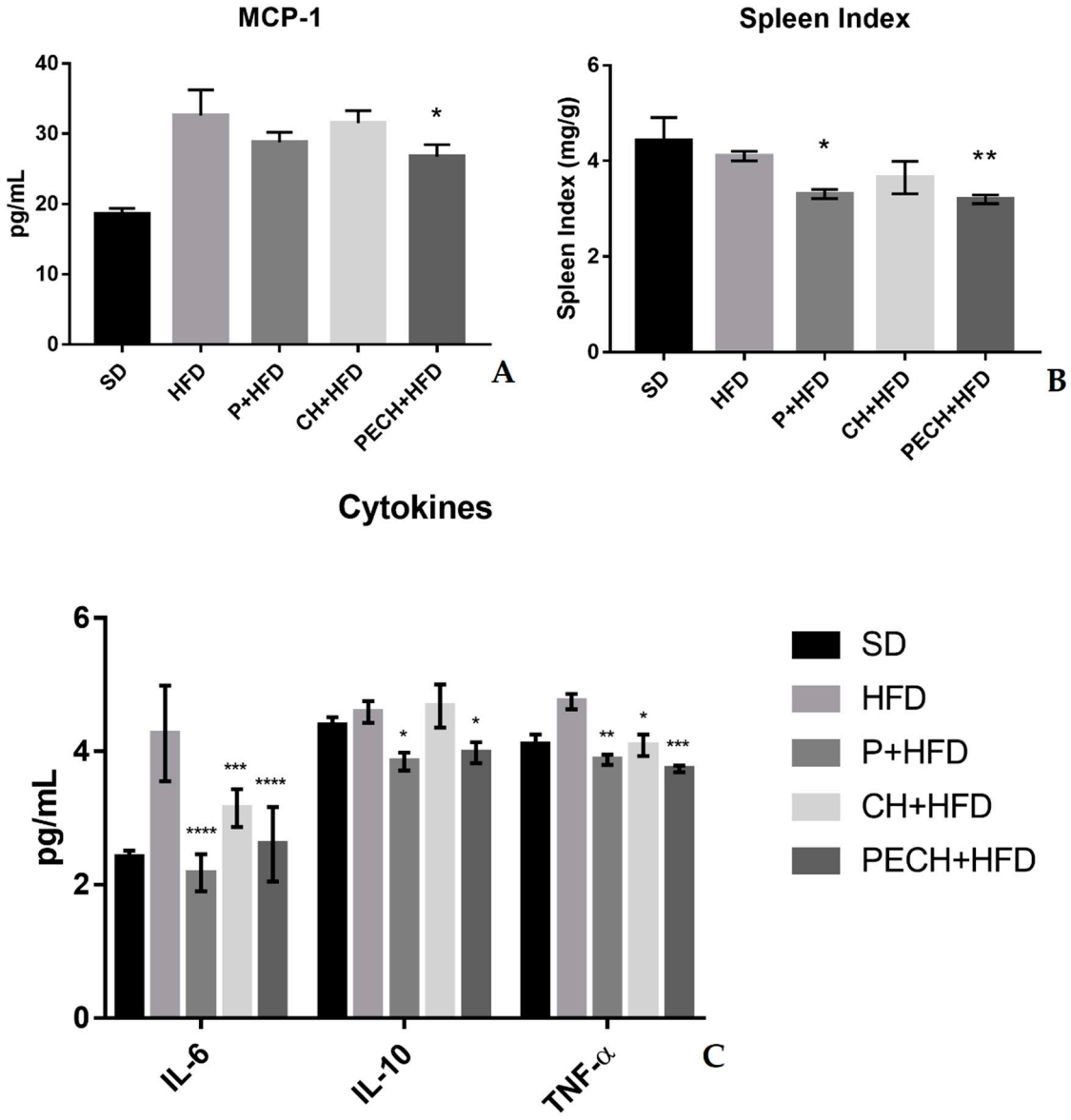


Figure 4. Effect of different diets on inflammatory biomarkers. (**A**) MCP-1 levels, (**B**) spleen index, (**C**) citokine levels (IL-6, IL-10, TNF-α). The different diets compared were standard diet (SD, $n = 4$), high-fat diet (HFD, $n = 8$), phenolic-enriched high-fat diet (P + HFD, $n = 8$), lyophilized cooked ham enriched high-fat diet (CH + HFD, $n = 8$) and lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH + HFD, $n = 8$). HFD mean results were compared against P + HFD, CH + HFD and PECH + HFD diets, significant differences are expressed as * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$.

In contrast, the consumption of the phenolics blend (P + HFD and PECH + HFD diets) decreased the concentration in plasma of cytokines IL-6, IL-10, TNF-α, and chemokine MCP-1 compared to HFD diet significantly. The global reduction of cytokines indicated lower levels of obesity-related systemic inflammation. Even the CH + HFD diet showed a lower concentration of IL-6 and TNF-α compared to HFD. In the case of the spleen index, measured as a systemic inflammatory marker, both diets, including the phenolic blend, showed significant differences (P + HFD and PECH + HFD). IL-1β was under detectable levels.

Results obtained from gut lysate analysis are expressed as pg/mL (Figure 5). TNF-α and MCP-1 were under the detection limit. Statistically significant differences were detected between SD and HFD ($p < 0.0001$) in IL-1β and IL-6 levels. No statistically significant differences were observed on IL-1 between samples. Both phenolic-enriched diets, P + HFD and PECH + HFD, could have substantial effects over inflammatory status, reducing the presence of IL-1β and IL-6 on mice gut significantly. CH+HFD also had an inhibitory effect over IL-6, as shown in plasma samples.

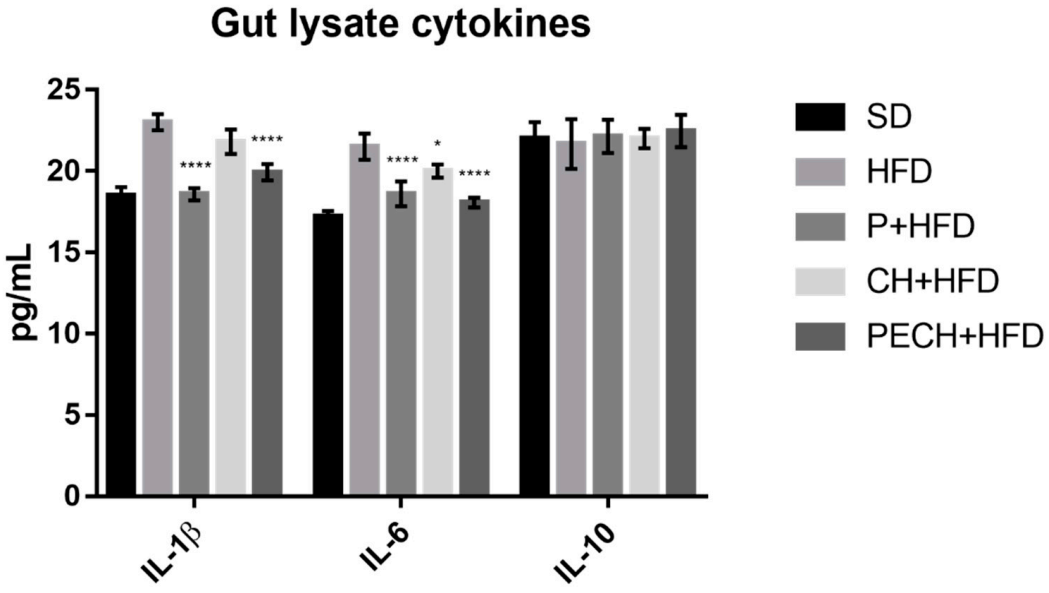


Figure 5. Effect of different diets on gut lysate cytokines. The different diets compared were standard diet, (SD, $n = 4$), high-fat diet (HFD, $n = 8$), phenolic-enriched high-fat diet (P + HFD, $n = 8$), lyophilized cooked ham enriched high-fat diet (CH + HFD, $n = 8$) and lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH + HFD, $n = 8$) on inflammatory biomarkers IL-1β, IL-6 and IL-10 in gut samples. HFD mean results were compared against P + HFD, CH + HFD and PECH + HFD diets, significant differences are expressed as * $p < 0.05$ **** $p < 0.0001$.

Cytokine levels in plasma did not report differences between CH + HFD and PECH + HFD diets, except for IL-10 ($p < 0,05$). However, the consumption of a polyphenol-enriched cooked ham compared with non-enriched one showed significative differences in gut lysate, reducing both inflammatory markers IL-1β and IL-6 ($p < 0,05$).

3.3. Plasma Antioxidant Capacity

Regarding plasma antioxidant capacity, the most relevant biomarkers were analysed (antioxidant enzymes were used as biomarkers of oxidative stress -PON1, GPx, Catalase- and FRAP for the overall antioxidant capacity of plasma), and represented in Figure 6 as affected for the different diets. As a general remark, the lowest levels of antioxidant enzymes indicate a poor oxidative homeostatic status. The consumption of both phenolic rich diets, P + HFD and PECH + HFD, showed increases in total antioxidant capacity of plasma measured by the FRAP method with significant differences between HFD diet (Figure 6D). Significant differences ($p < 0.001$) have been spotted in GPx and Catalase levels between SD and HFD, indicating that induced obesity can compromise antioxidant enzyme levels in plasma.

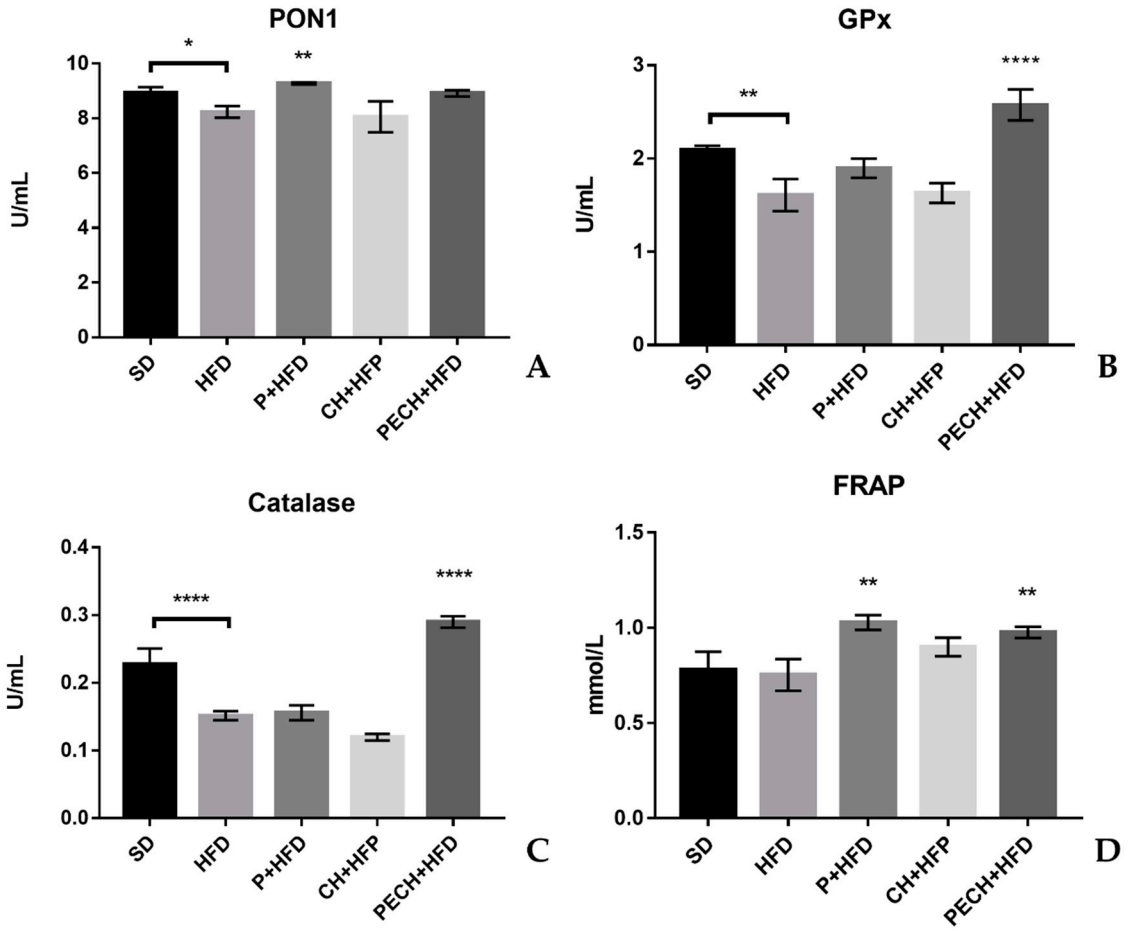


Figure 6. Effect of the different diets on antioxidant biomarkers. (**A**) PON1, (**B**) GPx, (**C**) Catalase, (**D**) FRAP. The different diets compared were standard diet, SD, $n = 4$), high-fat diet (HFD, $n = 8$), phenolic-enriched high-fat diet (P + HFD, $n = 8$), lyophilized cooked ham enriched high-fat diet (CH + HFD, $n = 8$) and lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH + HFD, $n = 8$). HFD mean results were compared against P + HFD, CH + HFD and PECH + HFD diets, significant differences are expressed as * $p < 0.05$ ** $p < 0.01$ **** $p < 0.0001$. When they exist, differences between SD and HFD were represented with "∩".

Accordingly, a polyphenol-rich diet also resulted in an increase in activity of different antioxidant enzymes such as catalase for both P + HFD and PECH + HFD diets, PON1 with P + HFD and GPx with PECH + HFD compared to HFD. Higher levels of antioxidants enzymes imply a better response against obesity-induced oxidative stress.

3.4. OxLDL Results

The analysis and quantification of oxLDL for each group is shown in Figure 7. Even if no significant differences were observed among groups there are two important trends in the levels of oxLDL: (1) There was a slight decrease in oxLDL levels after consumption of cooked ham (PECH + HFD) compared to the CH + HFD (that show a slight increase of oxLDL levels compared with SD) and (2) a decrease of oxLDL values after the consumption of bioactive compounds (P + HFD) compared to the SD group.

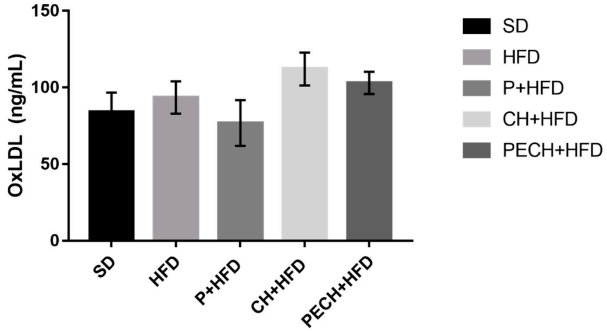


Figure 7. Effect of different diets on oxidized LDL (OxLDL) levels. The different diets compared were standard diet, (SD, *n* = 4), high-fat diet (HFD, *n* = 8), phenolic-enriched high-fat diet (P + HFD, *n* = 8), lyophilized cooked ham enriched high-fat diet (CH + HFD, *n* = 8) and lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH + HFD, *n* = 8).

3.5. Identification of Phenolic-Derived Metabolites in Urine

As expected, after the target screening strategy and the information provided by MS and MS/MS modes, six phenolic-derived metabolites were identified in urine samples from the P + HFD and PECH + HFD groups confirming the bioavailability of these phenolics (Table 2). The metabolites were detected after UPLC-QTOF analysis, although they were not quantified due to the low concentrations of the polyphenol metabolites in urine that were below their LOQ (15 nM for hydroxytyrosol glcuronide; 150 nM for 5-(3′,4′-dihydroxyphenyl-γ-valerolactone, and 4 nM for 5-(3′,4′-Dihydroxyphenyl-γ-valerolactone 3′-sulphate). No phenolic-derived metabolites were found in the rest of the groups. No phenolic-derived metabolites were detected in the remaining groups. It should be taken into account that epicatechin sulphate, 5-(3′,4′-Dihydroxyphenyl-γ-valerolactone, 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone 3′-sulphate and HXT glucuronide were detected in all urine samples from the two groups fed with the phenolic blend. However, ferulic acid 4-sulphate and feruloylquinic acid glucuronide were only identified in the group fed with the enriched phenolic compounds diet alone (P + HFD).

Table 2. Phenolic derived metabolites identified in urine.

Metabolites	RT (min)	Experimental *m/z*	MS/MS Fragments	Occurrence	
				P + HFD	PECH + HFD
Epicatechin sulphate	5.2	369.0286	125.0235; 109.0289; 289.0708	+	+
5-(3′,4′-Dihydroxyphenyl-γ-valerolactone	5.4	207.0663	163.0764; 122.0369	+	+
5-(3′,4′-Dihydroxyphenyl-γ-valerolactone 3′-sulphate	3.9; 5.3	303.0180	223.0605; 179.0702	+	+
Ferulic acid 4-sulphate	8.3	273.0074	193.0505; 134.0379	+	–
Feruoylquinic acid glucuronide	5.5	543.1355	367.1013; 498.9785; 173.0450	+	–
Hydroxytyrosol glucuronide	3.0	329.0878	123.0445; 153.0169; 176.0381; 131.0349	+	+

Analytical parameters for the determination of phenolic-derived metabolites in urine samples using UPLC-ESI-QTOF-MS. The different diets compared were phenolic-enriched high-fat diet (P+HFD) and lyophilized phenolic enriched cooked ham enriched high-fat diet (PECH+HFD, *n* = 8).

4. Discussion

One of the main drawbacks to link the beneficial health effects of phenolics with the dietary phenolics intake is the poor bioavailability of these compounds. Besides, most of these phenolics

are extensively metabolized by the gut microbiota to other derived metabolites that present higher absorption. Next, they undergo extensive phase-II metabolism yielding, mainly glucuronide and sulphate conjugates that can persist in systemic circulation up to 3–4 days after intake [37,38]. In our study, the identification of six phenolic-derived metabolite conjugates (Table 2) in the urine of mice fed with phenolic-enriched ham confirmed the bioavailability of phenolics and suggested that the effects observed could be mediated by their action.

Bodyweight changes were evaluated to prove the effectivity of high-fat diets, which were supposed to increase pathogenicity related biomarkers [7]. Changes complied with diet specification sheet and with previous studies [39,40]. Non statistically significant differences were found between diet-induced obesity groups regarding body weight. Body composition changes had corroborated higher adipose tissue in the high-fat diet groups. Adipose tissue directly correlated with the presence of inflammatory biomarkers such as IL-6 and TNF-α [41,42]. Variation in lipid infiltration in liver also showed the effectiveness of high-fat diets inducing obesity [43]

The spleen initiates the immune reaction to blood-borne antigens [44]. The spleen index reflects the prognosis of diseases and changes in this index respond to the nonspecific immunity of the organism [45]. Splenomegaly, which mainly occurs during active diseases [46], can be used as an inflammatory marker due to its increase in pro-inflammatory situations such as metabolic syndrome [47]. In the present study, the spleen index has shown significant differences between both polyphenol-enriched diets (P + HFD, PECH + HFD) and non-treated obese group.

Cytokines, measured as inflammatory mediators [48], have shown similar trends in inflammatory modulation. On the other hand, non-significant changes were observed in oxLDL levels. Cytokine IL-6 can be both inflammatory and anti-inflammatory, depending on certain conditions. Through metabolic syndrome conditions, higher levels of IL-6 have been linked with cardiovascular diseases, being people with the highest level of IL-6 twice to five times more likely to have a heart attack, stroke, or another cardiovascular episode [49,50]. In this study, all three treatment diets have shown a modulatory effect over IL-6 with statistically significant differences for the high-fat diet. While phenolic- enriched treatments showed the lowest IL-6 levels, especially in the gut, even the cooked ham enriched diet reduced its concentration. Those effects were previously corroborated on an *in-vitro* cell assay [51] and in mice gut [52]. While TNF-α is an inflammatory cytokine, which is downregulated through phenolic-enriched diets [53,54], IL-10 is a pleiotropic anti-inflammatory cytokine [55]. Its depletion, which was not as pronounced as those of TNF-α or IL-6, could be justified through the reduction of its expression by the treatments, but further studies, which should include PCR analysis, should be performed confirm these results. Chemokine MCP-1 is involved in macrophage recruitment. Local proliferation of macrophages has been shown to contribute to obesity-associated adipose tissue inflammation [56]. However, another study suggested that MCP-1 might be a necessary component of the inflammatory response for adipose tissue protection [57]. In this study, non-significant differences were observed through intervention in most of the cases, despite of PECH + HFD, which showed a slight, but significant reduction. The reduction of MCP-1 can result in a reduction of cardiovascular disease risk [58]. IL-1β is a pro-inflammatory cytokine produced by macrophages in response to TNF-α. Previous studies in mice gut have corroborated that polyphenols can have an inhibitory effect, resulting in a downregulation of inflammatory processes [59–61]

PON1 is an antioxidant enzyme synthesized primarily in the liver, and it is associated with high-density lipoproteins in serum [62]. Its role is to degrade toxic organophosphates and metabolize oxidized lipids [63], and lower levels of PON1 have been correlated with the metabolic disease [64]. Phenolic compounds have been proved to raise PON1 levels due to an aryl hydrocarbon receptor-dependent mechanism [65], and previous studies have demonstrated that polyphenol-enriched food increased PON1 levels [66,67]. While P + HFD has been shown to raise PON1 levels, no significant differences were achieved through PECH + HFD, which can be explained by the lower concentration of phenolic compounds. GPx is one of the main antioxidant enzymes, and its depletion is linked with the increase of free radicals [68]. Polyphenol supplementation raised GPx levels, preventing from

environmental injuries [69]. Higher levels of GPx have been associated with the prevention of some diseases such as cancer and cardiovascular disease [70]. FRAP results imply a measure of overall antioxidant capacity in plasma. Higher levels of phenolic compounds have been correlated with higher antioxidant capacity, which protects against previously mentioned diseases through neutralization of reactive oxygen species [71–74]. No differences were found between SD and HFD diet for the FRAP assay characteristics. FRAP detects free reducing compounds levels, and those are not supposed to change between a standard diet and a non-antioxidant-enriched high fat diet. Differences between HFD and CH + HFD could be explained by the antioxidants present in processed meat, such as vitamin C. Catalase is another antioxidant enzyme involved in the protection against oxidative damage through reactive oxygen species. Some studies have suggested that catalase plasma concentration can be reduced using some antioxidants like Vitamin E [75], but, in the present study, statistically significant differences were found between polyphenol enriched diets and high-fat diet. Catalase deficiency can worsen metabolic syndrome and lead to neurological and other disorders [76]. Therefore, the increase of catalase concentration in plasma, as observed in the present study, may have protective effects against age-related oxidative stress [77].

The differences observed between P + HFD and PECH + HFD on antioxidant enzymes modulation were also found in previous studies [44] and suggest that bringing the phenolic blend through processed food could have some effects due to processing (i.e., thermal processing, additives). Another difference between P + HFD and PECH + HFD is the presence of different peptides from cooked ham, which can also exert antioxidants effect [78]. Studying the mentioned factors could lead to exciting research about processed foods and polyphenol enrichment. To sum up, both P + HFD and PECH + HFD have shown significant modulation of inflammatory and oxidative status, changes which could lead to the prevention of metabolic syndrome derived diseases such as cardiovascular diseases and cancer.

5. Conclusions

Results in the murine induced obesity model, demonstrate a robust statistically significant improvement in key parameters related with obesity risk in the groups feed with a phenolic-enriched diet (P + HFD and PECH + HFD). In both groups there was an improvement in body composition parameters (Table 3), inflammatory biomarkers and antioxidant enzymes levels. Specifically, in the group feed with the phenolic enriched cooked ham (PECH + HFD) there was an improvement of total Fat volume, spleen index, plasmatic MCP1, IL-6, Il-10, TNF-α, gut IL-1β, gut IL-6 and GPx and catalase enzymes.

Table 3. Summary of comparisons between high fat diet group (HFD) and both phenolic enriched high fat diet (P+HFD) and phenolic enriched cooked ham high fat diet (PECH + HFD).

Oxidative and Inflammatory Markers		Does it Improve Compared to HFD Group in ...	
		P + HFD?	PECH + HFD?
Physiological parameters	Bodyweight	NO	NO
	Adipose tissue volume	YES ***	YES **
	Hepatic fat	YES ***	NO
	Spleen Index	YES *	YES **
Inflammatory markers (plasma)	MCP-1	NO	YES *
	IL-6	YES ****	YES ***
	IL-10	YES *	YES *
	TNF-α	YES **	YES ***
Inflammatory markers (gut)	IL-1β	YES ****	YES ****
	IL-6	YES ****	YES ****
	IL-10	NO	NO
Oxidative biomarkers	PON1	YES**	NO
	GPX	NO	YES ****
	CATALASA	YES *	YES ****
	FRAP	YES **	YES **
	PON1	NO	NO

Statistically significant differences are expressed as * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$.

Consuming foods rich in antioxidant compounds could be a new dietary strategy to mitigate the chronic inflammation and reducing oxidative stress associated with overweight or obesity and subsequent onset of other chronic non-communicable diseases. The dietary intake of some phenolic compounds, present only in certain plant foods, have shown significant antioxidant and anti-inflammatory activity through various possible mechanisms in different biological systems. Although some meat products also contain certain biopeptides with antioxidant activity, in addition to other essential nutrients, very little research has focused on studying the potential beneficial effects of consuming phenolic compounds in combination with processed meat products. The main findings of this study suggested that the consumption of a low fat and salt cooked ham and enriched with a mixture of dietary phenolic compounds exert a protective effect against the inflammatory status and the oxidative stress in a pre-clinical study of diet-induced obesity in C57/BL6J mice. These results suggest that the functionalized cooked ham with the appropriate dietary phenolics could result in the potential decrease of the risk of certain non-communicable chronic diseases. Those promising results should be further studied through human clinical assays to corroborate these potential health benefits.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/7/639/s1, Table S1: Phenolic compounds and derived metabolites searched in the urine samples by UPLC-ESI-QTOF-MS.

Author Contributions: All: writing of the manuscript; A.S. performed mice's treatment, sample collection, cytokine, chemokine, enzymatic, and antioxidant analysis. A.G.-V. developed the phenolic-enriched cooked ham model. A.G.-S. performed the OxLDL and UPLC-ESI-QTOF-MS analyses. A.A., F.A.T.-B., G.R.-B. and G.N. design of the experiments, interpretation of the results and revision of the article. All authors have read and agreed to the published version of the manuscript.

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Conclusión

Esta tesis ha demostrado que la incorporación de extractos naturales con propiedades antioxidantes y antiinflamatorias a los productos cárnicos procesados puede ofrecer una solución viable para mejorar la calidad nutricional de estos alimentos. A través de una exhaustiva investigación experimental, se ha evidenciado que los compuestos fenólicos, obtenidos de fuentes como el romero, el té verde y la semilla de uva, no solo mejoran la estabilidad antioxidante de los productos cárnicos, sino que también tienen un impacto positivo en la salud.

El uso de modelos celulares, en particular los macrófagos RAW 264.7 y los colonocitos Caco-2, ha permitido observar una significativa reducción de los niveles de especies reactivas de oxígeno (ROS) y citoquinas proinflamatorias, lo que indica un efecto inmunomodulador. Estos resultados sugieren que los productos cárnicos reformulados pueden jugar un papel clave en la reducción de los biomarcadores asociados a enfermedades crónicas, como el cáncer colorrectal y las enfermedades cardiovasculares.

Los ensayos preclínicos con un modelo animal de obesidad inducida también apoyan estos hallazgos, mostrando una mejora significativa en los niveles de inflamación sistémica y estrés oxidativo tras la inclusión de estos compuestos en la dieta. La reformulación con extractos fenólicos no solo benefició la respuesta inflamatoria a nivel intestinal, sino que también tuvo efectos positivos en otros órganos clave como el hígado y el tejido adiposo, reduciendo los factores de riesgo asociados al síndrome metabólico.

En términos de impacto industrial, esta tesis ofrece una propuesta innovadora para la producción de alimentos cárnicos funcionales. Las barreras tecnológicas para la implementación de estas reformulaciones parecen ser superables, lo que abre nuevas oportunidades para la industria alimentaria de cara a ofrecer productos más saludables sin comprometer el sabor ni la calidad.

Finalmente, este trabajo refuerza la importancia de un enfoque multidisciplinario en la mejora de la salud pública, integrando conocimientos de nutrición, tecnología de alimentos y medicina. Los resultados obtenidos sugieren que es posible desarrollar alimentos que no solo cumplan con los

estándares de calidad, sino que también ofrezcan beneficios para la salud a largo plazo.