UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

Estudio de los mecanismos fisiopatológicos del consumo sostenido de fructosa y del efecto protector de un extracto de granada en ratas *Wistar* mediante el uso de técnicas ómicas

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But if you never try, you'll never know

Índice

Glosario de términos

AGEs, productos finales de glicación avanzada (del inglés, *advanced glycation end-products*).

APOPs, productos avanzadas de oxidación proteica (del inglés, *advanced protein oxidation products*).

BAT, tejido adiposo marrón (del inglés, *brown adipose tissue*).

DNL, *de novo* lipogénesis.

EA, ácido elágico (del inglés, *ellagic acid*).

EII, enfermedad inflamatoria intestinal.

GIT, tracto gastrointestinal (del inglés, *gastrointestinal tract*).

GLUT2, transportador de glucosa 2 (del inglés, *facilitative glucose transporter 2*).

GLUT5, transportador de glucosa 5 (del inglés, *facilitative glucose transporter*).

HFCS, jarabe de maíz rico en fructosa (del inglés, *high-fructose corn syrup*).

MDA, malondialdehído.

MetS, síndrome metabólico (del inglés, *metabolic syndrome*).

NAFLD, enfermedad del hígado graso no alcohólico (del inglés, *non-alcoholic fatty liver disease*).

NASH, esteatosis hepática no alcohólica (del inglés, *non-alcoholic steatohepatitis*).

OMS, Organización Mundial de la Salud.

P, extracto de granada rico en punicalagina.

PU, punicalagina.

RAGE, receptor proinflamatorio de AGEs (del inglés, *receptor for advanced glycation end-products*).

RCS, especies reactivas carbonílicas (del inglés, *reactive carbonyl species*).

RNS, especies reactivas de nitrógeno (del inglés, *reactive nitrogen species*).

ROS, especies reactivas de oxígeno (del inglés, *reactive oxygen species*).

SAT, tejido adiposo subcutáneo (del inglés, *subcutaneous adipose tissue*).

SCFAs, ácidos grasos de cadena corta (del inglés, *short chain fatty acids*).

T2DM, diabetes *mellitus* tipo 2 (del inglés, *type 2 diabetes mellitus*).

VAT, tejido adiposo visceral (del inglés, *visceral adipose tissue*).

WAT, tejido adiposo blanco (del inglés, *white adipose tissue*).

3NT, 3-nitrotirosina.

α-AS, semialdehído α-aminoadípico (del inglés, α-*aminoadipic semialdehyde*).

γ-GS, semialdehído γ-glutámico (del inglés, *γ-glutamic semialdehyde*).

RESUMEN

Resumen

Algunos de los compuestos que ingerimos normalmente, como los azúcares reductores, promueven la aparición de estrés oxidativo en el lumen del tracto gastrointestinal (GIT, del inglés *gastrointestinal tract*). Es conocido el papel que el estrés oxidativo promovido *in vivo* tiene sobre diversos procesos patológicos en el GIT, como algunos tipos de cánceres gastrointestinales, colitis ulcerosa, enfermedad de Crohn, u otras enfermedades inflamatorias del intestino. El consumo de azúcares reductores favorece el aumento del estrés oxidativo luminal. La relación entre el aumento del consumo de azúcares reductores, como la fructosa, entre la población, así como la alta prevalencia de enfermedades metabólicas, como obesidad, hipertrigliceridemia, esteatosis hepática no alcohólica o enfermedad del hígado graso no alcohólico (NAFLD, del inglés *non-alcoholic fatty liver disease*), resistencia a la insulina o diabetes, entre otras, es un tema que preocupa enormemente a las autoridades sanitarias. Por esto, el conocimiento de los mecanismos que promueven el inicio y/o el desarrollo de estas patologías bajo consumos elevados de azúcares resulta imprescindible en el avance sobre estrategias nutricionales que permitan disminuir el efecto dañino.

Por otro lado, las proteínas de los alimentos que ingerimos son objetivo del estrés oxidativo luminal, y su interacción con los radicales libres podría dar lugar a la formación de compuestos no deseados cuyas consecuencias para los consumidores permanecen aún sin esclarecerse. La formación de estos compuestos en cualquiera de las fases de la digestión hace que el GIT y el resto de los órganos implicados en la asimilación de los alimentos queden expuestos al potencial efecto tóxico y mutagénico de algunas de estas especies. En la presente tesis doctoral, se ha evaluado cómo la presencia de glucosa y fructosa afecta a la digestibilidad de las proteínas durante su digestión, mediante un modelo *in vitro* de digestión y un ensayo *in vivo* en el que se analizaron los contenidos gastrointestinales de ratas *Wistar* expuestas a un consumo elevado de fructosa en el agua de bebida durante 10 semanas. Así mismo, en el experimento *in vivo*, se analizaron los tejidos gastrointestinales y hepático, así como el suero y la orina de los animales expuestos tanto a la suplementación con fructosa como a una suplementación con un extracto de granada rico en punicalagina. Las técnicas avanzadas utilizadas en la presente tesis doctoral han permitido analizar las bases de los mecanismos fisiopatológicos en los que se ve involucrada la fructosa y el efecto beneficioso del suplemento rico en punicalagina sobre la fisiopatología de los animales.

Palabras clave: fructosa, glicoxidación de proteínas, metabolómica, proteómica, punicalagina.

ABSTRACT

Abstract

Some of the compounds we commonly ingest, such as reducing sugars, promote the onset of oxidative stress in the lumen of the gastrointestinal tract (GIT). The role of the *in vivo*-promoted oxidative stress in several pathologies within the GIT, including gastrointestinal cancers, ulcerative colitis, Crohn's disease, or other inflammatory bowel diseases, is well known. The consumption of reducing sugars, such as fructose, promotes the increase of luminal oxidative stress. The association between increased reducing sugars consumption among the population and the high prevalence of metabolic diseases such as obesity, hypertriglyceridemia, non-alcoholic fatty liver disease (NAFLD), insulin resistance, or diabetes is a matter of great concern for health authorities. Therefore, understanding the mechanisms that promote the onset and/or development of the pathologies related to high sugars consumption is essential for improving nutritional strategies to reduce the harmful effects.

On the other hand, the food proteins we ingest are targets of the luminal oxidative stress, and their interaction with free radicals could lead to the formation of undesired compounds whose consequences for consumers remain unclear. The formation of these compounds in any phase of the digestion exposes the GIT and other organs to the potential toxic and mutagenic effects of some of these species. In this doctoral thesis, we assessed how the presence of glucose and fructose affects protein digestibility during digestion, using an *in vitro* digestion model and an *in vivo* assay in which the gastrointestinal contents of *Wistar* rats exposed to high fructose consumption in drinking water for 10 weeks were analyzed. Likewise, in the *in vivo* experiment, the gastrointestinal and hepatic tissues, as well as the serum and the urine from animals exposed to both fructose supplementation and supplementation with a punicalagin-rich pomegranate extract, were analyzed. The advanced techniques employed in this doctoral thesis have allowed for an analysis of the physiopathological mechanisms involving fructose and the beneficial effects of the polyphenol-rich supplement on the physiopathology of the animals.

Keywords: fructose, metabolomic, proteomic, protein glycoxidation, punicalagin.

INTRODUCCIÓN

1. Introducción

Los procesos de oxidación han sido reconocidos desde hace décadas como una de las causas no microbiológicas más importantes del deterioro de la calidad de carne y productos cárnicos, tanto desde el punto de vista sensorial, como tecnológico. Durante años, la comunidad científica ha estudiado en profundidad la oxidación de lípidos por sus negativas implicaciones tecnológicas, mientras que la oxidación de proteínas ha sido menos estudiada. Actualmente se trata de entender, cada vez con más precisión, los mecanismos en los que se basa la oxidación de proteínas en los alimentos por su potencial implicación en el deterioro de la textura de los mismos, la pérdida de funcionalidad o la menor digestibilidad de las proteínas *(*Lund *et al.*, 2011).

La ingesta de alimentos con proteínas oxidadas ha suscitado un enorme interés entre la comunidad científica en los últimos años. Así puede intuirse a partir del número de publicaciones y citaciones conectadas en relación con la digestión de alimentos ultraprocesados (Figura 1.1.).

Figura 1.1. Visualización de la red online de publicaciones y citaciones conectadas con el trabajo desarrollado por Van Hecke *et al.* (2018). Fuente: https://www.connectedpapers.com/

Sin embargo, los mecanismos moleculares implicados en los procesos de oxidación proteica durante la digestión de los alimentos permanecen siendo casi desconocidos. Teniendo en cuenta la potencial implicación de los fenómenos de oxidación de proteínas en algunos procesos, como en enfermedades neurodegenerativas relacionadas con el envejecimiento, tipo Alzheimer o Parkinson, enfermedades inflamatorias intestinales o diabetes *mellitus* tipo 2 (T2DM, del inglés *type 2 diabetes mellitus*), entre otras (Estévez, 2011; Estévez & Xiong, 2019; Oliphant & Allen-Vercoe, 2019; Rodríguez-Romero *et al.*, 2022), parece razonable cuestionarse en qué medida la oxidación de proteínas durante los procesos de digestión puede contribuir a la aparición o incluso al agravamiento de síntomas relacionados con algunas de estas patologías.

1.1. Concepto de oxidación y glicoxidación de proteínas

La oxidación es un proceso que se define como la cesión o pérdida de electrones de los átomos de una molécula. Esta reacción va unida a otra de ganancia de electrones por parte de otra molécula, la cual se denomina reducción. El conjunto de ambas se recoge bajo el término reacciones de oxidación-reducción (redox).

En los organismos vivos, las reacciones redox resultan fundamentales para los procesos de obtención de energía por parte de las células, a partir de los cuales se produce la formación de radicales libres o ROS (del inglés, *reactive oxygen species*). Sin embargo, el metabolismo de determinadas sustancias, así como por ejemplo la instauración de procesos inflamatorios, entre otras reacciones, originan igualmente la formación de radicales libres (Vona *et al.*, 2021).

En este contexto surge el concepto de estrés oxidativo. El estrés oxidativo es causado principalmente por un desequilibrio entre la generación de especies oxidantes y las defensas antioxidantes propias de los organismos biológicos (Davies, 2005). Las ROS son moléculas altamente prooxidantes que encuentran la estabilidad tras la substracción de electrones provenientes de los átomos de otras moléculas, como las proteínas. Adicionalmente, existen otras sustancias derivadas de distintas reacciones biológicas que son consideradas igualmente prooxidantes, como las RCS (del inglés, *reactive carbonyls species*), que se forman tras la interacción de azúcares reductores y el extremo amino de las proteínas; o las RNS (del inglés, *reactive nitrogen species*), que se forman a partir de sustancias nitrogenadas.

En todo caso, cuando las proteínas son sometidas a estrés oxidativo sufren procesos de fragmentación, agregación y polimerización (Figura 1.2), que se traducen en una alteración bioquímica y estructural que, a su vez, afectaría a su funcionalidad y digestibilidad (Lund *et al.*, 2011).

La carbonilación (Figura 1.2) es una de las modificaciones químicas más destacables que tienen lugar cuando se produce la oxidación de proteínas (Estévez, 2011). Cuando se habla de carbonilación de proteínas, deben tenerse en cuenta diferentes mecanismos:

- Carbonilación proteica primaria, la cual libera dos semialdehídos principalmente: α-aminoadípico (α-AS) y γ-glutámico (γ-GS), derivados de la oxidación de lisina y de arginina y prolina, respectivamente. Este tipo de carbonilación de proteínas está ligada a un estrés

oxidativo promovido por radicales libres o ROS. También recientemente se ha relacionado con la presencia de RCS, que son dicarbonilos como el glioxal o el metilglioxal, los cuales son derivados de reacciones tipo Maillard en las que participan azúcares reductores. Cuando se produce la oxidación de proteínas como consecuencia de la presencia de este tipo de RCS junto con la de ROS se acuña el término de glicoxidación de proteínas.

- Carbonilación proteica secundaria, mediante enlaces covalentes entre las proteínas y carbonilos lipídicos como el malonaldehído (MDA) (Estévez *et al.*, 2021).

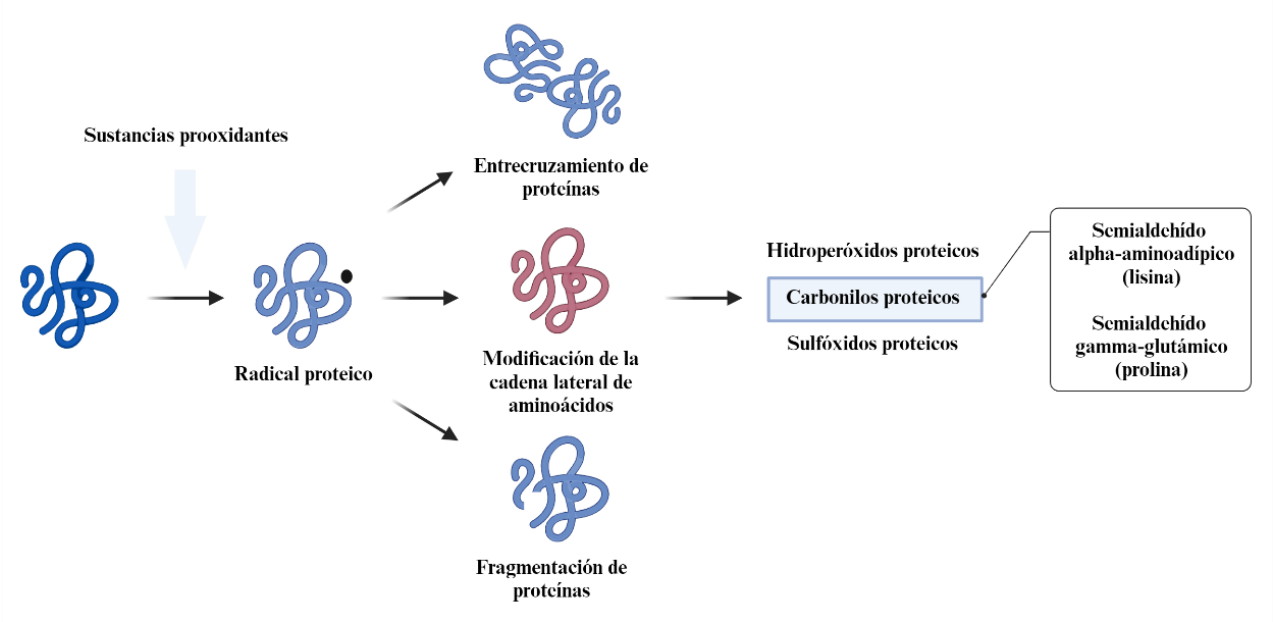

Figura 1.2. Modificaciones que pueden tener lugar en la estructura de las proteínas tras la formación de un radical proteico. Elaboración propia, basada en el trabajo de Lund *et al.* (2011).

Por otro lado, en presencia de azúcares reductores, las proteínas pueden verse envueltas en procesos de glicación. Este tipo de reacción fue descrita hace más de un siglo en alimentos por Maillard (Maillard, 1912), haciéndola responsable de las coloraciones marronáceas que aparecen en los alimentos tras ser sometidos a procesos térmicos. En ocasiones, determinados compuestos químicos son añadidos a los productos procesados buscando precisamente promover este tipo de reacciones, consiguiendo cambios en el aroma, el sabor o la textura de los alimentos deseables para los consumidores (Twarda-clapa *et al.*, 2022). A lo largo de los años, la investigación ha ido avanzando y ha pasado de identificar el acontecimiento de las reacciones de glicación en los alimentos a relacionar la glicación de proteínas *in vivo* con estadios de enfermedad tales como hiperglicemia, resistencia a la insulina o diabetes (Semchyshyn, 2013; Twarda-clapa *et al.*, 2022).

La glicación de proteínas consiste en una reacción no enzimática entre el grupo amino de aminoácidos, péptidos y/o proteínas y el grupo carbonilo de azúcares reductores como la glucosa o la fructosa. La condensación del grupo amino y el grupo carbonilo da lugar a la formación de bases de Schiff (aldiminas). Estos compuestos resultan bastante inestables, y tras una serie de reordenamientos pueden dar lugar a compuestos de glicación temprana, que resultan precursores de compuestos de glicación avanzada o AGEs (del inglés, *advanced glycation end-products*), totalmente estables. En función del azúcar reductor que se vea envuelto en estas reacciones, los compuestos de glicación temprana se denominan productos de Amadori, en el caso de la glucosa (glicación), y productos de Heyns, en el caso de la fructosa (fructación).

Tanto la formación de AGEs como la propia autooxidación de los azúcares origina la formación de ROS y RCS. También la degradación de las propias bases de Schiff contribuye a la formación de estas especies reactivas. Cada una de las rutas de formación de ROS y RCS a partir de fructosa (reacciones de fructación) aparecen plasmadas en la Figura 1.3.

El aumento tanto de AGEs como de ROS y RCS tiene un papel muy importante en el aumento del estrés oxidativo y carbonílico. Como se indica anteriormente, las proteínas pueden ser carboniladas tanto por la presencia de ROS como de RCS (Estévez *et al.*, 2021). En este contexto se acuña el término de glicoxidación de proteínas o estrés glicoxidativo (Luna & Estévez, 2019; Semchyshyn, 2013). Un acercamiento a la química de estas reacciones se describe en la Figura 1.3.

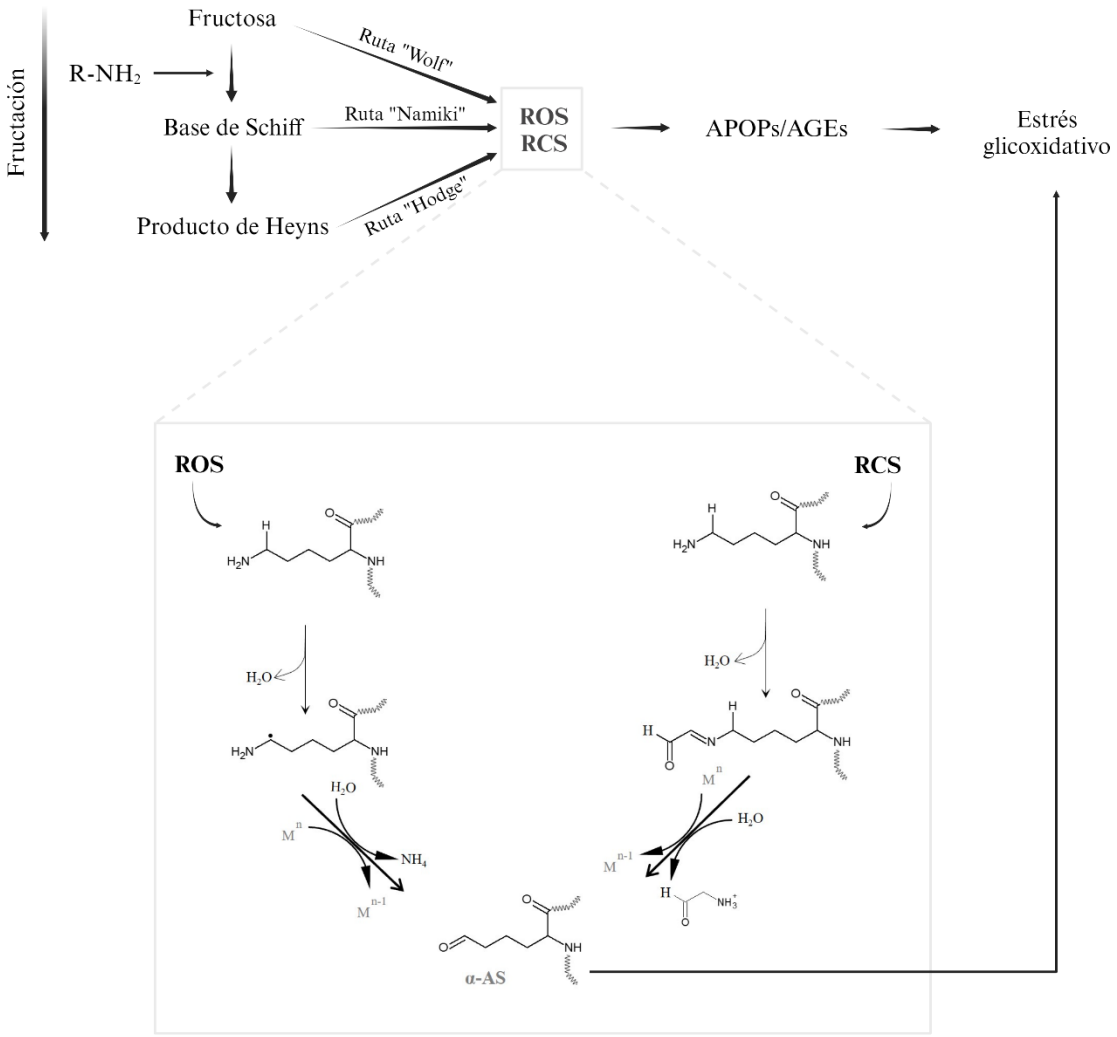

Figura 1.3. Mecanismo de glicoxidación de proteínas en presencia de fructosa. Elaboración propia, basada en los trabajos de Estévez *et al.* (2021) y Semchyshyn (2013).

La fructosa ha sido descrita como un monosacárido mucho más reactivo que la glucosa en las reacciones de Maillard, debido a la estabilidad de su cadena abierta y su grupo cetónico (Gugliucci, 2017; Semchyshyn, 2013). Cada reacción de fructación libera un radical superóxido, por lo que la fructosa genera 100 veces más ROS que la glucosa y promueve la apoptosis celular y la inflamación (Jiang *et al.*, 2021). Por ello, la presente tesis doctoral se centrará principalmente en las actuaciones del primero de estos compuestos.

1.2. Estrés oxidativo luminal y postprandial

Las circunstancias en las que la oxidación *in vivo* puede afectar a las proteínas son muy diferentes a las que subyacen a la oxidación de proteínas en la carne y los derivados cárnicos destinados al consumo humano a lo largo de su procesamiento y almacenamiento. Numerosas ROS generadas bajo las diferentes condiciones que tienen lugar a lo largo de la cadena de procesado (por ejemplo, altas temperaturas de procesado, bajo pH o autooxidación de azúcares reductores) y el posterior consumo, son capaces de iniciar cascadas de reacciones que pueden dar lugar a oxidación proteica y formación de AGEs durante el cocinado de la carne y durante su digestión (Martinez-Saez *et al.*, 2019; Rysman *et al.*, 2016; Twarda-clapa *et al.*, 2022; Van Hecke *et al.*, 2018).

A lo largo de los años, son varios los estudios que han investigado las reacciones de oxidación que tienen lugar en el animal vivo, debido a su tipo de crianza, dieta, genética, etc., como por ejemplo Moreno *et al.* (2020) o Ventanas *et al.* (2006), entre otros. Otros autores se han centrado en las reacciones de oxidación que tienen lugar durante el procesado *post mortem* de la carne y los productos cárnicos, como durante la manipulación (Soladoye *et al*., 2015), debido al efecto del almacenamiento (Feng *et al.*, 2016; Rysman *et al.*, 2016), procesos de maduración (He *et al.*, 2018; Ventanas et al., 2006), salmueras o como consecuencia del cocinado previo al consumo (Mitra *et al.*, 2018; Xiong *et al.*, 2020).

Probablemente, el campo menos explorado, aunque cada vez de más interés, es el referente a la oxidación *in situ* de proteínas durante la digestión de la carne y de los productos cárnicos (Figura 1.1).

Además de afectar negativamente a la calidad de los alimentos, la oxidación de proteínas promueve el estrés oxidativo dietético, que hace referencia a la alteración del estado redox *in vivo* como consecuencia de la dieta. La ingesta de alimentos con altos niveles de proteínas o lípidos oxidados promueve un aumento del estrés oxidativo en el lumen del tracto gastrointestinal (GIT, del inglés *gastrointestinal tract*), el cual favorece la oxidación de proteínas *in vivo*. Sin embargo, la ingesta conjunta de alimentos proteicos junto con azúcares reductores también podría promover estrés oxidativo luminal, que, en este caso concreto, podría denominarse estrés glicoxidativo luminal. En los organismos vivos, los sistemas antioxidantes de los propios tejidos se encuentran activos y protegen al organismo de los ataques de los radicales libres que constantemente se están generando. Sin embargo, estos mecanismos antioxidantes pueden verse sobrepasados cuando aumenta el estrés

oxidativo luminal (Estévez & Xiong, 2019). En todo caso, tanto el estrés oxidativo dietético como el consumo de compuestos que favorecen el estrés oxidativo luminal *in situ*, conllevan inevitablemente a un aumento del estrés oxidativo postprandial, que hace referencia a aquél que afecta a células y tejidos de órganos internos tras la ingesta de alimentos y que habitualmente se determina mediante la elevación de productos de oxidación en plasma (Kanner *et al.*, 2017).

El daño que puede causar la oxidación de proteínas *in vivo* sobre el funcionamiento y la integridad de células y tejidos a su paso por el GIT se describe con más detalle en los siguientes subapartados de la presente tesis doctoral. Elevados niveles de estrés oxidativo luminal de forma crónica pueden conllevar la activación de mecanismos proinflamatorios e inflamación de los tejidos en sí, lo cual a su vez agravaría paradójicamente el ambiente prooxidante del lumen (Vona *et al.*, 2021). Por tanto, resulta de gran relevancia conocer los cambios que tienen lugar en el ambiente luminal a lo largo del GIT con respecto al estrés oxidativo, dada su implicación en el daño a tejidos, y de cara a la multitud de enfermedades intestinales relacionadas con procesos inflamatorios del GIT, como las úlceras estomacales, la enfermedad inflamatoria intestinal (EII), la enfermedad de Crohn, la colitis ulcerosa, o el cáncer de colon (Bhattacharyya *et al.*, 2014; Scott *et al.*, 2013).

1.3. Oxidación de proteínas durante la digestión de los alimentos

1.3.1. Digestión de proteínas: estómago e intestino delgado

La digestión de las proteínas de la dieta comienza una vez el alimento llega al estómago. Las condiciones aerobias de este primer compartimento del GIT, así como la acidez que se alcanza en el mismo, hacen que el ambiente estomacal pueda ser considerado como un potente prooxidante (Kanner & Lapidot, 2001). Los jugos gástricos promueven el despliegue de la estructura proteica, asegurando el reconocimiento y acción por parte de las enzimas proteolíticas (Giromini *et al.*, 2019). Sin embargo, la desnaturalización de estas proteínas también podría aumentar la exposición de sus grupos hidrofóbicos a otras sustancias. Esto, junto con el ambiente prooxidante del estómago (Kanner & Lapidot, 2001), facilitaría la formación de enlaces cruzados y agregados proteicos (Lund *et al.*, 2011).

Los cambios que induce la oxidación de proteínas en la estructura de las mismas podría afectar a su digestibilidad, alterando la capacidad de las enzimas proteolíticas del GIT de reconocer y acceder al lugar de escisión (He *et al.*, 2018; Li *et al.*, 2017; Sante-Lhoutellier *et al.*, 2007). En este contexto, tanto las proteínas con modificaciones estructurales consumidas como tal, como las oxidadas *in situ*

en los primeros compartimentos del GIT, podrían pasar desapercibidas para las enzimas proteolíticas responsables de su degradación, presentes tanto en el estómago con en el intestino (Yin *et al.*, 2020). Una de las consecuencias directas de esto sería el avance sin degradar de estas proteínas alteradas hacía compartimentos posteriores como el colon (de La Pomélie, Santé-Lhoutellier, Sayd, *et al.*, 2018), como puede apreciarse en la Figura 1.4, rompiendo el patrón natural de digestión, y fomentando así la formación de compuestos nocivos para la salud humana (Kanner & Lapidot, 2001).

Sin embargo, también está documentado que elevados niveles de estrés oxidativo en el lumen podrían dañar los tejidos circundantes, no solo por la acción directa de ROS sobre ellos, sino también por la misma acreción inherente a los procesos de absorción de compuestos oxidados (Estévez & Luna, 2017; Estévez & Xiong, 2019; Soladoye *et al.*, 2015). Existen numerosos procesos patológicos gastroduodenales que cursan con inflamación relacionados directamente con el efecto del estrés oxidativo luminal sobre el epitelio gastrointestinal, como la úlcera péptica, la gastritis o el cáncer (Bhattacharyya *et al.*, 2014). En este contexto, son varios los autores que han investigado el daño a tejidos gástricos bajo condiciones de elevado estrés oxidativo luminal, como por ejemplo el ambiente que se genera durante la infección por *Helicobacter pilori* (Handa *et al.*, 2010; Li *et al.*, 2001; Van Hecke *et al.*, 2017).

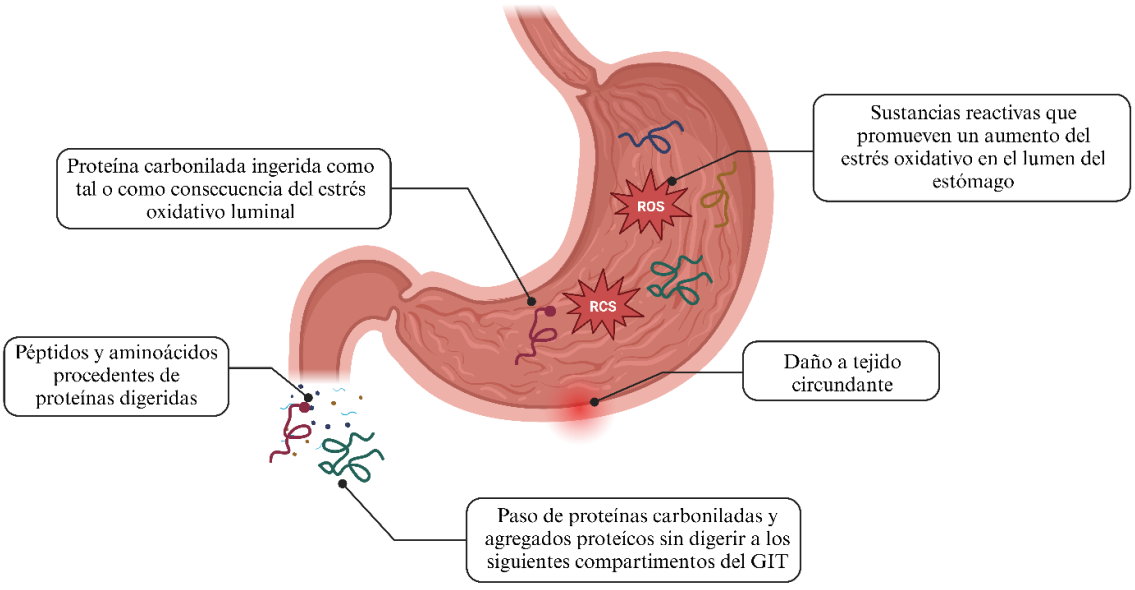

Figura 1.4. Resumen gráfico de las consecuencias de la ingesta de proteínas oxidadas y del estrés oxidativo luminal sobre las proteínas en el lumen gastrointestinal. Elaboración propia.

1.3.2. Fermentación de proteínas: colon

La microbiota, formada por unas 10^{11} células/ml de contenido colónico (Scott *et al.*, 2013), puede ser considerada en su conjunto como un órgano metabólico activo debido a su capacidad de biotransformar los componentes nutricionales procedentes de los alimentos en metabolitos microbianos (Rodríguez-Romero *et al.*, 2022). Los microorganismos más abundantes del intestino grueso humano pertenecen principalmente a los *phylum* Firmicutes, Bacteroidetes, Actinobacterias, Verrucomicrobia y Proteobacteria (Scott *et al.*, 2013). Cualquier cambio en la abundancia de los microorganismos que conforman la microbiota generaría una situación de disbiosis, la cual podría tener relación con un amplio rango de patologías, desde procesos inflamatorios intestinales hasta depresión, o incluso cáncer (Ding *et al.*, 2019; Richards *et al.*, 2016).

Ciertas bacterias colónicas son capaces de metabolizar una gran variedad de sustratos, mientras que otras están especializadas en la degradación de componentes más concretos de la dieta. Cambios en la abundancia y/o naturaleza de los sustratos que alcanzan el colon, promovidos por cambios en la frecuencia y/o cantidad de su ingesta o por cambios en su digestión en fases previas (estómago/intestino delgado), podrían favorecer el crecimiento de ciertos microorganismos sobre otros, alterando el equilibrio entre ellos y modificando así la composición de la microbiota.

La llegada al colon de mayores cantidades de proteínas, bien como consecuencia de un consumo excesivo o bien debido a una digestión enzimática alterada, podría originar cambios en la proporción de microorganismos que conforman la microbiota, favoreciendo el desarrollo de bacterias proteolíticas (Portune *et al.*, 2016). La utilización de proteínas como sustrato por parte de la microbiota conlleva en ocasiones connotaciones negativas (Oliphant & Allen-Vercoe, 2019). La presencia en el colon de este tipo de compuestos podría favorecer fermentaciones prolongadas por parte de la microbiota, lo cual podría ocasionar la producción de metabolitos nocivos para la salud (Van Hecke *et al.*, 2021). Como resultado del catabolismo microbiano de proteínas se generan metabolitos tóxicos como aminas, fenoles, indoles y compuestos azufrados, en función del perfil predominante de aminoácidos que actúe como sustrato (Fan *et al.*, 2015; Portune *et al.*, 2016; Yao *et al.*, 2016). Así, la descarboxilación de aminoácidos como estrategia catabólica por parte de ciertos microorganismos sobre la desaminación generaría cantidades mayores de amonio en el lumen colónico, cuyo exceso podría afectar negativamente al hospedador (Oliphant & Allen-Vercoe, 2019; Rodríguez-Romero *et al.*, 2022). El metabolismo por parte de la microbiota de aminoácidos

aromáticos como el triptófano promueve la formación de indoles (Rodríguez-Romero *et al.*, 2022), mientras que el metabolismo de aminoácidos azufrados como cisteína y metionina por parte de bacterias con las enzimas de degradación requeridas, como las pertenecientes al género *Clostridium*, puede ocasionar la prevalencia de sustancias azufradas en el colon (Oliphant & Allen-Vercoe, 2019). No obstante, hay que tener en cuenta que no todos los metabolitos microbianos que se generan como consecuencia del metabolismo de las proteínas tienen este carácter nocivo. Así, son muchas las bacterias que tienen la capacidad de producir ácidos grasos de cadena corta (SCFAs, del inglés *short-chain fatty acids*) a partir del metabolismo de aminoácidos, como por ejemplo el butirato, el cual destaca por su impacto positivo sobre la integridad barrera intestinal (Oliphant & Allen-Vercoe, 2019).

En este contexto, existe un creciente interés entre la comunidad científica en establecer relaciones directas entre géneros, e incluso especies, de bacterias concretas y metabolitos nocivos cuando se consumen tanto elevadas cantidades de proteínas como proteínas dañadas (Amaretti *et al.*, 2019; Shi *et al.*, 2020; Van Hecke *et al.*, 2021; Yin *et al.*, 2022). Esto resulta transcendental tanto de cara a identificar los potenciales desencadenantes de enfermedades prevalentes en la sociedad actualmente, como de cara a la aplicación de estrategias nutricionales que ayuden a paliar los efectos perjudiciales del consumo y la formación de ciertos compuestos nocivos durante la digestión.

1.4. Metabolismo intestinal de los azúcares reductores: fructosa

El empleo de fructosa en la industria alimentaria está ampliamente implantado a través del uso de sacarosa, formada por glucosa y fructosa, y jarabes de maíz ricos en fructosa (55% de fructosa), también conocidos como HFCS, del inglés *high-fructose corn syrup* (Shi *et al.*, 2021). El consumo de productos con altos contenidos en fructosa ha aumentado considerablemente en las últimas décadas a través de la ingesta de productos de bollería, bebidas azucaradas y snacks, entre otros, formulados con sacarosa y HFCS (Merino *et al.*, 2020). Existe numerosa bibliografía en la que se ha relacionado el consumo de este azúcar reductor con diversas alteraciones metabólicas como obesidad, adiposidad, dislipidemia, enfermedad del hígado graso no alcohólico (NAFLD, del inglés *non-alcoholic fatty liver disease*), resistencia a la insulina o T2DM, entre otras (Hannou *et al.*, 2018; Herman & Birnbaum, 2021; Muriel *et al.*, 2021), englobadas todas ellas bajo la patología que suscribe el síndrome metabólico (MetS, del inglés *metabolic syndrome*). La Organización Mundial de la Salud (OMS) recomienda desde hace años la limitación de la ingesta de azúcar a menos de un

10% de la ingesta energética total debido al riesgo documentado de enfermedades metabólicas (WHO, 2018). Dado el contenido en fructosa del azúcar, esto supondría no consumir más de 12 gramos de fructosa por día.

La capacidad de los azúcares reductores para inducir el inicio de reacciones de glicoxidación de proteínas durante su digestión ha sido documentada en varios estudios *in vitro* en los cuales se simularon las condiciones fisiopatológicas del estómago (Kanner & Lapidot, 2001; Oueslati *et al.*, 2016). La glicoxidación de proteínas implica la reacción de residuos de proteínas susceptibles con ROS y RCS (Estévez *et al.*, 2021; Hecker & Wagner, 2018; Luna & Estévez, 2019), formados en gran medida a partir de la autooxidación de azúcares reductores y de la formación de AGEs, como se ha descrito anteriormente en la presente tesis doctoral. La fructosa contribuye a crear un ambiente aún más prooxidante en el estómago a consecuencia de su autooxidación y de su papel en la formación de AGEs en los primeros compartimentos del GIT, como así lo recogen algunos ensayos *in vitro,* como el desarrollado por Bains *et al.* (2017) o Martinez-Saez *et al.* (2019). Como se ha descrito con anterioridad, la oxidación de proteínas deteriora la digestibilidad de las mismas modificando su composición aminoacídica, promoviendo de esta manera la incapacidad por parte de las enzimas proteolíticas de degradarlas y favoreciendo su paso por tanto a compartimentos posteriores del GIT, donde quedarían expuestas a largos procesos de fermentación por parte de la microbiota (Soladoye *et al.*, 2015). Algo menos se sabe del efecto de las reacciones de glicación sobre dichos fenómenos de digestibilidad y de efectos perjudiciales sobre la microbiota.

Cuando se consumen elevadas cantidades de fructosa, además de favorecer el estrés glicoxidativo luminal, también se favorece la llegada a colon y a hígado del azúcar reductor (Jang *et al.*, 2018). El intestino delgado es el órgano responsable de la primera degradación de fructosa a través del transportador de glucosa intestinal 5, también conocido como GLUT5 (del inglés, *facilitative glucose transporter* 5) (Jang *et al.*, 2018). El proceso de fructolisis intestinal y sus implicaciones metabólicas se explica con más detalle en el siguiente subapartado, por compartir la mayoría de las etapas con la fructolisis hepática. En este subapartado se intentará centrar la atención en las implicaciones que parece tener la presencia de fructosa en el lumen intestinal.

Según Jang *et al.* (2018), cuando se produce una ingesta superior a 1 g/kg de fructosa se podría saturar la capacidad del transportador encargado de la primera fase de su metabolismo. En este contexto, mayores cantidades de fructosa llegarían al colon y serían utilizadas por los

microorganismos, generando cambios sobre la microbiota y la funcionalidad del tracto que numerosos autores han intentado dilucidar (Beisner *et al.*, 2020; Guo *et al.*, 2021; Lambertz *et al.*, 2017; Mastrocola *et al.*, 2018; Song *et al.*, 2023; Wang *et al.*, 2022; Wang *et al.*, 2020). Así, la producción por parte de la microbiota de determinados metabolitos, como el ácido acético, se ha relacionado con la lipogénesis hepática debido al consumo excesivo de fructosa (Zhao *et al.*, 2020).

La formación de AGEs como consecuencia de la presencia del azúcar reductor en el lumen gastrointestinal podría tener un papel en la fisiopatología asociada al consumo de fructosa, desencadenando procesos inflamatorios que involucrarían al receptor proinflamatorio de productos finales de glicación avanzada (RAGE, del inglés *receptor for advanced glycation endproducts*) (Bains *et al.*, 2017). Son varios los autores que han centrado sus investigaciones en el estudio de la formación endógena de AGEs durante la digestión gástrica y duodenal a partir de modelos *in vitro* en los que la fructosa estuvo presente, como los mencionados Bains *et al.* (2017) o Martinez-Saez *et al.* (2019).

Dada la acumulación documentada de AGEs en determinados tejidos en situaciones de diabetes (Rahbar *et al.*, 1969), artritis reumatoide (Verzijl *et al.*, 2003) o enfermedad cardiovascular (Twarda-clapa *et al.*, 2022), esclarecer en qué medida la fructosa podría participar en la formación endógena de estos durante la digestión resulta de gran relevancia. Así, autores como Mastrocola *et al.* (2018) investigaron el papel de la fructosa sobre la formación de AGEs en un modelo *in vivo,* además de su su impacto sobre la microbiota. Los investigadores suministraron a ratones macho de la cepa C57B1/6J distintas dietas basadas en un aporte del 60% de fructosa durante 12 semanas, distinguiendo entre una dieta sólida enriquecida y una dieta líquida en la que fue el agua de bebida la que se enriqueció con el azúcar. Bajo este diseño experimental, observaron que la llegada a colon de mayores cantidades de fructosa promovió la acumulación de AGEs en los enterocitos y cambios en la microbiota intestinal, así como procesos de permeabilización de la membrana intestinal. Posteriormente, otros autores también dilucidaron en su estudio cambios en la expresión de proteínas involucradas en el mantenimiento de la integridad de la membrana intestinal, como consecuencia del estrés glicoxidativo promovido por la presencia de fructosa en el lumen (Cho *et al.*, 2021), lo cual podría favorecer el paso al torrente sanguíneo de sustancias perjudiciales que podrían desencadenar situaciones de endotoxemia.

1.5. Metabolismo hepático de fructosa: bases moleculares

La incapacidad del intestino de metabolizar fructosa cuando se consumen elevadas cantidades del azúcar (> 1 g/kg) podría afectar también al hígado. Desde hace décadas, se ha propuesto a este órgano como principal responsable del metabolismo de la fructosa tras su absorción intestinal (Softic *et al.*, 2016). Sin embargo, gracias al trabajo realizado por Jang *et al.* (2018), se empezó a reconocer el papel del intestino delgado en la degradación del azúcar reductor, implicando al metabolismo intestinal de fructosa en la fisiopatología asociada a su consumo excesivo y sostenido (Herman & Birnbaum, 2021; Muriel *et al.*, 2021). En la actualidad, la degradación hepática de fructosa mantiene una relación prácticamente consolidada con la instauración de esteatosis hepática no-alcohólica o NAFLD, cuyas bases moleculares están desarrolladas (Herman & Birnbaum, 2021; Softic *et al.*, 2020), la cual podría degenerar en procesos de fibrosis hepática (NASH, del inglés *non-alcoholic steatohepatitis*).

El consumo sostenido y elevado de fructosa se relaciona con un aumento de la adiposidad corporal, deposición de grasa hepática, alteración de marcadores lipídicos en sangre, elevadas concentraciones de ácido úrico en sangre y estados de resistencia a la insulina o prediabetes (Herman & Birnbaum, 2021). La presencia de fructosa en el lumen gastrointestinal y en el hígado desencadena estrés glicoxidativo, bien por los procesos de autooxidación, bien por la formación de AGEs (Mastrocola *et al.*, 2013; Semchyshyn, 2013), lo cual podría promover la activación de mecanismos defensivos (Gugliucci, 2017). Así, el consumo de fructosa también se relaciona con una respuesta inmunitaria alterada, así como con la activación de mecanismos proinflamatorios (Wang *et al.*, 2020).

Son varios los autores que han intentado concretar los mecanismos moleculares que inducen los daños hepáticos promovidos por la fructosa, consiguiendo un acercamiento importante a los mismos mediante experimentos *in vivo*, tanto humanos como animales, como García-Berumen *et al.* (2019), Hsieh *et al.* (2016), Janssens *et al.* (2017), Mamikutty *et al.* (2015), Novelle *et al.* (2021), Papadopoulos *et al.* (2023), Sellmann *et al.* (2015) o Zhang *et al.* (2008), junto con otros descritos a lo largo de la presente tesis doctoral.

A nivel molecular, el consumo excesivo y prolongado de fructosa se ha relacionado con alteraciones del metabolismo energético y lipídico tanto en intestino como en hígado (Muriel *et al.*, 2021). Existen estudios en los que se ha visto que la fructosa promueve los procesos de gluconeogénesis hepática (Hsieh *et al.*, 2016; Rajasekar & Anuradha, 2007). También está descrita la relación de

consumos abundantes de fructosa con procesos de lipogénesis y con una disminución de la β-oxidación de ácidos grasos a nivel hepático, lo cual podría estar detrás de la infiltración de grasa en el hígado que parece acontecer tras ingestas sostenidas y abundantes de fructosa (Herman & Birnbaum, 2021).

Tanto la fructolisis intestinal como la hepática comparten las mismas rutas metabólicas (Figura 1.5.). Así, la degradación de la fructosa en ambos casos tras su captación facilitada a través de los transportadores específicos (GLUT5 y GLUT2, respectivamente), comienza con la fosforilación de la fructosa a fructosa 1-fosfato (F1P) por parte de la quinasa fructoquinasa (KHK). Esta catálisis es un proceso ATP-dependiente que, además, no se regula mediante señales como la suficiencia energética o la concentración de insulina (Herman & Birnbaum, 2021). F1P posteriormente es degradada por la aldolasa B (ALDOB). Los productos de esta reacción son la dihidroxiacetona fosfato (DHAP) y el gliceraldehído, el cual requiere una posterior fosforilación por la enzima trioquinasa (TKFC), de la cual se escinde el gliceraldehído 3-fosfato (G3P). Tanto DHAP como G3P son sustratos conocidos por su papel en diversas rutas metabólicas como la producción de energía o la lipogénesis. El aumento del contenido de triosas fosfato podría explicar el incremento de las mencionadas rutas metabólicas, dando una base con la que explicar las alteraciones metabólicas que parece desencadenar la fructosa.

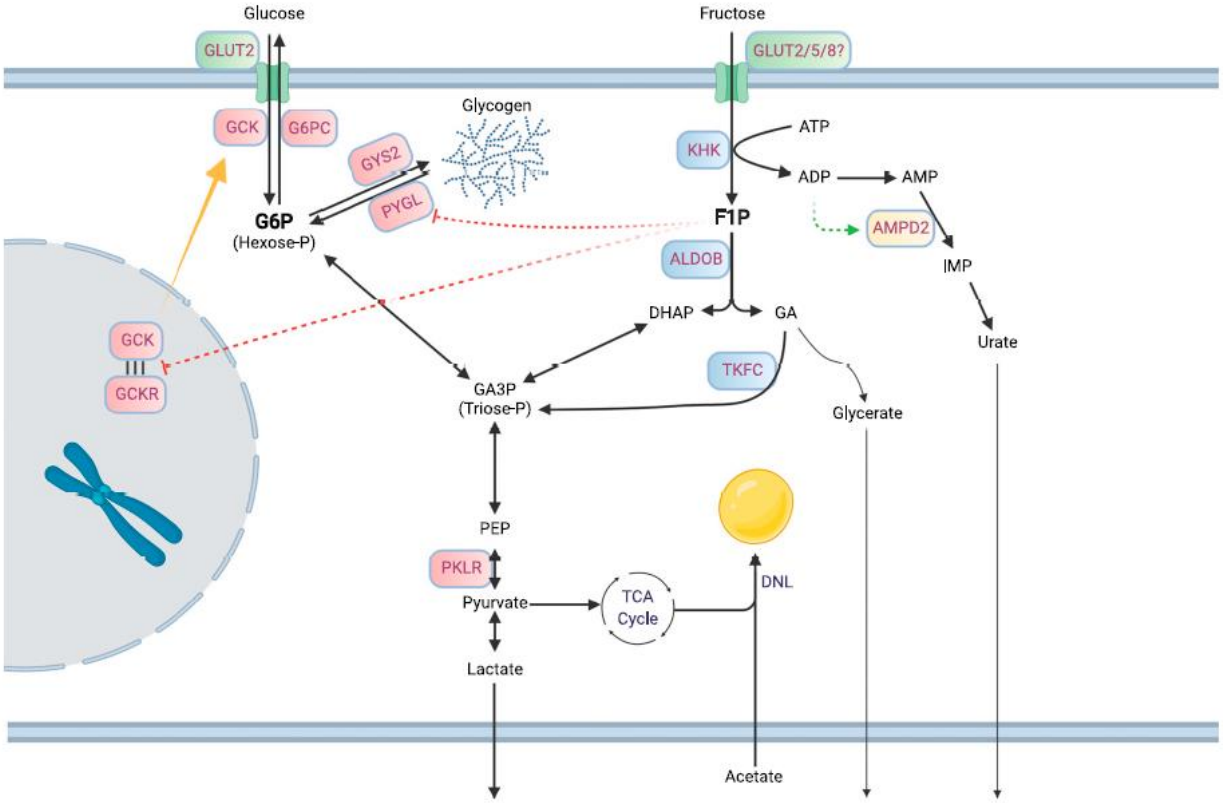

Figura 1.5. Fructolisis y bioquímica asociada. Fuente: Herman & Birnbaum (2021).

El aumento de estos compuestos podría incrementar, a su vez, la formación de sustratos involucrados en la lipogénesis hepática, como el citrato, estimulando la activación de la ruta (Softic *et al.*, 2016). Sin embargo, algunos autores han desestimado esta implicación de la fructosa en el metabolismo lipídico, hipotetizando sobre el posible papel de productos derivados de la degradación microbiana de la fructosa, como el ácido acético (Zhao *et al.*, 2020). El consumo prolongado e incrementado de fructosa también se ha relacionado con una mayor producción de ácido úrico (Muriel *et al.*, 2021), debido a la capacidad del azúcar de estimular la síntesis de purinas.

Dada las alteraciones promovidas por la fructosa a nivel celular, no resulta sorprendente que en última instancia el consumo elevado de fructosa conlleve daños mitocondriales, como han sugerido algunos autores como Jaiswal *et al.* (2015) o Mehta *et al.*, (2017).

En este contexto, es necesario señalar que el consumo excesivo de fructosa también podría afectar a otros órganos (Zhang *et al.*, 2017), como páncreas (Wang *et al.*, 2020), riñones (Rajesh & Sreekala, 2020), corazón (Zhang *et al.*, 2016) o cerebro (Cigliano *et al.*, 2018; Spagnuolo *et al.*, 2021). La

Figura 1.6 muestra de forma genérica los cambios ocasionados por el metabolismo de la fructosa a lo largo de su asimilación cuando se consume de forma excesiva, basados en la bibliografía consultada.

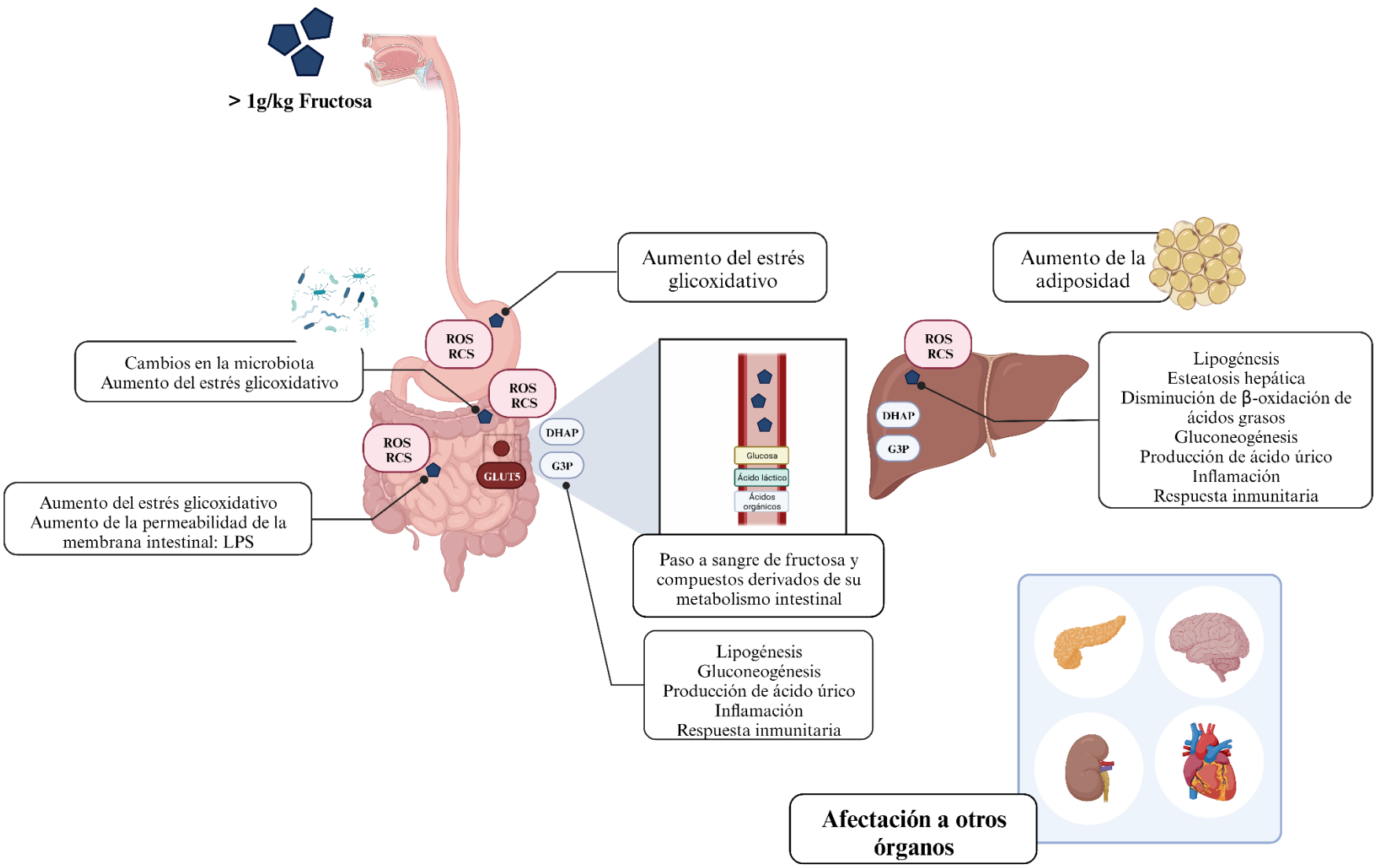

Figura 1.6. Resumen de los efectos de un consumo excesivo de fructosa basado en la bibliografía consultada. Elaboración propia.

1.6. Empleo de estrategias antioxidantes frente al estrés (glico)oxidativo

Desde la adición de sales nitrificantes hasta el uso de extractos vegetales, son muchas las estrategias antioxidantes que se han intentado llevar a cabo a lo largo de los años para evitar los efectos indeseables de la oxidación de proteínas y lípidos en la carne y los productos cárnicos, así como en otros productos procesados. Sin embargo, durante la digestión de los alimentos pueden originarse compuestos perjudiciales para el ser humano debido a la presencia de diversas moléculas derivadas de los antioxidantes y de las condiciones intraluminales. Por esto, desarrollar alternativas antioxidantes que maximicen los efectos esperados con un mínimo impacto sobre la salud del consumidor resulta prioritario para la investigación.

1.6.1. Nitritos

Las sales nitrificantes son usadas como aditivos desde tiempos remotos con el fin de prolongar la vida útil de los alimentos (Ruiz-Carrascal, 2015). En la industria cárnica, la adición de nitratos y nitritos durante la maduración de la carne es una práctica habitual, ya que regula de manera exitosa la oxidación de la mioglobina y el desarrollo de bacterias patógenas, además de participar en el desarrollo del sabor y aroma típico de los productos (de La Pomélie *et al.*, 2017; Karwowska & Kononiuk, 2020). Además de esto, los nitritos juegan un papel importante frente a los efectos indeseables de la oxidación de lípidos y proteínas, así como frente a diversos cambios químicos que experimentan algunos componentes de los alimentos (Karwowska & Kononiuk, 2020).

Los nitritos pueden actuar como agentes oxidantes, reductores y nitrosantes. Cuando se dan las condiciones de pH adecuadas (ácidas), se forma ácido nitroso (HNO_2) a partir del nitrito. A partir de HNO_2 puede formarse anhídrido nitroso (N_2O_3), el cual se encontraría en equilibrio con el óxido nítrico (NO) y el dióxido de nitrógeno (NO_2), ambos prooxidantes (Honikel, 2008). NO reacciona con la mioglobina, generando NO-mioglobina, responsable de la estabilización del color de los productos cárnicos (Ruiz-Carrascal, 2015). Sin embargo, la ingesta de nitratos y nitritos también puede desencadenar la formación de compuestos prooxidantes (RNS) en el lumen gastrointestinal dadas las condiciones ácidas del estómago. Cuando el organismo no tiene la capacidad de gestionar adecuadamente la formación de estas RNS, la situación puede desencadenar en una serie de efectos adversos, como el daño a proteínas, ADN, lípidos o hidratos de carbono (de La Pomélie *et al.*, 2019; Karwowska & Kononiuk, 2020).

Las proteínas pueden ser oxidadas y nitrosadas simultáneamente (Skibsted, 2011). Las alteraciones que promueven ambos procesos podrían afectar negativamente al valor nutricional de las mismas al modificar su composición aminoacídica. Como se ha descrito anteriormente en la presente tesis doctoral, las proteínas dañadas presentan una menor digestibilidad, lo cual las llevaría a pasar por el GIT intactas y a acumularse en el colon, donde serían fermentadas por la microbiota.

Por otra parte, los metales de transición son conocidos por su influencia catalítica en las reacciones de oxidación y nitrosación de proteínas. Así, son numerosos los trabajos que relacionan la capacidad del hierro hemo de la mioglobina con el desarrollo de estas reacciones durante la digestión de la carne y los productos cárnicos, a través de la formación de radicales libres (ROS) como consecuencia de su implicación en la cascada de reacciones conocida como química de Fenton (Bechaux *et al.*, 2018; de La Pomélie, Santé-Lhoutellier, & Gatellier, 2018; Oueslati *et al*., 2016; Villaverde *et al.*, 2014). Sin embargo, en presencia de nitritos, las interacciones en las que se ve envuelto este hierro hemo podrían verse estabilizadas por la propia interacción de los nitritos con la mioglobina (Bourassa *et al.*, 2001).

Bajo las diversas condiciones que se dan durante la manipulación y procesado de la carne y de los productos cárnicos, así como durante su digestión, como es el caso del ambiente ácido del estómago, los nitritos pueden también dar lugar a la formación de nitrosaminas a partir de aminas secundarias presentes en los alimentos, las cuales pueden tener efectos carcinogénicos (Karwowska & Kononiuk, 2020). Además, en presencia de iones metálicos, como Fe^{2+}/Fe^{3+}, estas interacciones pueden verse fomentadas (Ruiz-Carrascal, 2015). Existen varios autores que han relacionado la ingesta y formación endógena de nitrosaminas con el desarrollo de cáncer gastrointestinal (de La Pomélie, Santé-Lhoutellier, Sayd, *et al.*, 2018; Song *et al.*, 2015). Toda esta reactividad luminal en la que se ven envueltos los nitritos y los productos derivados de sus interacciones a lo largo del GIT, hicieron que en 2015 la Agencia Internacional de Investigaciones sobre el Cáncer (IARC, del inglés *International Agency for Research on Cancer*) clasificara los productos cárnicos procesados como carcinógenos para los humanos (grupo 1).

Por todo esto, resulta necesario tanto moderar el consumo de productos cárnicos procesados como regular la adición de nitratos y nitritos, la cual debe considerarse en función de la concentración final esperada en el producto, considerando tanto los cambios que se producen durante los procesos de maduración como los que se dan durante el almacenamiento de los productos (Reglamento (CE) Nº

1333/2008 del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre aditivos alimentarios).

1.6.2. Antioxidantes naturales como compuestos bioactivos: granada, punicalagina y urolitinas

El uso de extractos vegetales en la producción de alimentos está bastante extendido debido a las propiedades beneficiosas que presentan frente a la oxidación de lípidos y proteínas durante su procesado (Estévez *et al.*, 2021). Sin embargo, cada vez es mayor el interés que se presta a la utilización de compuestos antioxidantes derivados de extractos vegetales que mitiguen los efectos de los procesos oxidativos que se dan durante la digestión de los alimentos como consecuencia del estrés oxidativo.

La granada (*Punica granatum* L.) es una fruta rica en polifenoles que ha sido consumida desde tiempos ancestrales por su valor nutricional y por las propiedades beneficiosas para la salud que se le atribuyen (Kandylis & Kokkinomagoulos, 2020). Actualmente, la suplementación dietética a partir de extractos de granada supone un tema de interés entre la comunidad científica. Así, realizando una búsqueda sencilla en la base de datos *Scopus,* utilizando los descriptores "*pomegranate AND supplementation*", encontramos más de 200 trabajos en los últimos tres años basados en los efectos de la suplementación con granada. Entre ellos se pueden encontrar tanto estudios que utilizan la suplementación con granada para mejorar el rendimiento de la producción animal, como estudios *in vivo* tanto en animales como en humanos que analizan el impacto del consumo de este tipo de suplemento sobre determinadas situaciones de enfermedad.

Gracias a los compuestos fenólicos que naturalmente presenta la granada, como la punicalagina o el ácido elágico, la suplementación dietética a base de este fruto se ha relacionado con efectos beneficiosos para la salud, todos ellos relacionados con un posible impacto sobre el estrés oxidativo, como la mejora de procesos inflamatorios, efectos hepatoprotectivos, prevención de carcinogénesis y mejora de estados de diabetes, entre otros (Adachi *et al.*, 2020; Mandal *et al.*, 2017; Namdar *et al.*, 2023; Olvera-Sandoval *et al.*, 2022; Yanpar *et al.*, 2021).

La punicalagina (PU) es uno de los principales compuestos con actividad antioxidante que se relacionan con los efectos beneficiosos atribuidos a la suplementación con granada. Se trata de un compuesto perteneciente a la familia de los elagitaninos que ha sido y es objeto de estudio por sus remarcables actividades beneficiosas en el organismo. En la actualidad, son múltiples los autores

que han intentado descifrar las bases moleculares de sus efectos mediante la utilización de modelos animales y a través de técnicas avanzadas de estudio como la metagenómica o la metabolómica. Por ejemplo, Cao *et al.* (2020) analizó mediate técnicas enzimáticas e histopatológicas el efecto de una suplementación con PU sobre los daños hepáticos inducidos, tanto en un modelo *in vivo*, usando ratones macho C57BL/6J, como en un modelo *in vitro* a base de células humanas HepG2. Hua *et al.* (2022) investigaron el efecto de este compuesto sobre las consecuencias de nefropatía diabética inducida usando ratones macho C57BL/6J, mientras que Liu *et al.* (2023) usaron técnicas metabolómicas y transcriptómicas para analizar los efectos de PU en el colon de diferentes cepas de ratones macho a los que se indujo colitis, química y microbiológicamente. También otros grupos de trabajo han puesto su atención en los posibles efectos beneficiosos que este polifenol podría tener sobre la función cognitiva durante procesos patológicos, como Chen *et al.* (2023). En todos los casos, se pudo ver que la suplementación con PU mejoró los efectos de los diversos estados patológicos inducidos, si bien algunos autores señalan las limitaciones de algunos de estos estudios por no poder asociar el efecto del compuesto a la reducción del estrés oxidativo directamente Chen *et al.* (2023).

La biodisponibilidad de la mayoría de los elagitaninos es muy baja, por lo que existe evidencia de que este tipo de compuestos, como la PU, no son absorbidos como tal en el GIT y sufren diversas transformaciones por parte de la microbiota intestinal (Yin *et al.*, 2023).

Los efectos beneficiosos que se atribuyen a PU parecen tener relación con su interacción con determinadas especies de la microbiota intestinal, las cuales biotransformarían el polifenol en urolitinas bioactivas (Tomás-Barberán *et al.*, 2017). La producción de urolitinas varía considerablemente entre individuos (Zhang *et al.*, 2023). Además, algunos autores han documento diferentes tipos de urolitinas, las cuales normalmente difieren en su estructura (García-Villalba *et al.*, 2016). La PU parece actuar como precursor del ácido elágico, promoviendo su formación a través de interacciones con la microbiota. Sin embargo, uno de los aspectos más inexplorados en la actualidad es el de los géneros de bacterias que dan lugar a la síntesis de estos compuestos bioactivos cuando la PU está presente en el lumen gastrointestinal. A pesar de que los diferentes tipos de urolitinas podrían ser sintetizados por diferentes bacterias a partir de ácido elágico, existe bibliografía en la que se relaciona la formación final de estos compuestos con *Gordonibacter urolithinfaciens* y *Gordonibacter pamelaceae* (Selma *et al.*, 2014), al igual que con diversos géneros de la familia *Eggerthellaceae* (Zhang *et al.*, 2023).

Los principales efectos que se le atribuyen a las urolitinas comprenden capacidad antioxidante, antiinflamatoria, anticancerígena o su aptitud para modular la señalización celular y el metabolismo energético, entre otras, según algunos experimentos *in vitro* (Cerdá *et al.*, 2005; González-Sarrías *et al.*, 2017). Sin embargo, en la actualidad las evidencias directas a partir de ensayos *in vivo* acerca de las propiedades beneficiosas de las urolitinas se encuentra bajo debate (García-Villalba *et al.*, 2022), por lo que resulta necesaria más información en este ámbito.

1.7. Metodologías para el estudio de la digestión y metabolismo de los alimentos

La creciente evidencia sobre la relación entre la alimentación y algunas enfermedades inflamatorias intestinales ha desencadenado un creciente interés en la investigación de la digestión de los alimentos en los últimos años. En este ámbito, son varias las metodologías que se utilizan para el estudio de los cambios que se producen durante los procesos de digestión, distinguiendo entre procesos *in vitro* y procesos *in vivo*.

1.7.1. Ensayos in vitro

Los métodos de digestión *in vitro* han sido extensivamente utilizados para investigar el comportamiento de ciertos componentes alimenticios durante la digestión. Pueden dividirse en métodos de digestión estáticos y dinámicos (Dupont *et al.*, 2019). Sin embargo, no todos ellos resultan óptimos para el estudio de las reacciones bioquímicas que tienen lugar durante la digestión de los alimentos, como la oxidación o la nitrosación de proteínas, por lo que algunas de ellas además de ventajas también presentan inconvenientes.

Las plataformas de digestión *in vitro* estáticas se caracterizan por su simplicidad. A pesar de esto, este tipo de métodos proporcionan gran información acerca de las modificaciones que algunas moléculas como las proteínas pueden sufrir durante su exposición a las condiciones gastrointestinales, lo que facilita una amplia comprensión de las reacciones.

Durante años se han desarrollado diferentes métodos de digestión. No obstante, dada la dificultad a la hora de comparar los resultados entre laboratorios, se ha propuesto un modelo de digestión *in vitro* estándar por parte de un grupo internacional de expertos en el campo, mediante el COST INFOGEST NETWORK (Minekus *et al.,* 2014), recientemente actualizado (INFOGEST 2.0) (Brodkorb *et al.*, 2019).

Minekus *et al.* (2014) proponen un modelo de digestión *in vitro* que simula la digestión enzimática del tracto superior del GIT, a través de una etapa de digestión gástrica y otra intestinal para llevarla a cabo. Para cada una de las etapas propuestas, así como para una primera etapa salival, los autores describen en detalle la composición iónica tanto del fluido salival, como del gástrico y del intestinal, que deben utilizarse, de forma que cualquier investigador que desee poner a punto el método tendrá como referencia este preciso punto de partida. También se detallan las concentraciones de enzimas que deben utilizarse en cada una de las etapas (amilasa, pepsina, tripsina, lipasa pancreática, etc.), la concentración de los reactivos de origen óptimos para elaborarlas, así como la forma de llevar a cabo su utilización durante la digestión. A lo largo del trabajo desarrollado por los expertos, se describe también cómo proceder para adquirir y almacenar de forma adecuada distintas alícuotas de los digeridos durante el proceso. Todo esto, junto con la actualización llevada a cabo por Brodkorb *et al.* (2019), en la que incluye modificaciones con respecto al empleo de la fase oral y al uso de lipasa gástrica, hacen del método un robusto patrón que sin duda facilitará no solo llevar a cabo una digestión *in vitro*, si no extrapolar datos para una adecuada comparación entre laboratorios.

Sin embargo, dada la incapacidad de este sistema estático de simular algunas condiciones dinámicas que tienen lugar durante la digestión *in vivo*, como la concentración o la cantidad en que se liberan algunas enzimas digestivas, los movimientos peristálticos del GIT o la permeabilidad de la membrana intestinal, así como los tiempos de vaciado gástrico, se han propuesto métodos de digestión *in vitro* dinámicos. Estos novedosos sistemas tienen la capacidad de influir sobre el rango de pH del bolo, mediante bombas que regulan la secreción de sustancias ácidas o básicas para ajustar. También actúan sobre la cinética de las concentraciones y el volumen de las soluciones enzimáticas, con relación al tipo de alimento simulado. Otro aspecto que tienen en cuenta es la dinámica del transporte de los contenidos a lo largo del GIT simulado, así como la absorción mediante membranas de diálisis (Dupont *et al.*, 2019).

Sin embargo, implantar este conglomerado de sistemas puede llegar a resultar muy caro, a la vez que se necesitan instalaciones adecuadas para su uso y unas revisiones y mantenimiento adecuados a lo largo de su vida útil.

A lo largo de la elaboración de la presente tesis doctoral se ha recurrido a numerosos estudios *in vitro* que han servido de base para los ensayos que se han desarrollado. Dado el carácter preliminar del primer ensayo que aborda esta tesis, se optó por un experimento *in vitro* simplificado basado en

el trabajo de Santé-Lhoutellier *et al.* (2008), el cual se describe detalladamente en la sección *3. Diseño experimental*.

1.7.2. Ensayos in vivo: animales de experimentación

El uso de modelos animales para este tipo de estudios ha sido éticamente criticado, a pesar de ser el modelo que proporciona la información más relevante y extrapolable a los procesos que tienen lugar en los humanos.

La experimentación animal ha sido clave en el avance de la investigación científica, pero cuenta con una connotación negativa debido al uso que en el pasado se les dio a los animales como meros utensilios de los ensayos. Sin embargo, en la actualidad, el uso que se hace de los animales en la experimentación animal está muy alejado del que despertaron estos prejuicios. Existen normativas europeas que regulan y aseguran mediante adaptaciones nacionales y regionales el bienestar de los animales durante los experimentos, basándose en el principio de las 3R: reemplazo, reducción y refinamiento. Así, desde la Directiva 2010/63/UE, relativa a la protección de los animales utilizados para fines científicos, hasta el Real Decreto 53/2013, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia; pasando por la Ley 32/2007, para el cuidado de los animales, en su explotación, transporte, experimentación y sacrificio, se conforma el cuadro legislativo que específicamente regula el uso de los animales de experimentación.

Sin embargo, siempre que sea posible, se debe acudir a métodos alternativos oficialmente reconocidos para evitar el uso de animales. Cuando se hace imperativo el uso de éstos, se recurre a la reducción del número de animales utilizados, considerando en todo momento que este tiene que ser el suficiente para la aplicabilidad estadística de los resultados. Esto se puede conseguir por ejemplo mediante el uso de softwares específicos que se han diseñado para calcular el número de animales concreto que sería recomendable usar en función de la robustez de los resultados que se esperan conseguir. Por último, se asegurará durante todos los tipos de ensayos el refinamiento de los procesos, vigilando en todo momento los signos de dolor que pudieran mostrar los animales y su intensidad, asegurando el bienestar de los mismos para minimizar al extremo el impacto de la investigación en su fisiología y calidad de vida.

A lo largo de la elaboración de la presente tesis doctoral se han consultado numerosos ensayos realizados sobre animales de experimentación, como puede apreciarse en la extensa bibliografía

descrita. La consulta de estos trabajos, además de la de trabajos *in vivo* realizados sobre muestras humanas, ha resultado trascendental a la hora de sacar las principales conclusiones. A pesar de que la validación de métodos alternativos cada vez involucra más ensayos, el uso de metodologías *in vivo* resulta fundamental para seguir avanzando en las investigaciones sobre los estados de enfermedad, principalmente aquellos en los que su cronificación o su fatal desenlace aminoran la calidad de vida de las personas que los sufren. No obstante, en ningún caso se pone en cuestión la relevancia de los resultados obtenidos mediante la utilización de metodologías *in vitro*.

1.7.3. Ciencias ómicas: genómica, metabolómica y proteómica

Las ciencias ómicas permiten el estudio de un gran número de moléculas implicadas en el funcionamiento de los organismos. Dado el grado de desarrollo que han alcanzado en los últimos años, estas tecnologías permiten en la actualidad estudios a gran escala de genes codificados en el ADN (genómica), de ARN (transcriptómica), de proteínas sintetizadas (proteómica) y de metabolitos (metabolómica) (Figura 1.7).

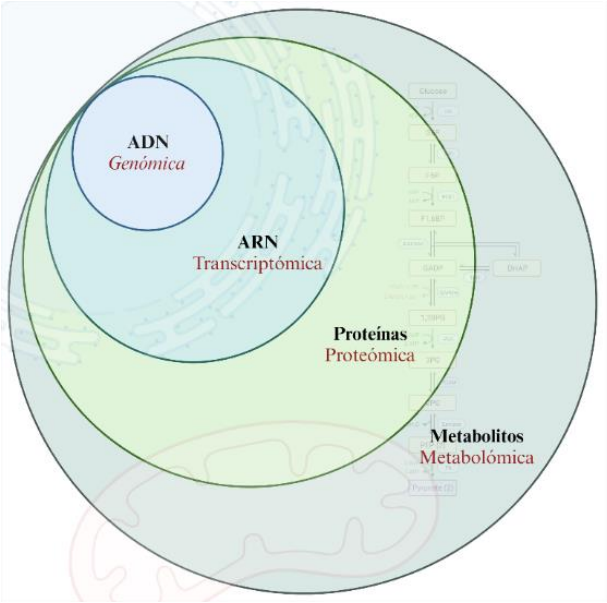

Figura 1.7. Vista global de las diferentes ciencias ómicas y el objeto de análisis de cada una de ellas dentro de la célula. Elaboración propia.

La aplicación de estos avances resulta muy valiosa en el estudio de ciertas enfermedades, pudiendo utilizarse y optimizarse para la obtención de diagnósticos tempranos que permitan prevenir el desarrollo de ciertas enfermedades o diseñar tratamientos personalizados.

Durante la presente investigación se ha combinado el uso de algunas de estas técnicas para poder descifrar los mecanismos que subyacen tanto a los efectos negativos asociados al consumo de fructosa como al efecto protector que un suplemento a base de extracto de granada podría tener sobre ellos. Así, para el desarrollo de la presente tesis doctoral se han utilizado técnicas no dirigidas de metagenómica, proteómica y metabolómica.

Dentro de la genómica, que hace referencia a la secuenciación de genes, la metagenómica supone una herramienta potencial para estudiar la diversidad microbiana de diferentes muestras. Implica la secuenciación masiva en paralelo de marcadores moleculares seleccionados, junto con el análisis bioinformático utilizando bases de datos públicas. Esto permite la detección de organismos cultivables y no cultivables presentes en una muestra total de ADN. Entre los marcadores moleculares más útiles para este tipo de análisis se encuentra el gen 16S rRNA, que permite la identificación de diferentes especies bacterianas, ya que su secuencia está altamente conservada entre las distintas especies de microorganismos. La aplicación de esta técnica ha simplificado considerablemente la realización de estudios centrados en explorar la composición microbiana y las dinámicas de la microbiota relacionadas con estados de salud y enfermedad (Kim *et al.*, 2017).

Para entender mejor los cambios que se producen en las comunidades de microorganismos que habitan el tracto gastrointestinal se recurre al cálculo de diferentes índices de diversidad. En el año 1972, Whittaker describió tres tipos de diversidad: la diversidad α, que se refiere a la variedad de especies dentro de un área o ecosistema en particular; la diversidad β, que hace referencia a la variedad de especies entre diferentes ecosistemas; y la diversidad γ, que se refiere a la medida de la diversidad de especies a escala geográfica (Whittaker & Whittaker, 1972). En este contexto, los índices de diversidad a los que se hace referencia a lo largo de la presente tesis doctoral son los basados en la diversidad α de la microbiota. A pesar de que no hay un consenso establecido sobre cuál es el mejor índice de diversidad, resultan muy útiles para las comparaciones entre las muestras de los grupos tratados y no tratados dentro del mismo ensayo (Kim *et al.*, 2017). Los índices de diversidad α que más comúnmente se usan para el análisis de la microbiota son el índice de Shannon-Wiever (H) y el índice de Simpson (D). Ambos consideran tanto la riqueza de especies como la uniformidad, pero el índice de Simpson está más influenciado por la uniformidad que por la riqueza en comparación con el índice de Shannon. La riqueza hace referencia al número total de especies diferentes presentes en la comunidad, pero le da el mismo valor a cada una de ellas sin tener en cuenta la abundancia en la que se encuentran en la muestra. Sin embargo, la uniformidad hace

referencia a la equidad en la distribución de las abundancias de las diferentes especies presentes en la comunidad bajo estudio. Esto quiere decir que indica cómo las diferentes especies están distribuidas de manera relativa en el ecosistema concreto objeto de estudio, en este caso el microbioma. Con la expresión de estos índices se puede orientar hacia la variedad de especies que conforman la microbiota, así como hacia su abundancia relativa, la cual ayuda en la detección de especies dominantes y/o minoritarias.

A pesar de la valiosa información que proporciona la secuenciación masiva de genes, cada vez es más evidente que contar con esa única fuente información no es suficiente para descifrar los fenómenos que ocurren a nivel celular, de órganos y del sistema (Yarmush & Jayaraman, 2002). El término proteoma se refiere al equivalente proteico del genoma. La proteómica no dirigida incluye el conjunto completo de productos génicos que son sintetizados por el genoma, es decir, las proteínas. Sin embargo, el campo de la proteómica resulta bastante desafiante, ya que el proteoma es sumamente dinámico, en contraste con la información estática que proporciona el genoma. A pesar de ser complejo, el proteoma define en gran medida el comportamiento y la función celular real, pues a diferencia del genoma proporciona información que incluye los cambios postraduccionales que han podido sufrir las proteínas una vez expresadas.

El metaboloma, por su parte, se refiere al conjunto completo de pequeñas moléculas metabólicas presentes en un organismo en un momento determinado. En combinación con los análisis proteómicos, resulta útil para la comprensión de los procesos biológicos que están ocurriendo en un momento determinado en un organismo concreto. También ambas técnicas pueden ser utilizadas para la identificación de biomarcadores y para explorar las distintas alteraciones metabólicas asociadas con enfermedades. A pesar de que existen diversas técnicas para el análisis tanto del proteoma como del metaboloma, para los estudios que conforman la presente tesis doctoral se han utilizado técnicas de espectrometría de masas para la detección de aminoácidos y metabolitos, respectivamente, combinadas con potentes softwares y bases de datos para la identificación, cuantificación, análisis estadístico de los datos e interpretación de los resultados.

Figura 1.8. *Wordcloud* sobre algunos de los softwares y bases de datos utilizados para el análisis y la interpretación de los resultados arrojados por la espectrometría de masas en lo referente a la proteómica y a la metabolómica. Elaboración propia.

PLANTEAMIENTO Y OBJETIVOS

2. Planteamiento y objetivos

El consumo de azúcares reductores tiene un potencial efecto sobre la funcionalidad de las proteínas durante su digestión. Además, tanto estos azúcares como los compuestos derivados de su degradación (dicarbonilos), podrían desempeñar un papel importante en situaciones de disbiosis y sobre el metabolismo hepático. Se desconocen, sin embargo, muchos de los mecanismos moleculares y fisiopatológicos implicados en estos efectos adversos para la nutrición y la salud. Por ello, la presente tesis doctoral tiene como planteamiento general dilucidar de qué manera la ingesta de azúcares reductores afecta a la digestibilidad de las proteínas durante la digestión y sus posibles consecuencias, partiendo de la hipótesis de que los azúcares reductores promueven la glicoxidación de proteínas y, por tanto, pueden tener efectos negativos sobre la salud en cuanto a su impacto sobre el metabolismo de diferentes nutrientes. Por otra parte, partiendo de la experiencia del grupo en la evaluación del efecto beneficioso de compuestos antioxidantes sobre el estrés oxidativo en ensayos *in vitro* que se han realizado de forma paralela, se pensó que sería pertinente hipotetizar sobre los efectos protectores que un suplemento de granada rico en punicalagina podría tener sobre los efectos perniciosos del consumo de fructosa. Para alcanzar los objetivos marcados, se llevó a cabo un análisis comparativo de la expresión de diversos marcadores de estrés oxidativo, apoyado por el uso de diferentes técnicas ómicas: transcriptómica, metabolómica y proteómica.

Dichos objetivos son:

1. Dilucidar el efecto de la glucosa y de los productos derivados de las reacciones en las que participa, así como de los nitritos como compuestos antioxidantes, sobre reacciones de oxidación, glicación y nitrosación de proteínas durante la fase gástrica de la digestión mediante un estudio *in vitro*.
2. Entender el impacto de una dieta suplementada con fructosa sobre el metabolismo a diferentes niveles tras el desarrollo de un ensayo *in vivo:* metabolismo gastrointestinal, impacto sobre la microbiota y metabolismo hepático.
3. Conocer los efectos potencialmente beneficiosos del consumo de un extracto comercial de granada rico en punicalagina sobre la fisiopatología promovida por el consumo de fructosa en los animales.

4. Profundizar en los mecanismos moleculares que subyacen las reacciones anteriores mediante el uso de ciencias ómicas.

DISEÑO EXPERIMENTAL

3. Diseño experimental

La presente tesis doctoral está dividida en cuatro capítulos bien diferenciados (Figura 3.2). En el primer capítulo se evalúa el efecto de la glucosa como azúcar reductor y algunos productos propios de las reacciones en las que participa, como el glioxal, sobre proteínas miofibrilares durante una digestión *in vitro*. Además, mediante un análisis paralelo, se analiza el efecto de la adición de nitritos como compuestos antioxidantes sobre dichas proteínas en presencia de los mismos compuestos. Este ensayo se describe con más detalle en el subapartado 3.1. de esta tesis doctoral. Los tres capítulos posteriores engloban los resultados obtenidos del análisis de un elevado consumo de fructosa sobre el proceso de digestión de proteínas dietéticas durante una digestión *in vivo*, usando animales de experimentación, así como las consecuencias de este consumo sobre órganos internos como el hígado. Por otro lado, los dos últimos capítulos evalúan el efecto de un suplemento de granada como compuesto antioxidante sobre los efectos atribuidos al consumo de fructosa (Figura 3.2). Igualmente, una descripción del ensayo *in vivo* se desarrolla con más detalle en el subapartado 3.2. de la presente tesis doctoral.

3.1. Experimento in vitro

Para el desarrollo del presente ensayo se propuso la digestión *in vitro* de un sistema modelo cárnico a base de proteínas miofibrilares (MP, del inglés *myofibrillar proteins*) en presencia de diversos compuestos, tales como mioglobina, glucosa, glioxal y nitritos. Las proteínas fueron extraídas y purificadas según las pautas descritas en el trabajo de Estévez *et al.* (2008).

En primer lugar, se elaboró una suspensión a partir de estas MP de 100 µg/ml de concentración en 100 mM de un tampón fosfato pH 6.5 con 0.6 M NaCl, con el fin de garantizar una dispersión homogénea de las proteínas. Posteriormente, la suspensión fue sonicada en un baño con hielo durante 3 minutos junto con 30 mg de trioleína, que se añadió para simular la porción lipídica que se atribuye en la carne fresca, usando un U 50 Control Ikasonic Sonicator (Janke & Kunkel GmbH & Co. KG, Staufen, Alemania).

Se consideraron seis grupos experimentales según la adición de diversos compuestos, estableciendo un grupo control que solo constó de proteínas miofibrilares. Así, en función de las especies químicas añadidas los grupos experimentales, elaborados por triplicado, fueron:

- Proteínas miofibrilares (CONTROL).
- Mioglobina (Mb; 10 mg/ml).
- Glucosa (GLU; 10 mg/ml).
- Glioxal (GLY; 10 mg/ml).
- Mioglobina + Glucosa (Mb + GLU; 10 mg/ml de cada especie).
- Mioglobina + Glioxal (Mb + GLY; 10 mg/ml de cada especie).

La concentración utilizada para cada especie fue seleccionada basándose en la concentración estimada que presentan en los alimentos. Al mismo tiempo, se preparó un conjunto adicional de lotes usando las mismas combinaciones de compuestos, pero añadiendo además 0.1 mg/ml de nitrito sódico (lotes [N+]), también por triplicado:

- CONTROL [N+].
- Mb [N+].
- GLU [N+].
- GLY [N+].
- Mb + GLU [N+].
- Mb + GLY [N+].

Una vez que todas las disoluciones estuvieron preparadas, se dispusieron 10 ml de cada una en viales de policarbonato con tapa roscada para ser sometidos al proceso de digestión descrito por Santé-Lhoutellier *et al.* (2008). Este proceso incluyó la adición de 10 ml de un tampón de glicina 33 mM a pH 1.8 y 5 U/mg MP de pepsina proveniente de mucosa gástrica porcina. La mezcla se incubó durante 2 horas a 37 ºC, y el proceso de digestión se detuvo con la adición de una solución de ácido tricloroacético (TCA, del inglés *trichloroacetic acid*) al 15 % (p/v). Se tomaron muestras antes de empezar el proceso de digestión y después, y estas fueron congeladas a -80 ºC hasta la realización de los análisis posteriores. Estos análisis se enumeran en detalle en la Figura 3.2.

3.2. Experimento in vivo

Veintiún ratas macho de la cepa *Wistar,* pertenecientes a la especie *Rattus novergicus,* fueron utilizadas en el experimento *in vivo* siguiendo los requerimientos legales españoles (Real Decreto 53/2013). El diseño de los procedimientos que conformaron el proyecto contó con la aprobación

tanto del Comité de Bioética de la Universidad de Extremadura (137-2020) como de la Junta de Extremadura (EXP20200904).

Todos los procedimientos pautados, así como la manipulación y eutanasia de los animales fueron llevados a cabo por personal con los requerimientos prescritos por la Dirección General de Sanidad Animal de la Junta de Extremadura.

Los animales fueron suministrados por el Servicio de Animalario de la Universidad de Extremadura (Cáceres, España), y todos los procedimientos fueron llevados a cabo en sus instalaciones.

Al principio del ensayo, los animales eran machos de unas 6-7 semanas de vida y pesaron entre 123-234 gramos. Durante una semana, y tras ser individualmente identificadas mediante una perforación en el pabellón auditivo Figura 3.1, las ratas fueron mantenidas en cajas ventiladas, con agua y pienso *ad libitum* y bajo condiciones climáticas controladas: 20-22 ºC de temperatura, 40-50 % de humedad y ciclos de luz-oscuridad de 12 y 12 horas. Los animales se agruparon en jaulas marcadas como A, B y C. Cada jaula contenía de 2 o 3 animales, y cada animal se identificó como 1, 2 o 3 en función de la marca de la oreja. Así, los animales que no tenían marca eran las ratas 1, los que tenían la marca en la oreja derecha eran los animales 2, y los que tenían la marca en la oreja izquierda eran los animales 3.

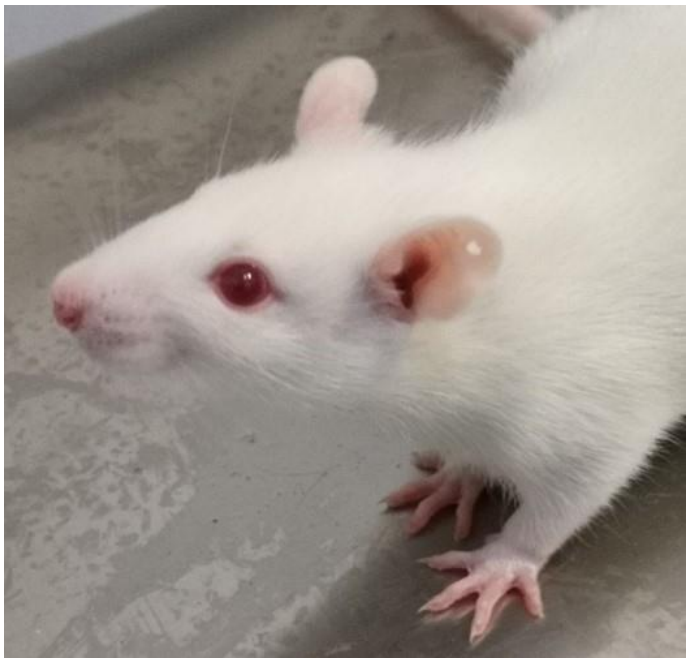

Figura 3.1. Rata *Wistar* con marca distintiva en el pabellón auditivo izquierdo (animal 3).

Una vez terminó el periodo de adaptación, los animales fueron asignados al azar a cada uno de los tres grupos de experimentación: i) grupo control (C) (n = 7), el cual recibió un pienso base junto con agua de bebida durante todo el ensayo; ii) grupo fructosa (F) (n = 7), el cual se alimentó del mismo pienso base junto con un 30 % (p/v) de fructosa en el agua de bebida; y iii) grupo fructosa y suplemento de granada rico en punicalagina (F+P) (n = 7), cuyo agua de bebida fue reemplazada por una solución al 30 % (p/v) de fructosa junto con un 0.2 % (p/v) de P, la cual acompañó al pienso durante su alimentación a lo largo del estudio.

A lo largo de todo el periodo experimental se utilizó el mismo pienso base para todos los animales, "*Teklad Global Diet 2014*", con un 14.3 % de proteína cruda, suministrado por ENVIGO (Madison, WI, USA). El extracto de granada rico en punicalagina "*Granatum PLUS"* fue suministrado por "Antioxidantes Naturales del Mediterráneo S.L." (Murcia, España).

El ensayo fue llevado a cabo durante 10 semanas. De forma diaria se supervisó a los animales para asegurar su bienestar y salubridad. A lo largo de todo el estudio, tanto el consumo de pienso como de las soluciones de bebida fue monitorizado de forma gravimétrica con el fin de calcular la ingesta energética y el consumo de los suplementos. Este proceso se llevó a cabo normalmente cada dos o tres días, dependiendo de las necesidades de los animales. Igualmente, los animales fueron pesados semanalmente para registrar la evolución del aumento de peso corporal esperado.

Al final del periodo experimental, las ratas fueron eutanasiadas con una edad aproximada de 16-17 semanas y un peso de entre 371 a 513 gramos. La eutanasia se llevó a cabo mediante exanguinación por punción cardiaca. Previamente, los animales fueron anestesiados mediante la inhalación de un 5% de isoflurano. Toda la sangre se recolectó en tubos con EDTA, y posteriormente fue almacenada a temperaturas de -80 ºC. Posteriormente se realizó una necropsia reglada de cada animal durante la cual se obtuvieron muestras de distintos tejidos. Se retiraron los depósitos grasos de los animales, los cuales fueron pesados de acuerdo con su tipo y localización, en línea con las descripciones de Chusyd *et al.* (2016). Así, tanto el tejido adiposo subcutáneo (SAT, del inglés *subcutaneous adipose tissue*) como el visceral (VAT, del inglés *visceral adipose tissue*) fueron separados cuidadosamente y pesados de forma individual. El VAT fue obtenido a partir de los tejidos grasos de diferentes localizaciones del abdomen de las ratas: retroperitoneal, perirrenal, gonadal e inguinal. La suma de SAT and VAT fue considerada como tejido adiposo blanco (WAT, del inglés *white adipose tissue*).

También los depósitos de grasa de la región interescapular de los animales fueron extraídos y pesados bajo la denominación de tejido adiposo marrón (BAT, del inglés *brown adipose tissue*).

Por otra parte, el tracto gastrointestinal de los animales (GIT, del inglés *gastrointestinal tract*) fue rápidamente diseccionado y fijado para evitar pérdidas de material intraluminal. El estómago, yeyuno, ciego y colon se muestrearon asépticamente, y el contenido digerido de cada uno (incluyendo las heces del recto) se extrajo y dispuso en tubos tipo *Eppendorf* bajo las mismas condiciones. Los tejidos fueron limpiados con agua destilada fría una vez vacíos. Finalmente, tanto los contenidos como los correspondientes tejidos se almacenaron inmediatamente a -80 ºC hasta su posterior uso. El hígado de cada animal también fue extraído e inmediatamente almacenado en contenedores adecuados a -80 ºC. Una porción de cada tejido fue fijada en un 5% de formalina para los posteriores análisis histopatológicos e inmunohistoquímicos. Todos los análisis realizados sobre estas muestras aparecen enumerados en la Figura 3.2.

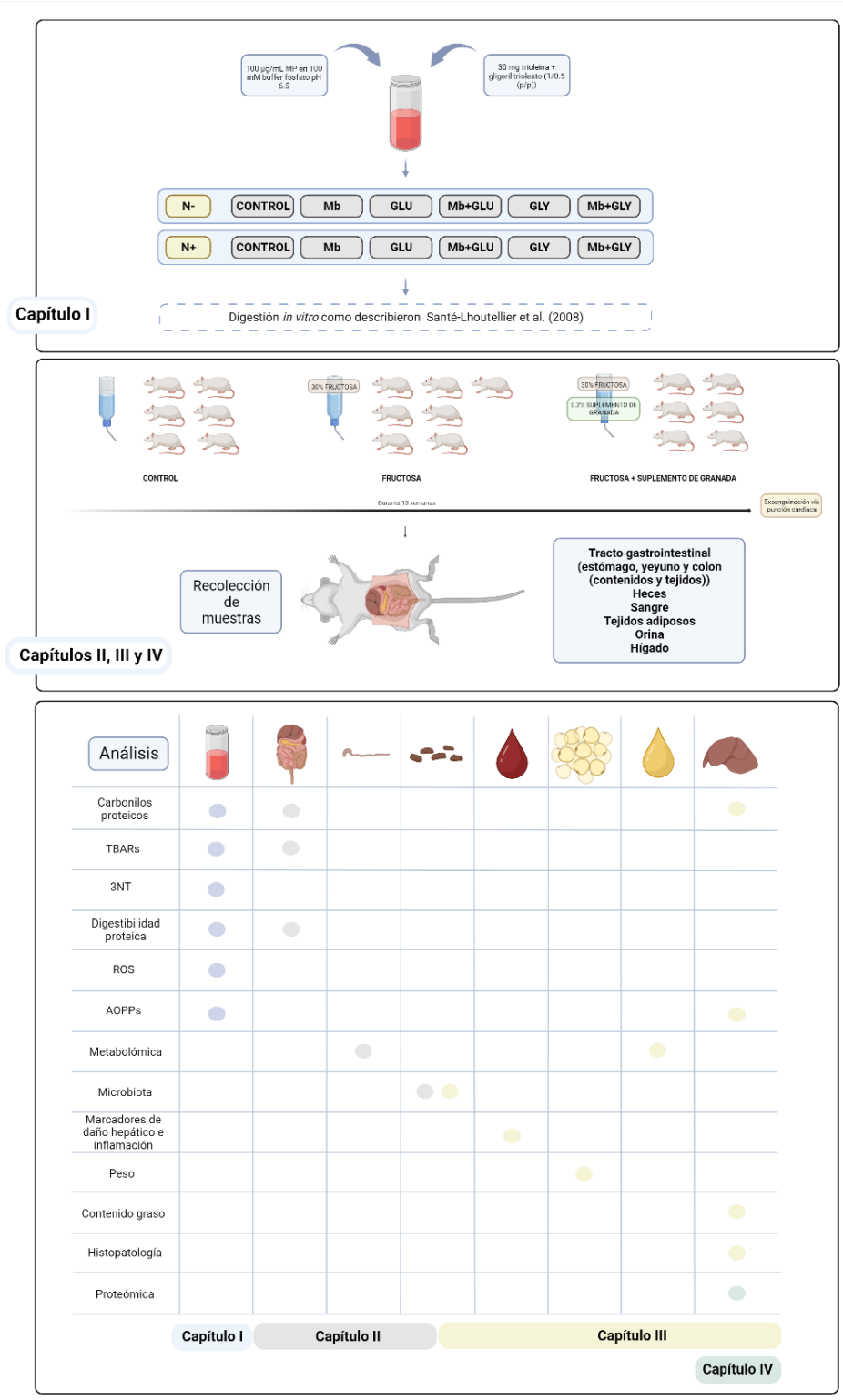

Figura 3.2. Visión general del diseño experimental de la presente tesis doctoral. Elaboración propia.

RESULTADOS

4. Resultados

Para la adecuada comprensión de los resultados de la presente tesis doctoral, estos se han agrupado en cuatro capítulos. Cada capítulo corresponde a un trabajo publicado/pendiente de publicar. Así, tanto el trabajo del Capítulo I como el del Capítulo II se encuentran publicados en las revistas *Food Chemistry* y *Journal of Agricultural and Food Chemistry*, respectivamente, ambas enmarcadas en el primer cuartil del *Journal Citation Reports* ^{TM} (JCR, 2022) en el campo de conocimiento de "*Food Science and Technology*". El trabajo que forma el Capítulo III se encuentra en estado de revisión por parte de la revista *Journal of Nutritional Biochemistry*, mientras que el del Capítulo IV lo está igualmente por parte de la revista *Molecular Nutrition and Food Research*.

- Capítulo I. *Glucose boosts protein oxidation/nitration during simulated gastric digestion of myofibrillar proteins by creating a severe pro-oxidative environment*.
- Capítulo II. *Impact of sustained fructose consumption on gastrointestinal function and health in Wistar rats: glycoxidative stress, impaired protein digestion and shifted faecal microbiota*.
- Capítulo III. *Pomegranate supplementation alleviates the dyslipidemia and the onset of non-alcoholic fatty liver disease caused by chronic dietary fructose in Wistar rats.*
- Capítulo IV. *Molecular mechanisms underlying the protective effect of pomegranate supplementation against fructose-induced liver damage: the mitochondria in the eye of the storm.*

Capítulo I

Glucose Boosts Protein Oxidation/Nitration During Simulated Gastric Digestion of Myofibrillar Proteins by Creating a Severe Pro-oxidative Environment

La glucosa promueve la oxidación / nitrosación de proteínas miofibrilares durante una digestión gástrica simulada por medio de la creación de un intenso ambiente pro-oxidativo

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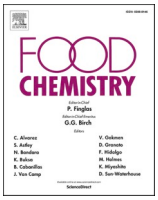

Glucose boosts protein oxidation/nitration during simulated gastric digestion of myofibrillar proteins by creating a severe pro-oxidative environment

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ABSTRACT

The severe pro-oxidative environment in the stomach promotes oxidation of dietary components. The pro-oxidant molecular mechanisms of reducing sugars on this environment are unknown. To investigate the mechanisms involved in protein oxidation and nitration during a simulated gastric digestion (porcine pepsin, 37 °C, 2 h) of meat proteins, these were exposed to several dietary reactive components namely myoglobin, glucose, glyoxal, myoglobin + glucose and myoglobin + glyoxal. Two versions of each experimental unit were prepared depending on the addition or absence of nitrite. Compared to control (only meat proteins), myoglobin + glucose showed the highest pro-oxidative and pro-nitrosative effect ($p < 0.001$), likely caused by an increase in ROS derived from the degradation of glucose during assay. Nitrite promoted the occurrence of protein nitration but decreased protein oxidation in myoglobin-added groups ($p < 0.001$) by, plausibly, stabilizing heme iron. These results indicate the relevant role of glyco-oxidation during digestion of red meat with other dietary components such as reducing sugars.

1. Introduction

The occurrence of oxidative reactions during food digestion has become a topic of increasing interest. The investigation of postprandial events enables a more realistic approach in the assessment of food impact on nutrition and health, as food components are severely oxidized during the digestion phases (Oueslati et al., 2016). From a nutritional standpoint, lipid oxidation leads to the loss of essential fatty acids and vitamins while protein oxidation involves the irreversible degradation of essential amino acids. Protein oxidation has also been linked to a further nutritional loss, as oxidized food proteins may not be fully degraded by digestive enzymes and hence, undigested oxidized proteins may progress to the colonic phase where they are eventually fermented (de La Pomélie et al., 2018).

The intake of oxidized food components has also been linked to safety and health concerns. Studies have shown that the intake of oxidized lipids leads to an increase of oxidation markers in blood and other internal organs of experimental animals and humans (Estévez, Li, Soladoye, & Van-Hecke, 2017; Estévez & Xiong, 2019). The role played by oxidized lipids in the pathogenesis is usually linked to the cytotoxicity and mutagenicity potential of species such as 4-hydroxy-2-nonenal –HNE– or malondialdehyde –MDA–) (Van Hecke et al., 2014). Recent articles have also collected scientific evidence of the pathological effects of several oxidized amino acids in target locations such as the gastrointestinal tract (GIT) (Díaz-Velasco et al., 2020, 2022), the liver (Estévez & Luna, 2017; Estévez & Xiong, 2019) or the pancreas (Estaras et al., 2020).

Understanding the chemistry behind these oxidative processes is essential to minimize their occurrence and their adverse biological consequences in the GIT. Most of recent studies on food digestion have focused on Fenton-like radical-mediated mechanisms (de La Pomélie et al., 2018; Oueslati et al., 2016). On this line, red meat has been identified as a highly susceptible food owing to the occurrence of pro-oxidant heme and non-heme iron that may induce the formation of reactive oxygen species (ROS) (de La Pomélie et al., 2018; Oueslati et al., 2016). Within the gastrointestinal tract, the stomach acts as a pro-oxidative bioreactor owing to the oxygenated environment and acidic pH (Kanner & Lapidot, 2001). Under these conditions, ferrous iron (Fe^{2+}) from myoglobin (Mb) reacts with oxygen to form superoxide radical (O_2^{*-}) [Reaction 1].

$$Mb\ (Fe^{2+}) + O_2 \rightarrow Mb\ (Fe^{3+}) + O_2^{*-} \quad \text{(Reaction 1)}$$

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At low pH, the formation of hydrogen peroxide readily occurs [Reaction 2], with this peroxide being subsequently decomposed into hydroxyl radical through the Fenton reaction [Reaction 3].

$$2\ O_2^{*-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Reaction 2)}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^* + OH^- \quad \text{(Reaction 3)}$$

These species initiate the oxidative deamination of alkaline amino acids, which leads to the formation of protein carbonyls (Estévez, 2011). Protein carbonylation, normally used as an expression of the oxidative damage to proteins, has been reported to increase severely during gastric digestion of meat (de La Pomélie et al., 2018; Oueslati et al., 2016).

Other radical and non-radical species such as reducing sugars and their degradation products (e.g. glyoxal; GLY) and reactive nitrogen species (RNS) may be able to induce modifications (glycation/nitration, respectively) in food proteins (Luna & Estévez, 2019; Skibsted, 2011). The implication of these dietary components in oxidative and nitrosative reactions during digestion of meat and meat products needs to be investigated. First, it is ignored whether reducing sugars, such as glucose, are able to induce protein oxidation/nitration during gastric digestion of meat proteins. Second, the role of nitrite on the occurrence of these glyco-oxidative reactions in a gastric-simulated environment is wholly ignored. We hypothesize, according to evidence from previous studies, that glucose could play a major role in the oxidative and nitrosative damage to meat proteins and that the underlying mechanisms may not only involve Maillard-mediated ones but actually the formation of ROS.

The present study was designed to investigate the effect of meat (myoglobin) and non-meat dietary components (glucose, glyoxal and nitrite) on the nature and intensity of oxidative and nitrosative reactions occurred during simulated gastric digestion of emulsions prepared with meat lipids and myofibrillar proteins.

2. Material and methods

2.1. Chemicals, reagents, and materials

All chemicals and enzymes were supplied from Panreac (Panreac Química, S.A., Barcelona, Spain), Merck (Merck, Darmstadt, Germany), and Scharlau (Barcelona, Spain). Pepsin from porcine gastric mucosa (with empirical protease activity of 3450 U/mg protein and CAS No 9001–75-6), glyceryl trioleate (>99 % purity and CAS No 122–32-7), trilinoleate (>98 % purity and CAS No 537–40-6) and myoglobin from equine skeletal muscle (>95 % purity and CAS No 309–705-0) were purchased from Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany). The water used in the experiments was purified by passage through a Milli-Q system (Millipore Corp., Bedford, MA, USA).

Protein carbonyls standards (α-aminoadipic and γ-glutamic semialdehydes; AAS and GGS, respectively) were synthesized in the laboratory following the procedure described by Akagawa et al. (2005).The yield (>90 %), authentication of the standard compounds and their purity (>95 %) were evaluated by LC-MS^2 and NMR as described by Estévez, Ollilainen, & Heinonen (2009).

The procedure for myofibrillar protein extraction and purification described by Estévez, Kylli, Puolanne, Kivikari, & Heinonen (2008) was applied to a bovine muscle *longissimus dorsi*, which was purchased from a local slaughterhouse. The final protein isolate (48 mg/mL) was assessed for its purity (<80 %) by SDS-PAGE electrophoresis. Bands corresponding to myosin HC (230 kDa), actin (42 kDa) and other myofibrillar proteins were detected.

2.2. Experimental design

Myofibrillar proteins (MP) (100 μg/mL) were suspended in 100 mM phosphate buffer pH 6.5 with 0.6 M NaCl to guarantee a homogenous dispersion. 30 mg of a mixture of triolein and glyceryl trioleate (1/0.5; w/w) was added to protein suspensions that were sonicated in an ice bath for 3 min with a U 50 Control Ikasonic Sonicator (Janke & Kunkel GmbH & Co. KG, Staufen, Germany). Depending on the addition of various chemical species, namely, myoglobin (Mb; 10 mg/mL); glucose (GLU; 10 mg/mL); Mb + GLU (10 mg/mL of each species); glyoxal (GLY; 10 mg/mL) and GLY + Mb (10 mg/mL of each species), six experimental groups were considered, along with a CONTROL group without reactants added. An additional set of experimental units was prepared using the same setting but adding in each of the experimental units 0.1 mg/mL of sodium nitrite. Both set of experimental units (6 nitrite free; [N-] and 6 with added nitrite [N +]) were prepared in triplicate in three independent experimental assays. The concentration of MP, lipids and Mb was selected based on their occurrence in fresh beef. The concentration of GLU, GLY and nitrite was selected based on food-occurring concentrations of such species. All experimental units (10 mL, total volume per unit) were dispensed in 25 mL screw-capped polycarbonate vials and subjected to a simulated gastric digestion in accordance with the procedure described by Santé-Lhoutellier et al. (2008) with minor modifications. 10 mL of 33 mM glycine buffer at pH 1.8 (gastric pH) and pepsin from porcine gastric mucosa (5 U/mg MP) were added to the experimental units and the mixture was allowed to incubate for 2 h at 37 °C. Digestion was terminated by addition of 15 % (final concentration) of trichloroacetic acid (TCA). Samples were taken before the simulated gastric digestion (BD), and immediately after digestion (AD) and all of them were kept at −80 °C for further analyses.

2.3. Analytical methods

2.3.1. Determination of thiobarbituric acid reactive substances (TBARS) numbers

TBARS were quantified using the method described by Ganhão, Estévez, & Morcuende, (2011) with some modifications. 1 mL of sample was homogenized with 8.5 mL perchloric acid (3.86 %) and 0.5 mL butylated hydroxytoluene (BHT) (4.2 % in ethanol). While homogenization, the plastic tubes were immersed in an ice bath to minimize the development of oxidative reactions. The slurry was filtered and centrifuged (600 g for 4 min) and 2 mL aliquots were mixed with 2 mL thiobarbituric acid (0.02 M) in test tubes. The test tubes were placed in a boiling water bath (100 °C) for 45 min together with the tubes from the standard curve. After cooling, the absorbance was measured at 532 nm in a SHIMADZU UV Spectrophotometer (UV-1800). The standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) solution in 3.86 % perchloric acid. Results were calculated as mg MDA per L of sample.

2.3.2. Analysis of protein carbonyls

The extraction, identification and quantification of protein carbonyls was carried out following the fluorescent HPLC procedure originally developed and optimized by Utrera, Morcuende, Rodríguez-Carpena, & Estévez (2011). 200 μL of sample were dispensed in 2-mL Eppendorf tubes and treated with 1 mL 10 % TCA. Each tube was vortexed and then subjected to centrifugation at 2000 g for 30 min at 4 °C. The supernatant was removed, and the pellet was treated again with 1 mL 5 % TCA and subsequently centrifuged at 5000 g for 5 min at 4 °C. The pellets were mixed with the following solutions (freshly prepared): 0.5 mL 250 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 % sodium dodecyl sulfate (SDS) and 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL 50 mM ρ-aminobenzoic acid (ABA) in 250 mM MES buffer pH 6.0 and 0.25 mL 100 mM sodium cyanoborohydride ($NaBH_3CN$) in 250 mM MES buffer pH 6.0. The tubes were vortexed and then incubated in an oven at 37 °C for 90 min. The samples were stirred every 15 min. After derivatization, samples were treated with 1 mL 50 % TCA solution and centrifuged at 5000 g for 10 min. The pellet was then washed twice with 1.5 mL 10 % TCA and diethyl ether-ethanol (1:1). The washed pellets were hydrolyzed with 1.5 mL 6 N HCl and kept in an oven at 110 °C for 18 h. The hydrolysates were dried in a centrifugal

evaporator *in vacuo*. The hydrolysates were redissolved with 200 μL milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 μm pore size, Pall Corporation, USA) for HPLC analysis.

A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Kyoto, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS on-line degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence detector (FLD), and a CBM-20A system controller, was used. An aliquot (1 μL) from the reconstituted protein hydrolysates was injected and analyzed in the above mentioned HPLC equipment. ABA-derivatized proteins, namely AAS-ABA and GGS-ABA, were eluted in a Cosmosil 5C18-AR-II RP-HPLC column (5 μm, 150 × 4.6 mm) equipped with a guard column (10 × 4.6 mm) packed with the same material. The flow rate was kept at 1 mL/min and the temperature of the column was maintained constant at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standards (0.1 μL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from the standard compounds. The peaks corresponding to AAS-ABA and GGS-ABA were manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 nM (Figure S1, available as Supplementary material) as proposed by Utrera, Rodríguez-Carpena, Morcuende, & Estévez (2012) with a regression coefficient of 0.999. Results (the sum of derivatized carbonyls) are expressed as nmol of carbonyl compound per mg of protein. Supplementary Figure S2 shows FLD chromatograms of standard compounds (A) and two additional chromatograms illustrating the detection of such compounds in two different samples (B and C).

2.3.3. Analysis of reactive oxygen species (ROS)

To check the potential formation of ROS in the experimental units, superoxide radical was assessed in experimental units following the method reported by Susanto et al. (2006) as follows. For each experimental unit, three reaction mixtures were prepared i) one with superoxide dismutase (SOD) (100 U/mL), ii) other with diethylthiocarbamate (DETCA) (3 mM), and finally iii) with NADH (100 mM) and DETCA (3 mM). The test tubes were incubated for 1 h at 37 °C with regular shaking. The supernatant was mixed with cytochrome *c* (20 μM) and absorbance was immediately read at 550 nm using a Hitachi U-2000 spectrophotometer. The absorbance differences between sample with or without SOD were used to calculate the release of superoxide radical by using the molecular extinction coefficient for cytochrome *c* of 21 mM^{-1} $cm^{-}1$.

2.3.4. Analysis of advanced oxidation protein products (AOPPs)

AOPPs were analyzed in accordance with the method originally described and optimized by Estévez et al. (2008) using a LS-55 Perkin-Elmer fluorescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.). Prior to the analysis, digests were diluted with 100 mM sodium phosphate buffer, pH 7.4. AOPPs were excited at 350 nm, and the emitted fluorescence was recorded from 400 to 500 nm. The excitation and emission slits were both set to 10 nm and scanning speed was 500 nm/min. Results are expressed as fluorescence intensity (Area units). Supplementary Figure S3 shows fluorescence spectra of corresponding to AOPPs in the tested samples.

2.3.5. Analysis of 3-NitroTyrosine (3NT)

3NT was analyzed by spectrophotometry according to the technique reported by Yang, Zhang, & Pöschl (2010). Briefly, 5 mL of digests was treated with 10 % cold TCA and then centrifuged at 670 g for 5 min. Protein pellets were reconstituted with 1 mL of 8 M urea in 100 mM sodium phosphate buffer at pH 3.5. Tyrosine and 3NT were assessed at 280 and 357 nm, respectively, in a SHIMADZU UV Spectrophotometer (UV-1800). The extent of protein nitration was expressed as nitrosation degree (ND), which is defined as the average number of 3NT residues divided by the total number of tyrosine residues in a protein molecule.

2.3.6. Digestion of proteins

The extent to which proteins were hydrolyzed during the simulated gastric digestion was measured by quantifying the non-protein nitrogen (NPN) as follows. Five grams of digest was homogenized twice with 20 mL deionized water and centrifuged at 5000 g, 4 °C for 10 min. Combined supernatants were filtered through Whatman No. 1 filter paper and subsequently mixed with 10 mL of 20 % trichloroacetic acid. Samples were allowed to stand at room temperature for 30 min and then centrifuged at 5000 g at 4 °C for 10 min. Supernatants were filtered through Whatman No. 4 filter paper. NPN was quantified using the Kjeldahl method (AOAC, 2000). Results are expressed as mM.

2.4. Statistical analysis

All experimental groups were made in triplicate (3 true replicates) and each individual experimental unit was analyzed three times for each measurement (3 technical replicates). The effect of the addition of reactants on the concentration of TBARS, protein carbonyls, free radicals, AOPPs and 3NT was analyzed by a one-way Analysis of Variance (ANOVA). The effect of digestion (BD vs AD-N+ / AD-N-) was assessed by a repeated measures ANOVA. The Tukey's test was used for multiple comparisons of the means. The significance level was set at $p < 0.05$.

3. Results

3.1. Lipid oxidation

As indicators of the extent of lipid oxidation, TBARS were measured before and after the *in vitro* digestion of the studied samples (Table 1). Before digestion, TBARS numbers were similar for all groups and quantitively low, ranging from 0.09 mg MDA/L in the CONTROL group to 0.15 mg MDA/L in Mb + GLU samples.

The levels of TBARS significantly increased during the *in vitro*

Table 1
Concentration of TBARS (mg TBARS/L sample) in experimental units before (BD) and after digestion in the absence (AD_N-) or presence (AD_N +) of nitrite (means ± standard deviations).

	BD	AD_N-	AD_N+	p^A
CONTROL	$0.09^{y,b} \pm 0.02$	$0.23^{x,d} \pm 0.08$	$0.21^{x,cd} \pm 0.07$	**
Mb	$0.12^{y,ab} \pm 0.03$	$0.45^{x,c} \pm 0.10$	$0.16^{y,d} \pm 0.07$	**
GLU	$0.11^{y,ab} \pm 0.02$	$0.65^{x,b} \pm 0.11$	$0.57^{x,a} \pm 0.15$	**
Mb + GLU	$0.15^{z,a} \pm 0.03$	$1.29^{x,a} \pm 0.21$	$0.27^{y,c} \pm 0.07$	***
GLY	$0.10^{y,ab} \pm 0.03$	$0.51^{x,bc} \pm 0.13$	$0.53^{x,a} \pm 0.12$	***
Mb + GLY	$0.12^{z,ab} \pm 0.03$	$0.59^{x,bc} \pm 0.12$	$0.39^{y,b} \pm 0.09$	***
p^B	*	**	*	

BD: analysis performed in freshly prepared experimental units (before digestion). Means and deviations were calculated from both, nitrite-free and added nitrite set of samples, after observing no significant effect of nitrite in BD samples.
AD_N-: analysis performed in nitrite-free experimental units after simulated gastric digestion.
AD_N+: analysis performed in experimental units with added nitrite after simulated gastric digestion.
Experimental groups: CONTROL: MP suspension (100 μg/ mL) without reactants. Mb: MP + Mb; 10 mg/mL; GLU: MP + glucose, 10 mg/mL; Mb + GLU: MP + myoglobin + glucose; 10 mg/mL of each species); GLY: MP + glyoxal, 10 mg/mL; GLY + Mb: MP + glyoxal + myoglobin, 10 mg/mL of each species).
p^A: significance level of the effect of digestion in ANOVA: *: $p < 0.05$; **: $p < 0.01$; $p < 0.001$. Means with different letters (x-z) within a row (type of experimental unit) are significantly different in post-hoc Tukey tests.
p^B: significance level of the effect of reactants in ANOVA. Means with different letters (a-d) within a column (type of digestion) are significantly different in post-hoc Tukey tests.

digestion in all groups, except for samples containing myoglobin with added nitrite. Among all tested groups, Mb + GLU samples showed the highest TBARS values after *in vitro* digestion (1.29 mg MDA/L) ($p < 0.01$), followed by the GLU group (0.65 mg MDA/L), which was, in turn, higher than those from Mb + GLY and GLY (0.59 and 0.51 mg MDA/L, respectively). The lowest levels of TBARS were shown by the Mb samples ($p < 0.01$). Nitrite-added groups containing Mb had significantly lower lipid oxidation levels compared to their nitrite-free counterparts. Among treated groups, Mb [N +] and Mb + GLU [N +] samples had similar TBARS values (0.16 mg MDA/L and 0.27 mg MDA/L, respectively) to that of control (0.21 mg MDA/L).

3.2. Protein oxidation

Table 2 shows the concentration of protein carbonyls in experimental units before and after the *in vitro* digestion assay. Protein carbonyls are commonly used as indicators of protein oxidation in meat systems and other biological samples (Estévez, 2011; Estévez et al., 2021). Even before the *in vitro* digestion process, the exposure to the tested compounds had significant effects on the extent of protein carbonylation ($p < 0.01$), the GLY samples showing the highest amount of protein carbonyls (0.86 nm carbonyls/mg protein). Carbonyl levels significantly increased in the groups without nitrites after the *in vitro* digestion assay ($p < 0.001$). Thus, values before *in vitro* digestion ranged from 0.17 – 0.86 nmol carbonyls per mg of protein, while after the experimental digestion test oscillated between 1.75 and 20.21 nmol carbonyls/mg protein. The Mb + GLU group showed the highest level of protein oxidation after *in vitro* digestion (20.21 nmol carbonyl/mg protein) ($p < 0.001$). Among treated samples, Mb showed the significantly lowest level of carbonyls after the *in vitro* digestion process (2.98 nmol/mg protein).

Nitrite addition significantly decreased protein oxidation levels after *in vitro* digestion in samples with myoglobin ($p < 0.001$). The decrease in carbonyls in Mb + GLU [N +] samples (from 20.21 to 10.89 nmol carbonyls /mg protein) was the most remarkable when compared with the nitrite-free [N-] counterparts. Nitrite addition had no significant impact on the extent of protein carbonylation in GLY [N +] and GLU [N +] groups after *in vitro* digestion in contrast to their nitrite-free counterparts.

Table 2
Concentration of protein carbonyls (nmol carbonyls/mg protein) in experimental units before (BD) and after digestion in the absence (AD_N-) or presence (AD_N +) of nitrite (means ± standard deviations).

	BD	AD_N-	AD_N+	p^B
CONTROL	$0.17^{y,d} \pm 0.04$	$1.75^{x,e} \pm 0.25$	$1.45^{x,d} \pm 0.20$	**
Mb	$0.34^{z,c} \pm 0.07$	$2.98^{x,d} \pm 0.36$	$1.34^{y,d} \pm 0.18$	***
GLU	$0.41^{y,c} \pm 0.08$	$8.56^{x,c} \pm 0.99$	$7.98^{x,c} \pm 0.84$	***
Mb + GLU	$0.58^{z,b} \pm 0.10$	$20.21^{x,a} \pm 2.27$	$10.89^{y,b} \pm 1.33$	***
GLY	$0.86^{y,a} \pm 0.11$	$15.99^{x,b} \pm 1.64$	$14.84^{x,a} \pm 1.57$	***
Mb + GLY	$0.57^{z,b} \pm 0.07$	$16.05^{x,b} \pm 1.40$	$13.02^{y,a} \pm 1.18$	***
p^B	**	***	***	

BD: analysis performed in freshly prepared experimental units (before digestion). Means and deviations were calculated from both, nitrite-free and added nitrite set of samples, after observing no significant effect of nitrite in BD samples.
AD_N-: analysis performed in nitrite-free experimental units after simulated gastric digestion.
AD_N+: analysis performed in experimental units with added nitrite after simulated gastric digestion.
Experimental groups: CONTROL: MP suspension (100 μg/ mL) without reactants. Mb: MP + Mb; 10 mg/mL; GLU: MP + glucose, 10 mg/mL; Mb + GLU: MP + myoglobin + glucose; 10 mg/mL of each species); GLY: MP + glyoxal, 10 mg/mL; GLY + Mb: MP + glyoxal + myoglobin, 10 mg/mL of each species).
p^A: significance level of the effect of digestion in ANOVA: **: $p < 0.01$; $p < 0.001$. Means with different letters (x-z) within a row (type of experimental unit) are significantly different in post-hoc Tukey tests.
p^B: significance level of the effect of reactants in ANOVA. Means with different letters (a-d) within a column (type of digestion) are significantly different in post-hoc Tukey tests.

3.3. Radical production

No significant differences were found among experimental units for the amount of radicals before the *in vitro* digestion (Table 3.A). In three groups, in fact, the concentration of these radical species was below the detection limit.

After digestion, the production of radical species significantly increased in the groups without added nitrite. The highest levels of superoxide radicals were found in Mb + GLU samples (523 μM) ($p < 0.001$), while the GLU and Mb groups reached 298 μM and 210 μM of superoxide radicals, respectively ($p < 0.001$).

When nitrites were added, the concentration of radicals significantly decreased in samples with Mb + GLU [N +] (212 μM) and Mb [N +] (112 μM), compared to their nitrite-free counterparts (523 μM and 210 μM, respectively).

3.4. AOPPSs

Table 3.B shows the levels of Advanced Oxidation Protein Products (AOPPs) in the different experimental units, before and after the *in vitro* digestion process.

Before digestion, results showed significant differences in the levels of AOPPs among groups ($p < 0.05$). Samples from GLY and Mb + GLY groups displayed the highest levels of AOPPs (186 and 179 fluorescence units, respectively) compared to the other treatments. After *in vitro*

Table 3
Concentration of radical species (μM) (A) and AOPPs (fluorescence units) (B) in experimental units before (BD) and after digestion in the absence (AD_N-) or presence (AD_N +) of nitrite (means ± standard deviations).

(A)	BD	AD_N-	AD_N+	p^A
CONTROL	<LOD	$69^{e} \pm 15$	$72^{d} \pm 21$	–
Mb	$45^{z} \pm 15$	$210^{x,c} \pm 33$	$112^{y,d} \pm 19$	***
GLU	$56^{y} \pm 18$	$298^{x,b} \pm 30$	$302^{x,a} \pm 30$	***
Mb + GLU	$69^{z} \pm 26$	$523^{x,a} \pm 42$	$212^{y,b} \pm 32$	***
GLY	<LOD	$116^{d} \pm 26$	$111^{c} \pm 28$	–
Mb + GLY	<LOD	$256^{bc} \pm 32$	$110^{c} \pm 29$	–
p^B	–	***	**	
(B)	BD	AD_N-	AD_N+	p^A
CONTROL	$112^{y,b} \pm 18$	210x,e ± 34	192x,d ± 40	*
Mb	$120^{z,b} \pm 23$	337x,d ± 39	247y,c ± 41	*
GLU	$135^{y,b} \pm 30$	446x,c ± 55	469x,b ± 114	***
Mb + GLU	$128^{z,b} \pm 30$	822x,b ± 109	423y,b ± 108	***
GLY	$186^{y,a} \pm 31$	1147x,a ± 189	1002x,a ± 190	***
Mb + GLY	$179^{y,a} \pm 28$	1220x,a ± 199	1103x,a ± 205	***
p^B	*	***	***	

BD: analysis performed in freshly prepared experimental units (before digestion). Means and deviations were calculated from both, nitrite-free and added nitrite set of samples, after observing no significant effect of nitrite in BD samples.
LOD: Limit of detection.
AD_N-: analysis performed in nitrite-free experimental units after simulated gastric digestion.
AD_N+: analysis performed in experimental units with added nitrite after simulated gastric digestion.
Experimental groups: CONTROL: MP suspension (100 μg/ mL) without reactants. Mb: MP + Mb; 10 mg/mL; GLU: MP + glucose, 10 mg/mL; Mb + GLU: MP + myoglobin + glucose; 10 mg/mL of each species); GLY: MP + glyoxal, 10 mg/mL; GLY + Mb: MP + glyoxal + myoglobin, 10 mg/mL of each species).
p^A: significance level of the effect of digestion in ANOVA: **: $p < 0.01$; $p < 0.001$. Means with different letters (x-z) within a row (type of experimental unit) are significantly different in post-hoc Tukey tests.
p^B: significance level of the effect of reactants in ANOVA. Means with different letters (a-d) within a column (type of digestion) are significantly different in post-hoc Tukey tests.

digestion assay, AOPPs significantly increased in all groups. Likewise, GLY and Mb + GLY samples showed the highest levels of this parameter (1147 and 1220 fluorescence units, respectively), followed by the Mb + GLU group (822 fluorescence units).

When nitrite was added, Mb + GLU and Mb samples showed the most remarkable change in their AOPPs levels as compared to the groups without nitrite, decreasing from 822 to 423 fluorescence units in the former, and from 337 to 247 in the latter ($p < 0.05$). Among N + samples, GLY and Mb + GLY groups had, once again, the highest levels of AOPPs after the *in vitro* digestion process.

3.5. Protein nitration

Table 4 shows the extent of protein nitration expressed as nitrosation degree (ND) in the different experimental units, before and after the *in vitro* digestion process.

The addition of the compounds under study had no significant effect on the ND of the samples before the *in vitro* digestion. After the *in vitro* digestion assay, all samples significantly increased the ND values. The Mb + GLU [N-] group displayed the highest ND values after *in vitro* digestion, with these values increasing from 0.12 in the undigested samples to 0.31 in the digested counterparts ($p < 0.05$). When nitrite was added, the ND significantly increased in all cases as compared to the nitrite-free counterparts. Among N + samples, GLY, Mb + GLY and GLU had the highest ND followed by Mb + GLU, while samples with Mb had similar ND than controls.

3.6. Digestibility

The digestibility of the samples, measured as the concentration of non-protein nitrogen (NPN) after the *in vitro* digestion test of the different experimental units, is shown in Table 5.

The compounds under study significantly reduced the digestibility of myofibrillar proteins as compared to CONTROL in the nitrite-free digestion. Therefore, CONTROL [N-] group displayed the significantly highest NPN values (135 mM) followed by the Mb, GLU and the two glyoxal groups (GLY and Mb + GLY). Mb + GLU samples showed the lowest NPN values (66.8 mM) with these values being similar to those displayed by GLY groups. No significant differences were found between samples with and without added nitrites except for CONTROL where a significant reduction was observed.

Table 4
Nitrosation degree (dimensionless) in experimental units before (BD) and after digestion in the absence (AD_N-) or presence (AD_N +) of nitrite (means ± standard deviations).

	BD	AD_N-	AD_N+	p^A
CONTROL	0.10z ± 0.03	0.21y,b ± 0.07	0.35x,c ± 0.08	**
Mb	0.12z ± 0.03	0.22y,b ± 0.04	0.42x,c ± 0.10	**
GLU	0.11z ± 0.02	0.26y,ab ± 0.05	0.79x,a ± 0.11	**
Mb + GLU	0.12z ± 0.03	0.31y,a ± 0.09	0.65x,ab ± 0.11	***
GLY	0.10z ± 0.03	0.23y,b ± 0.04	0.34x,c ± 0.13	***
Mb + GLY	0.12z ± 0.04	0.25y,ab ± 0.06	0.41x,c ± 0.16	***
p^B	ns	*	**	

BD: analysis performed in freshly prepared experimental units (before digestion). Means and deviations were calculated from both, nitrite-free and added nitrite set of samples, after observing no significant effect of nitrite in BD samples.
AD_N-: analysis performed in nitrite-free experimental units after simulated gastric digestion.
AD_N+: analysis performed in experimental units with added nitrite after simulated gastric digestion.
Experimental groups: CONTROL: MP suspension (100 μg/ mL) without reactants. Mb: MP + Mb; 10 mg/mL; GLU: MP + glucose, 10 mg/mL; Mb + GLU: MP + myoglobin + glucose; 10 mg/mL of each species); GLY: MP + glyoxal, 10 mg/mL; GLY + Mb: MP + glyoxal + myoglobin, 10 mg/mL of each species).
p^A: significance level of the effect of digestion in ANOVA: ns: no significant; **: $p < 0.01$; $p < 0.001$. Means with different letters (x-z) within a row (type of experimental unit) are significantly different in post-hoc Tukey tests.
p^B: significance level of the effect of reactants in ANOVA. Means with different letters (a-d) within a column (type of digestion) are significantly different in post-hoc Tukey tests.

Table 5
Non-protein nitrogen (mM) after digestion in the absence (AD_N-) or presence (AD_N +) of nitrite (means ± standard deviations).

	AD_N-	AD_N+	p^A
CONTROL	135.0^{a} ± 21.3	109.0^{a} ± 16.2	*
Mb	105.0^{b} ± 15.5	99.8^{ab} ± 12.7	ns
GLU	89.6^{bc} ± 9.62	84.2^{bc} ± 12.8	ns
Mb + GLU	66.8^{d} ± 7.81	73.2^{c} ± 14.3	ns
GLY	77.1^{cd} ± 0.13	76.9^{c} ± 12.6	ns
Mb + GLY	72.5^{cd} ± 0.16	81.0^{bc} ± 11.6	ns
p^B	**	*	

AD_N-: analysis performed in nitrite-free experimental units after simulated gastric digestion.
AD_N+: analysis performed in experimental units with added nitrite after simulated gastric digestion.
Experimental groups: CONTROL: MP suspension (100 μg/ mL) without reactants. Mb: MP + Mb; 10 mg/mL; GLU: MP + glucose, 10 mg/mL; Mb + GLU: MP + myoglobin + glucose; 10 mg/mL of each species); GLY: MP + glyoxal, 10 mg/mL; GLY + Mb: MP + glyoxal + myoglobin, 10 mg/mL of each species).
p^A: significance level of the effect of digestion in ANOVA: *: $p < 0.05$; **: $p < 0.01$; $p < 0.001$. Means with different letters (x-z) within a row (type of experimental unit) are significantly different in post-hoc Tukey tests.
p^B: significance level of the effect of reactants in ANOVA. Means with different letters.

4. Discussion

4.1. Effect of pro-oxidative environments on the (glyco)-oxidative damage to MP during simulated gastric digestion

Overall, lipid and protein oxidation markers were found to increase after *in vitro* gastric digestion, illustrating that dietary proteins undergo, upon intake, further oxidative damage. This is in agreement with previous *in vitro* (Oueslati et al., 2016) and *in vivo* (Van Hecke et al., 2021) studies in which a considerable increase in oxidation products was reported in meat digests. The stomach has been defined as a bioreactor in which pro-oxidative factors of the gastric fluids (e.g. low pH) facilitate the occurrence of oxidative and nitrosative reactions (de La Pomélie et al., 2018). Dietary components play a major role in the nature and intensity of these reactions in the GIT and red meat has been regarded as a pro-oxidative dietary component (Macho-González et al., 2020). Heme iron has been unequivocally identified as a major catalyst of oxidative and nitrosative reactions in the GIT and is thought to be responsible for assorted pathological conditions linked to the intake of red meat (Tappel, 2007).

In our study, Mb significantly promoted lipid and protein oxidation and the formation of AOPPs in myofibrillar proteins. Previous studies showed that Mb boosted the formation of TBARS and protein carbonyls during simulated gastrointestinal digestion of muscle foods (Oueslati et al., 2016). The redox chemistry of heme iron has been profusely documented and Mb is known to form ferric protein radicals, which are able to induce oxidation of biomolecules such as lipids and proteins (Kröger-Ohlsen et al., 2003). Mb-mediated ROS formation is accelerated at low pH, which supports the pro-oxidative environment created in the gastric lumen upon red meat intake (Kröger-Ohlsen et al., 2003).

The molecular basis of the pro-oxidative action of Mb during digestion has been recently reviewed by Bechaux et al. (2018). The Fenton-like chemistry described by these authors can be reasonably ascribed to the present assay as the oxidative damage of Mb occurred along with an increase in ROS.

Yet, glucose induced more severe oxidative changes in MP than those

caused by Mb. The concentration of lipid and protein carbonyls and AOPPs was significantly higher in experimental units incubated with glucose than in those exposed to the pro-oxidant action of Mb. While reducing sugars are not typically described as promoters of oxidative reactions in food systems, several studies have shown the ability of reducing sugars to promote protein carbonylation by a Maillard-type mechanism (Villaverde & Estévez, 2013). Luna & Estévez (2019) reported that glucose and their degradation products (i.e., glyoxal and methylglyoxal) promoted protein oxidation to a greater extent than a hydroxyl-radical generating system. While Maillard-derived dicarbonyls are known to induce carbonylation via a definite oxidative deamination mechanism on lysine residues, the authors hypothesized whether the generation of ROS from glucose in the presence of iron could have also contributed to the severe oxidative damage. The present study confirms the efficient role of glucose as inductor of (glyco)-oxidative damage to proteins and supports the hypothesis that such damage is also mediated by ROS. The oxidation levels found in GLU systems are consistent with the formation of ROS and furthermore, the combination of glucose with Mb intensified both, ROS generation and the subsequent oxidative damage. The generation of ROS through the autoxidation of reducing sugars under physiological conditions, have been experimentally documented by Wang et al. (2016). The present results originally emphasize the relevant role of glucose as inducers of ROS-mediated degradation of biomolecules during gastric digestion of proteins and shows that such effect is enhanced by heme iron. Interestingly, GLY is able to induce intense protein carbonylation of AOPPs formation in the absence of Mb plausibly through the Maillard-like pathways described by Luna & Estévez (2019). While an increase of ROS was also found in GLY systems compared to CONTROL samples, ROS-mediated mechanisms may have played a minor role in GLY units. In fact, the addition of Mb to these systems did not increase the oxidative potential of the dicarbonyl, which was, at some point, expected. It is therefore, proposed that in a complex food system consisting of meat proteins, Mb and reducing sugars, the oxidative reactions would mainly be promoted by the reactive products from sugars, dicarbonyls and ROS, in an indefinite proportion (Fig. 1).

4.2. *Effect of nitrite of the oxidative and nitrosative damage to MP*

The addition of nitrite significantly diminished the extent of the oxidative damage to MP. The observed lipid antioxidative effect of nitrite during *in vitro* digestion is in agreement with previous findings by (Van Hecke et al., 2014), and it is consistent with the well-known antioxidative effect of nitrites in meat products during processing and subsequent storage (Ruiz-Carrascal, 2016). In the present experiment, however, this effect depended on the occurrence of Mb. Nitrite was found to inhibit TBARS formation and protein carbonyls accretion in experimental units with added Mb. The effect of nitrite in the other experimental units was negligible. These results clearly indicate that nitrite could be counteracting the pro-oxidant action of Mb by reacting with and hence, stabilizing the heme pigment. In the presence of nitrite, Mb was unable to promote the generation of ROS, providing strength to the hypothesis of nitrite providing oxidative stability to heme iron. This protective effect was also observed against the formation of AOPPs, except for Mb-GLY systems. As already reported, Mb did not seem to affect the glyco-oxidative effects of GLY, and hence, the addition of nitrite had a limited effect in diminishing the damage caused by the dicarbonyl. Interestingly, nitrite reduced the concentration of ROS in Mb-GLY systems, which indicates that the formation of AOPPs by GLY occurs independently of radical-mediated mechanisms.

The stabilizing effect of nitrite on Mb has been known for some time. The initial reduction of nitrite to nitric oxide (NO), which readily occurs in the stomach at low pH, is followed by the covalent linkage between NO and heme iron [Reaction 4], leading to the formation of nitrosylmyoglobin (Mb[Fe^{2+}]NO) (Skibsted, 2011).

$$Mb(Fe^{3+}) + NO \rightarrow Mb(Fe^{2+})NO \quad \text{(Reaction 4)}$$

The formation of NO from nitrite can also be catalysed by oxymyoglobin (Mb[Fe^{2+}]) which is oxidised into metmyoglobin (Mb[Fe^{3+}]) [Reaction 5] (Skibsted, 2011).

$$Mb(Fe^{2+}) + NO_2^- + H_2O \rightarrow Mb(Fe^{3+}) + NO + 2OH^- \quad \text{(Reaction 5)}$$

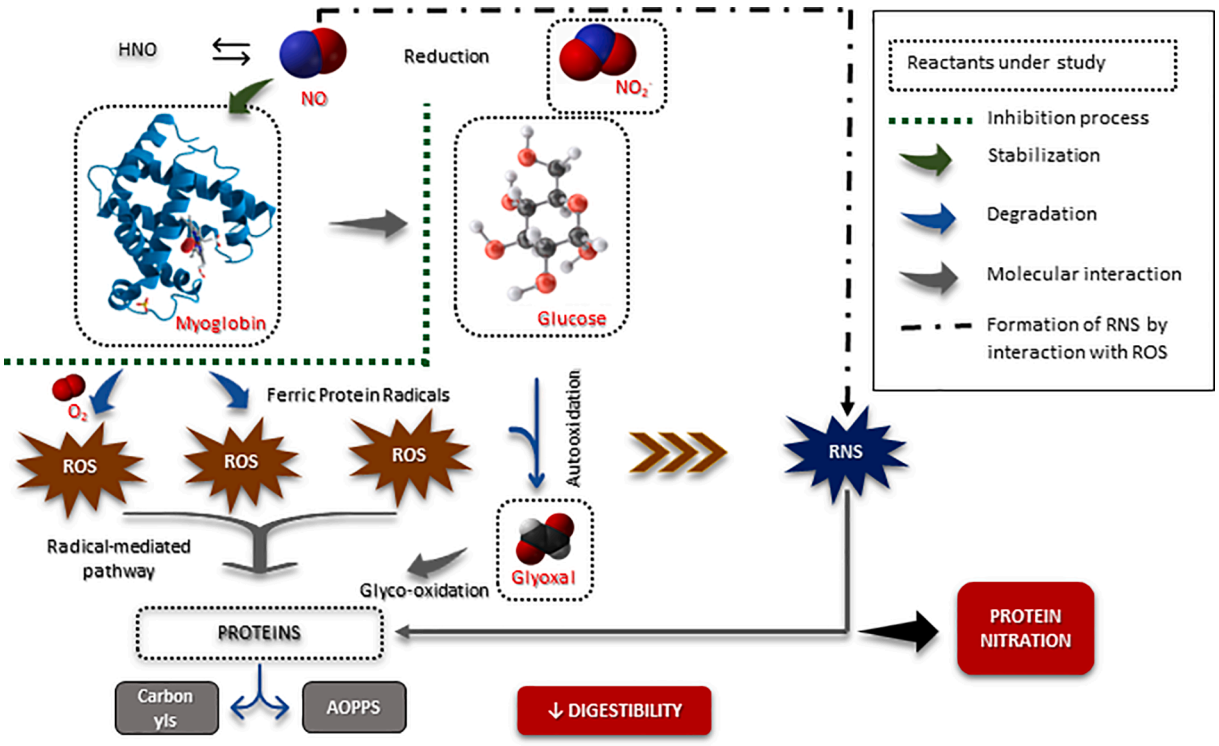

Fig. 1. Schematic representation of the proposed oxidative and nitrosative reactions would mainly be promoted by the reactive products from reactants under study.

This pigment is known to be highly stable in meat products and its reactivity in redox reactions is strongly inhibited (Ruiz-Carrascal, 2016; Skibsted, 2011). Furthermore, Mb(Fe^{2+})NO can actually act as antioxidant through a couple of mechanisms, namely, dissociation of NO [Reaction 6] and direct neutralization of radical species [LOO* in Reaction 7] (Skibsted, 2011).

$$Mb(Fe^{2+})NO \rightarrow Mb(Fe^{2+}) + NO \quad \text{(Reaction 6)}$$

$$Mb(Fe^{2+})NO + LOO^{*} \rightarrow \text{non-radical products} \quad \text{(Reaction 7)}$$

The antioxidant action ascribed to nitrite in cured meat products (Skibsted, 2011) could actually be, at least, partly attributed to the low reactivity of Mb(Fe^{2+})NO and to its antioxidant actions. Though these mechanisms could be applicable to the conditions of the present experiment and hence, explain the protection of nitrite against the oxidation of MP, the actual formation of this nitrosated pigment in the current simulated gastric *in vitro* conditions or *in vivo*, is pending to be proven.

The occurrence of protein nitrosation in systems with no added nitrite could respond to the reactivity of residual nitrite in the experimental units. Previous studies have also found nitrosated amino acids in *in vitro* systems without added nitrite (Villaverde et al., 2014).

As expected, the digestion of the MP isolates in the presence of nitrite led to significant increases of the nitrosation degree with the highest values being found in experimental units treated with glucose and Mb + GLU. These experimental units were also found to have the highest concentrations of ROS, which emphasizes the timely connection between these oxygen-containing reactive species and reactive nitrogen species (RNS) as previously stated by Skibsted (2011). The latter are described to be responsible for the nitrosation of lipids and proteins in biological systems and may be formed from ROS and reactive forms of nitrogen such as nitrite and nitric oxide. While the formation of 3-nitrotyrosine and other nitrosated species such as nitrosamines are known to take place during the gastric digestion of cured meats owing to the occurrence of the reactants and favourable conditions (e.g. low pH) (de La Pomélie et al., 2018), the present study contributes to understanding the underlying molecular mechanisms. In this regard, glucose is originally identified as promoter of nitrosative reactions plausibly through the generation of ROS under acidic conditions. In this regard, nitrite may play a dual role. On the one hand, it is a required reactant in nitrosative reactions, and its addition leads to increased nitrosated proteins. On the other hand, nitrite counteract the pro-oxidant action of Mb, with this component being a leading promoter of oxidative reactions during digestion of red meat. As a result, nitrite has a protective effect against the occurrence of Mb-promoted oxidative reactions and the unavoidable formation of nitrosated proteins in cured meat systems may also be diminished in the presence of Mb owing to the limited formation of Mb-radical species. Furthermore, Mb has been described to decompose RNS such as peroxynitrite, which may contribute to block the reaction of such species with other proteins (Bourassa et al., 2001). While nitrite may have this protective effect in Mb-containing systems, glucose and particularly, Maillard dicarbonyls remain unaffected by nitrite. Reducing sugars and their degradation products greatly boost glyco-oxidative reactions during gastric digestion of meat proteins.

4.3. Impact of oxidative and nitrosative damage on protein digestibility

From a nutritional point of view, the impaired digestibility of oxidized proteins has been long described as a major consequence of the oxidative damage to food proteins (Estévez, 2011). Though scientific literature reveals contradictory results on this topic, as recently revised by Estévez et al. (2021) it is generally accepted that severely oxidized proteins, such as those found in ultra-processed muscle foods, are not fully digested. Carbonylation and other severe chemical modifications occurred in oxidized proteins impede the recognition of such proteins by peptin, trypsin and other proteases, leading to an impaired digestion (de La Pomélie et al., 2018). These mechanisms seem to be applicable to the present results since the experimental conditions causing the highest levels of protein oxidation (Mb + GLU) also led to the lowest levels of protein degradation, followed by GLY and Mb + GLY. The extent of digestibility, as measured by the concentration of non-protein nitrogen (NPN), was reduced to half in MP subjected to Mb-GLU as compared to CONTROL samples. These three experimental groups (Mb + GLU, GLY, Mb + GLY) also had the highest levels of AOPPs, suggesting that such advanced glycation products may also contribute to impair protein digestibility. Certainly, previous works have suggested that the accretion of fluorescent protein aggregation products could cause protein resistance to degradation by proteases as well as impeding function to approach and catalyze protein degradation (Suyama et al., 2002). In fact, in the present study, the correlation between protein degradation (as measured by NPN) and AOPPs ($r = 0.74$; $p < 0.05$) was higher than that between NPN and protein carbonyls ($r = 0.59$; $p < 0.05$). In consistency with our results, Luna & Estévez (2019) observed how the glyco-oxidation of MP by GLY and other glucose degradation products caused decreased susceptibility of proteins to digestive proteases. On the same line, Feng et al. (2015) reported a direct connection between oxidation-related damage in whey proteins and decreased protein digestibility. To similar results and conclusions came Duque-Estrada et al. (2019) working on soy protein matrices. It is worth emphasizing that in a more realistic and complex food system, the extent of oxidative damage during digestion and therefore, its impact on digestibility may not only depend on the dietary components but also in the previous culinary practice (Ferreira et al., 2018). The authors reported that processing may also play a relevant role on redox post-prandial events, who indicated that severe oxidative damage caused in protein during ultra-processing of chicken patties seriously affected protein digestibility and the overall nutritional value of the product.

Overall, the addition of nitrite to the experimental units, did not significantly affect the extent of protein digestibility. Only in CONTROL samples, the occurrence of nitrite led to a significant decrease in protein digestibility. Since the extent of protein nitration in this group was not particularly severe, it is unlikely that the nitrosation degree had a relevant impact on protein digestibility. These results contrast with those reported by de La Pomélie et al. (2018) and Théron et al. (2018) who observed that nitrite addition to previously cooked meat caused a significant reduction in protein digestibility. Yet, notable differences between both studies could explain this divergence including the origin of the protein, the concentration of nitrite, the occurrence of other reagents such as ascorbate and the means for the assessment of protein nitrosation and protein digestibility. According to the authors, the nitrosation of aromatic amino acids such as tryptophan or tyrosine could affect the action of pepsin on MP. This was not observed in the present study.

In addition to the depletion of the nutritional value of dietary proteins owing to an impaired digestibility, the occurrence of protein oxidation and nitration as well as the formation of AOPPs in foods has consequences in terms of food safety and health. On the one hand, the accumulation of oxidized and undigested proteins in colon can lead to large fermentation processes by the gut microbiome, that can produce several harmful metabolites at this location which are related with the development of colorectal cancer (Rombouts et al., 2017). On the other hand, the occurrence and accumulation of protein oxidation products in tissues are related with aging and age-related diseases and type II diabetes (Arcanjo et al., 2018) and it is known that the intake of oxidized lipids and proteins contributes to the onset of *in vivo* oxidative stress, both in the GIT and in internal organs (Estévez & Luna, 2017; Estévez & Xiong, 2019). In recent studies, specific dietary protein oxidation products have been found to induce oxidative stress and toxicity in intestinal cells (Díaz-Velasco et al., 2020) and alter the digestive function of pancreatic cells (Estaras et al., 2020). AOPPs can also contribute to the development of several diabetic complications and the presence of Maillard reaction's final products in the intestine may cause

inflammatory processes (Estévez & Luna, 2017).

5. Conclusions

This study originally describes the relevant role of glyco-oxidative reactions in the oxidative and nitrosative damage to meat proteins during simulated gastric digestion. Mb promotes the severe pro-oxidative actions of glucose while nitrite tends to inhibit the pro-oxidative effects of heme iron. Taking into account the results from the present study, reducing sugars and their oxidation products should be regarded as potentially harmful dietary components when digested with red meat as their ability to promote the glyco-oxidative and nitrosative in meat proteins is here proven. The results from the present study prove that glucose induces protein carbonylation not only by Maillard-mediated mechanisms, as ROS are also produced in the tested conditions. *In vivo* studies should be carried out to corroborate the negative influence of glucose and GLY during gastric digestion of red meat.

CRediT authorship contribution statement

G. Sánchez-Terrón: Data curation, Methodology, Formal analysis, Writing – original draft. **D. Morcuende:** Data curation, Methodology, Formal analysis, Validation, Writing – review & editing. **R. Martínez:** Data curation, Methodology, Formal analysis, Validation, Writing – review & editing. **J. Ruiz-Carrascal:** Data curation, Methodology, Supervision, Validation, Writing – review & editing. **M. Estévez:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133805.

References

Akagawa, M., Sasaki, D., Kurota, Y., & Suyama, K. (2005). Formation of α-aminoadipic and γ-glutamic semialdehydes in proteins by the Maillard reaction. *Annals of the New York Academy of Sciences, 1043*, 129–134. https://doi.org/10.1196/annals.1333.016

Arcanjo, N. M. O., Luna, C., Madruga, M. S., & Estévez, M. (2018). Antioxidant and pro-oxidant actions of resveratrol on human serum albumin in the presence of toxic diabetes metabolites: Glyoxal and methyl-glyoxal. *Biochimica et Biophysica Acta - General Subjects, 1862*(9), 1938–1947. https://doi.org/10.1016/j.bbagen.2018.06.007

Bechaux, J., de La Pomélie, D., Théron, L., Santé-Lhoutellier, V., & Gatellier, P. (2018). Iron-catalysed chemistry in the gastrointestinal tract: Mechanisms, kinetics and consequences. *A review. Food Chemistry, 268*(June), 27–39. https://doi.org/10.1016/j.foodchem.2018.06.018

Bourassa, J. L., Ives, E. P., Marqueling, A. L., Shimanovich, R., & Groves, J. T. (2001). Myoglobin catalyzes its own nitration. *Journal of the American Chemical Society, 123*(21), 5142–5143. https://doi.org/10.1021/ja015621m

de La Pomélie, D., Santé-Lhoutellier, V., Sayd, T., & Gatellier, P. (2018). Oxidation and nitrosation of meat proteins under gastro-intestinal conditions: Consequences in terms of nutritional and health values of meat. *Food Chemistry*, *243*(July 2017), 295–304. https://doi.org/10.1016/j.foodchem.2017.09.135.

Díaz-Velasco, S., Delgado, J., Peña, F. J., & Estévez, M. (2022). Protein oxidation marker, α-amino adipic acid, impairs proteome of differentiated human enterocytes: Underlying toxicological mechanisms. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1870*(7), Article 140797. https://doi.org/10.1016/j.bbapap.2022.140797

Díaz-Velasco, S., González, A., Peña, F., & Estévez, M. (2020). Noxious effects of selected food-occurring oxidized amino acids on differentiated CACO-2 intestinal human cells. *Food and Chemical Toxicology, 144*(July), 1–8. https://doi.org/10.1016/j.fct.2020.111650

Duque-Estrada, P., Berton-Carabin, C. C., Nieuwkoop, M., Dekkers, B. L., Janssen, A. E. M., & Van Der Goot, A. J. (2019). Protein Oxidation and in Vitro Gastric Digestion of Processed Soy-Based Matrices. *Journal of Agricultural and Food Chemistry, 67*(34), 9591–9600. https://doi.org/10.1021/acs.jafc.9b02423

Estaras, M., Ameur, F. Z., Estévez, M., Díaz-Velasco, S., & Gonzalez, A. (2020). The lysine derivative aminoadipic acid, a biomarker of protein oxidation and diabetes-risk, induces production of reactive oxygen species and impairs trypsin secretion in mouse pancreatic acinar cells. *Food and Chemical Toxicology, 145*(July). https://doi.org/10.1016/j.fct.2020.111594

Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science, 89*(3), 259–279. https://doi.org/10.1016/j.meatsci.2011.04.025

Estévez, M., Díaz-Velasco, S., & Martínez, R. (2021). Protein carbonylation in food and nutrition: A concise update. *Amino Acids*. https://doi.org/10.1007/s00726-021-03085-6

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., & Heinonen, M. (2008). Fluorescence spectroscopy as a novel approach for the assessment of myofibrillar protein oxidation in oil-in-water emulsions. *Meat Science, 80*(4), 1290–1296. https://doi.org/10.1016/j.meatsci.2008.06.004

Estévez, M., Li, Z., Soladoye, O. P., & Van-Hecke, T. (2017). Health Risks of Food Oxidation. *Advances in Food and Nutrition Research, 82*, 45–81. https://doi.org/10.1016/bs.afnr.2016.12.005

Estévez, M., & Luna, C. (2017). Dietary protein oxidation: A silent threat to human health? *Critical Reviews in Food Science and Nutrition, 57*(17), 3781–3793. https://doi.org/10.1080/10408398.2016.1165182

Estévez, M., Ollilainen, V., & Heinonen, M. (2009). Analysis of protein oxidation markers α-Aminoadipic and γ-Glutamic semialdehydes in food proteins using liquid chromatography (LC)-Electrospray ionization (ESI)-Multistage tandem mass spectrometry (MS). *Journal of Agricultural and Food Chemistry, 57*(9), 3901–3910. https://doi.org/10.1021/jf804017p

Estévez, M., & Xiong, Y. (2019). Intake of Oxidized Proteins and Amino Acids and Causative Oxidative Stress and Disease: Recent Scientific Evidences and Hypotheses. *Journal of Food Science, 84*(3), 387–396. https://doi.org/10.1111/1750-3841.14460

Feng, X., Li, C., Ullah, N., Cao, J., Lan, Y., Ge, W., … Chen, L. (2015). Susceptibility of whey protein isolate to oxidation and changes in physicochemical, structural, and digestibility characteristics. *Journal of Dairy Science, 98*(11), 7602–7613. https://doi.org/10.3168/jds.2015-9814

Ferreira, V. C. S., Morcuende, D., Madruga, M. S., Silva, F. A. P., & Estévez, M. (2018). Role of protein oxidation in the nutritional loss and texture changes in ready-to-eat chicken patties. *International Journal of Food Science and Technology, 53*(6), 1518–1526. https://doi.org/10.1111/ijfs.13733

Ganhão, R., Estévez, M., & Morcuende, D. (2011). Suitability of the TBA method for assessing lipid oxidation in a meat system with added phenolic-rich materials. *Food Chemistry, 126*(2), 772–778. https://doi.org/10.1016/j.foodchem.2010.11.064

Kanner, J., & Lapidot, T. (2001). The stomach as a bioreactor: Dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology and Medicine, 31*(11), 1388–1395. https://doi.org/10.1016/S0891-5849(01)00718-3

Kröger-Ohlsen, M. V., Østdal, H., & Andersen, M. L. (2003). The effect of pH on the oxidation of bovine serum albumin by hypervalent myoglobin species. *Archives of Biochemistry and Biophysics, 416*(2), 202–208. https://doi.org/10.1016/S0003-9861(03)00317-5

Luna, C., & Estévez, M. (2019). CEFormation of allysine in β-lactoglobulin and myofibrillar proteins by glyoxal and methylglyoxal: Impact on water-holding capacity and in vitro digestibility. *Food Chemistry*, *271*(April 2018), 87–93. https://doi.org/10.1016/j.foodchem.2018.07.167.

Macho-González, A., Garcimartín, A., López-Oliva, M. E., Bastida, S., Benedí, J., Ros, G., … Sánchez-Muniz, F. J. (2020). Can meat and meat-products induce oxidative stress? *Antioxidants, 9*(7), 1–22. https://doi.org/10.3390/antiox9070638

Oueslati, K., De La Pomélie, D., Santé-Lhoutellier, V., & Gatellier, P. (2016). Impact of the Fenton process in meat digestion as assessed using an in vitro gastro-intestinal model. *Food Chemistry, 209*, 43–49. https://doi.org/10.1016/j.foodchem.2016.04.041

Rombouts, C., Hemeryck, L. Y., Van Hecke, T., De Smet, S., De Vos, W. H., & Vanhaecke, L. (2017). Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. *Scientific Reports*, *7*(January 2017), 1–13. https://doi.org/10.1038/srep42514.

Ruiz-Carrascal, J. (2016). *Cured Foods: Health Effects* (B. Caballero, P. M. Finglas, & F. B. T.-E. of F. and H. Toldrá (eds.); pp. 338–342). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384947-2.00207-5.

Santé-Lhoutellier, V., Astruc, T., Marinova, P., Greve, E., & Gatellier, P. (2008). Effect of meat cooking on physicochemical state and in vitro digestibility of myofibrillar

proteins. *Journal of Agricultural and Food Chemistry, 56*(4), 1488–1494. https://doi.org/10.1021/jf072999g

Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide - Biology and Chemistry, 24*(4), 176–183. https://doi.org/10.1016/j.niox.2011.03.307

Susanto, A. P., Firdhaosh, M., Wang, D.-A., & Moe, K. T. (2006). Superoxide detection by chemiluminescence, fluorescence, and spectrophotometry methods. In *ICBPE 2006 - Proceedings of the 2006 International Conference on Biomedical and Pharmaceutical Engineering* (pp. 537–540). https://doi.org/10.1109/ICBPE.2006.348652

Suyama, K., Akagawa, M., & Sasaki, T. (2002). Oxidative deamination of lysine residue in plasma protein from diabetic rat: α-dicarbonyl-mediated mechanism. In *International Congress Series* (Vol. 1245, Issue C). https://doi.org/10.1016/S0531-5131(02)01019-1.

Tappel, A. (2007). Heme of consumed red meat can act as a catalyst of oxidative damage and could initiate colon, breast and prostate cancers, heart disease and other diseases. *Medical Hypotheses, 68*(3), 562–564. https://doi.org/10.1016/j.mehy.2006.08.025

Théron, L., Chambon, C., Sayd, T., De La Pomélie, D., Santé-Lhoutellier, V., & Gatellier, P. (2018). To what extent does the nitrosation of meat proteins influence their digestibility? *Food Research International, 113*, 175–182. https://doi.org/10.1016/j.foodres.2018.06.071

Utrera, M., Morcuende, D., Rodríguez-Carpena, J. G., & Estévez, M. (2011). Fluorescent HPLC for the detection of specific protein oxidation carbonyls - α-aminoadipic and γ-glutamic semialdehydes - in meat systems. *Meat Science, 89*(4), 500–506. https://doi.org/10.1016/j.meatsci.2011.05.017

Utrera, M., Rodríguez-Carpena, J. G., Morcuende, D., & Estévez, M. (2012). Formation of lysine-derived oxidation products and loss of tryptophan during processing of porcine patties with added avocado byproducts. *Journal of Agricultural and Food Chemistry, 60*(15), 3917–3926. https://doi.org/10.1021/jf3001313

Van Hecke, T., Vanden Bussche, J., Vanhaecke, L., Vossen, E., Van Camp, J., & De Smet, S. (2014). Nitrite curing of chicken, pork, and beef inhibits oxidation but does not affect N-nitroso compound (NOC)-specific DNA adduct formation during in vitro digestion. *Journal of Agricultural and Food Chemistry, 62*(8), 1980–1988. https://doi.org/10.1021/jf4057583

Van Hecke, T., Vossen, E., Goethals, S., Boon, N., De Vrieze, J., & De Smet, S. (2021). In vitro and in vivo digestion of red cured cooked meat: oxidation, intestinal microbiota and fecal metabolites. *Food Research International*, *142*(September 2020), 110203. https://doi.org/10.1016/j.foodres.2021.110203.

Villaverde, A., & Estévez, M. (2013). Carbonylation of myofibrillar proteins through the Maillard pathway: Effect of reducing sugars and reaction temperature. *Journal of Agricultural and Food Chemistry, 61*(12), 3140–3147. https://doi.org/10.1021/jf305451p

Villaverde, A., Parra, V., & Estévez, M. (2014). Oxidative and nitrosative stress induced in myofibrillar proteins by a hydroxyl-radical-generating system: Impact of nitrite and ascorbate. *Journal of Agricultural and Food Chemistry, 62*(10), 2158–2164. https://doi.org/10.1021/jf405705t

Wang, Q., Durand, E., Elias, R. J., & Tikekar, R. V. (2016). Generation of reactive oxidative species from thermal treatment of sugar solutions. *Food Chemistry, 196*, 301–308. https://doi.org/10.1016/j.foodchem.2015.09.044

Yang, H., Zhang, Y., & Pöschl, U. (2010). Quantification of nitrotyrosine in nitrated proteins. *Analytical and Bioanalytical Chemistry, 397*(2), 879–886. https://doi.org/10.1007/s00216-010-3557-3

Capítulo II

Impact of Sustained Fructose Consumption on Gastrointestinal Function and Health in Wistar Rats: Glycoxidative Stress, Impaired Protein Digestion and Shifted Faecal Microbiota

Impacto del consumo sostenido de fructosa sobre la función gastrointestinal y la salud de ratas Wistar: estrés glicoxidativo, digestión proteica afectada y microbiota fecal alterada

Impact of Sustained Fructose Consumption on Gastrointestinal Function and Health in *Wistar* Rats: Glycoxidative Stress, Impaired Protein Digestion and Shifted Faecal Microbiota

Guadalupe Sánchez-Terrón, Remigio Martínez, Jorge Ruiz, Carolina Luna and Mario Estévez*

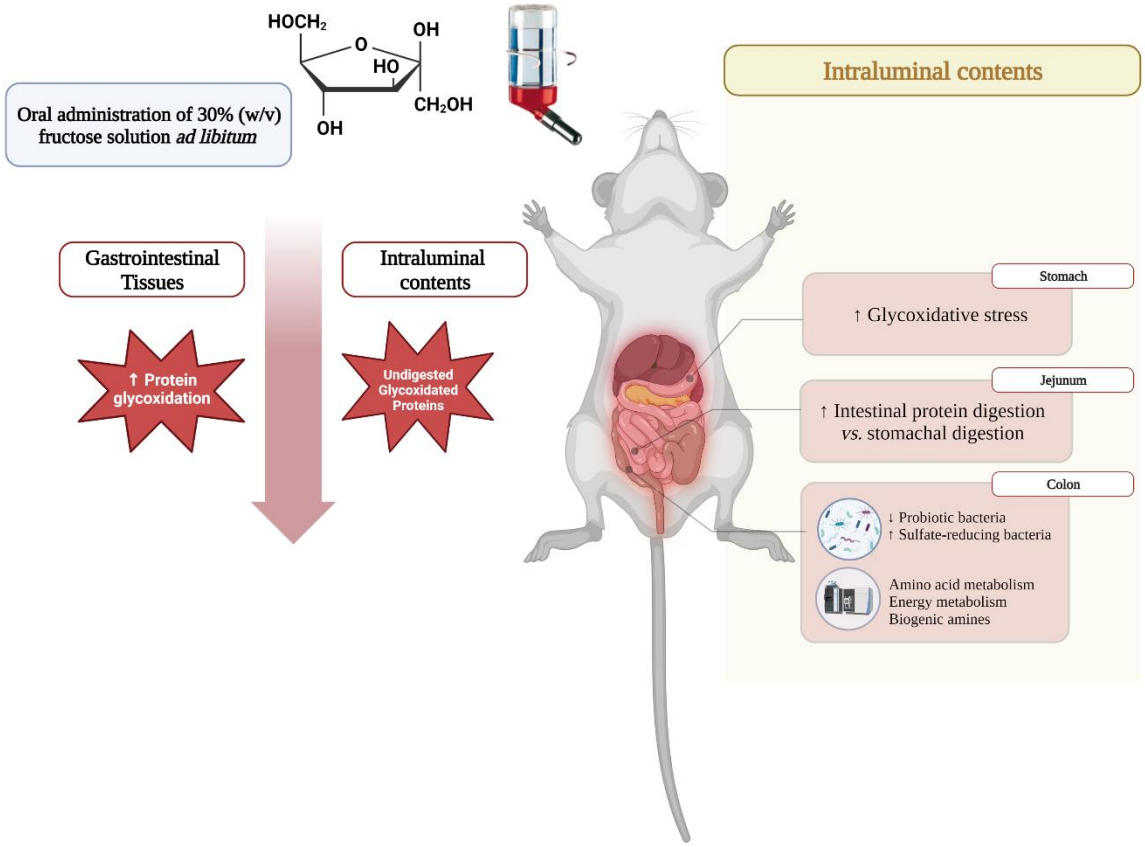

Figura 4.1. Resumen gráfico del Capítulo II. Elaboración propia.

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Impact of Sustained Fructose Consumption on Gastrointestinal Function and Health in *Wistar* Rats: Glycoxidative Stress, Impaired Protein Digestion, and Shifted Fecal Microbiota

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ABSTRACT: The gastrointestinal tract (GIT) is the target of assorted pathological conditions, and dietary components are known to affect its functionality and health. In previous *in vitro* studies, we observed that reducing sugars induced protein glycoxidation and impaired protein digestibility. To gain further insights into the pathophysiological effects of dietary sugars, *Wistar* rats were provided with a 30% (w/v) fructose water solution for 10 weeks. Upon slaughter, *in vivo* protein digestibility was assessed, and the entire GIT (digests and tissues) was analyzed for markers of oxidative stress and untargeted metabolomics. Additionally, the impact of sustained fructose intake on colonic microbiota was also evaluated. High fructose intake for 10 weeks decreased protein digestibility and promoted changes in the physiological digestion of proteins, enhancing intestinal digestion rather than stomach digestion. Moreover, at colonic stages, the oxidative stress was harmfully increased, and both the microbiota and the intraluminal colonic metabolome were modified.

KEYWORDS: *fructose, metabolomic, microbiota, protein digestibility, protein glycoxidation*

1. INTRODUCTION

The World Health Organization (WHO) has recommended for more than a decade limiting free sugar intake to less than 10% of the total energy intake based on the evidence showing that a higher consumption of sugars increases the risk of metabolic diseases.[1]

Sucrose (50% fructose) is the most used sugar in the food industry. According to WHO recommendations, a healthy individual should not consume more than 25 g of sucrose per day, which corresponds to a recommended daily intake of less than 12 g.[1] However, consumption of elevated levels of dietary fructose is currently an established daily habit through the consumption of sugar-sweetened beverages, snacks, and baked goods formulated with sucrose or commercial high-fructose corn syrup (HFCS) (55% fructose).[2] There is a body of evidence that excessive fructose consumption is responsible for several metabolic impairments, which are associated with metabolic syndrome (MetS) due to the disturbance of liver metabolism. The main manifestations of these impairments are adiposity, dyslipidaemia, nonalcoholic fatty liver disease (NAFLD), insulin resistance, and type 2 diabetes (T2D).[3]

Besides the high caloric value of sugars (proadiposity) and their ability to induce insulin resistance (prodiabetic), the molecular basis of the noxious effects of increased levels of circulating sugar is related to the onset of oxidative stress mechanisms.[4,5] In particular, the reactive carbonyl moiety in reducing sugars such as fructose plays a pivotal role in the pathophysiological effects of these species. Reducing sugars and reactive carbonyl species (RCS) formed from their degradation (i.e., dicarbonyls such as glyoxal and methylglyoxal) are known to induce oxidative damage to proteins and other biomolecules (glycoxidation).[6] Protein carbonylation is an early manifestation of glycoxidation, which is known to take place, for instance, in individuals suffering from insulin resistance and enduring hyperglycemia.[7,8] In a recent study, we were able to reproduce the entire carbonylation pathway (lysine−allysine−aminoadipic acid) in human plasma proteins under simulated hyperglycemic conditions.[9] Protein carbonyls and other sugar-derived reactive species are implicated in the formation of advanced glycation end products (AGEs), which are accumulated in target tissues leading to physiological impairments.[10] The ability of sugars and their dicarbonyls to induce oxidative stress in several organs such as the intestine,[11] liver,[12] pancreas,[13] and brain[14] is thought to be associated with the onset of various of the aforementioned related diseases (NALD, T2D, aging, etc.).[3]

While the impact of dietary fructose on the physiology of the liver, pancreas, and various other internal organs is well known,[3] fructose may interact, prior to intestinal uptake and organic distribution, with other dietary components, with microbiota and epithelial cells from the GIT leading to noxious effects at this location. However, the postprandial effects of fructose consumption are poorly understood. A previous *in vitro* study revealed the severe deleterious effects of glucose on the oxidative stability and digestibility of dietary proteins when allowed to react in the pro-oxidative environment of the

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stomach.[15] Under simulated physiological conditions, glucose enhances the glycoxidative damage to meat proteins, leading to impaired digestibility and a loss of nutritional value. *In vivo* studies on the impact of glucose, fructose, and other sugars with highly reactive carbonyls on the onset of luminal or tissue oxidative stress in the GIT are scarce. In a recent study, it was observed that fructose consumption led to disturbance of intestinal microbiota, and that, in turn, with abnormal immune response.[16] The onset of enduring oxidative stress in the lumen, which may eventually transfer to the epithelium of the GIT, along with severe microbiota disturbance (dysbiosis), has been hypothesized to contribute to the onset of numerous pathological conditions, such as inflammatory bowel disease (IBD) and colorectal cancer (CRC).[17]

Given the many complex mechanisms by which fructose may affect gut health and, in turn, organic homeostasis, this study was designed to gain a deeper understanding of the effects of fructose intake on protein digestibility and the occurrence of oxidative stress using an *in vivo* model (*Wistar* rats). The impact of sustained fructose consumption on gut microbiota and colonic metabolome was also studied.

2. MATERIALS AND METHODS

2.1. Chemicals. All reagents, chemicals, and standard compounds were obtained from Sigma Chemicals (Sigma-Aldrich, Stheinheim, Germany), Fisher (Fisher Scientific S.L., Madrid, Spain), and Panreac (Panreac Qumica, S.A., Barcelona, Spain). Ultrapure water was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA).

2.2. Animals, Feeds, and Other Materials. *Wistar* breed rats of the *Rattus novergicus* species were used in our experiment according to Spanish legal requirements (RD 53/2013), the bioethics committee of the University of Extremadura (137-2020), and approval of the Board of Extremadura (EXP20200904). The design and performance of the experiment, including animal manipulation and euthanasia, were carried out by licensed veterinarians with all requirements by legal authority (Dirección General de Sanidad Animal de Junta de Extremadura). Twelve male rats were used in the present study. The rats were supplied and maintained during the whole assay at the Animal Facilities Service of the University of Extremadura (Cáceres, Spain), and at the beginning of the assay, they were 6−7 weeks old and weighed 186 g on average. During the entire study period, the same rodent basal feed used was the "Teklad Global Diet 2014", supplied by ENVIGO (Madison, WI), with a crude protein content of 14.3%.

2.3. Experimental Design. The animals were subjected to a 1 week adaptation period. During this period, the rats were maintained in ventilated cages, with water and feed *ad libitum*, under controlled climatic conditions (20−22 °C temperature, 40−50% humidity and 12−12 h light/dark cycle). Individual identification of animals was performed during the adaptation period by means of a perforation code in the auditory pavilion.

After the adaptation period was concluded, we divided the animals into two experimental groups (n = 6 in each group): (i) a control group (C) that received the basal feed and drinking water during the entire assay and (ii) a fructose group (F), which consumed basal feed and 30% w/v fructose water solution. The rats coexisted in subgroups of three animals per cage. On average, rats from the F group had 9 g of fructose/kg of live bodyweight/day. The 30% (w/v) fructose solution is selected based on the literature that reported significant oxidative stress in *Wistar* rats induced by the dietary intake of such an amount of sugar.[18] Additionally, the 30% of fructose we applied is in the range between 20% (equivalent to the top 5% of American consumers) and 63% of free fructose concentrations in the diet.[19]

The experiment was conducted for 10 weeks. The animals were visited and checked daily to ensure their safety and well-being. During the assay, food and water consumption were gravimetrically monitored every time they were filled, depending on the demand of the animals (every 2 or 3 days, approximately), and bodyweights were registered weekly (Table S2).

2.4. Slaughter, Necropsy and Sampling. Both food and drink were *ad libitum* available to experimental animals until slaughter. *Wistar* rats were euthanized at the end of the experimental period at an approximate age of 16−17 weeks old and an average weight of 437 g. Euthanasia was performed by exsanguination via cardiac puncture. Previously, the animals were anesthetized using 5% inhaled isoflurane. The GIT of the animals was readily dissected from corpses and clamped to avoid loss of intraluminal material. The stomach, small intestine (jejunum), cecum, and large intestine (distal colon) were aseptically sampled. Under the same conditions, the intraluminal material (digests) at each of the aforementioned locations was gently removed, dispensed in Eppendorf tubes, and stored immediately at −80 °C until analyses were performed. Feces from the rectum were also aseptically collected and stored at −80 °C until analyses were performed. Once emptied, the tissue from each location was thoroughly cleaned with cold distilled water. A portion of each location was dispensed in *Eppendorf* tubes and stored at −80 °C until analyses were performed.

2.5. Analytical Procedures. *2.5.1. Assessment of Glycoxidative Stress in Digests and Gut Tissues. 2.5.1.1. Protein Carbonylation.* The accretion of protein carbonyls in the feeds, luminal contents, and tissues was assessed as previously described,[20] with slight modifications. The quantification of specific protein carbonyls, namely, α-aminoadipic and γ-glutamic semialdehydes (α-AS and γ-GS, respectively), was carried out using an HPLC analysis attached to a fluoresce detector. GIT digests and tissues were thoroughly homogenized. For contents, 250 mg of the stomach, jejunum, cecum, and colon digests, as well as feces, were individually mixed and homogenized with 1 mL of PBS in Eppendorf tubes in a mixer mill. On the other hand, 500 mg of the respective tissues were homogenized with 0.5 mL of PBS. Results from the quantification of α-AS and γ-GS were expressed as total primary protein carbonyls (PPCs) as nmol carbonyl/mg protein. The remaining steps of the procedure were exactly as those reported by the above-mentioned authors.[20]

2.5.1.2. Advanced Protein Oxidation Products (APOPs). APOPs were analyzed using fluorescent spectroscopy (PerkinElmer, Beaconsfield, U.K.), as reported.[9] Thoroughly homogenized samples were diluted with 100 mM sodium phosphate buffer, pH 7.4, with 2 M guanidine chlorhydrate. APOPs were excited at 350 nm, and the emitted fluorescence was recorded from 400 to 500 nm. The excitation and emission slits were both set to 10 nm, and the scanning speed was 500 nm/min. The fluorescence results were applied to a correction factor (Cf = Pt/Pp) where Pt is the total average of the amount of protein from all samples and Pp is the content of protein in each sample. Results are expressed as arbitrary fluorescence intensity (area units) (FU).

2.5.1.3. Thiobarbituric Acid Reactive Substances (TBARSs). Malondialdehyde (MDA) and other TBARSs were extracted from feeds, luminal contents, and tissues and subsequently quantified following the procedure reported by Ganhão et al.[21] with some modifications. Samples extracted from sample homogenates were treated with 8 volumes of perchloric acid (3.86%) and 0.5 volumes of butylated hydroxytoluene (BHT) (4.2% in ethanol) to avoid further peroxidation. Upon a reaction with 0.02 M thiobarbituric acid (TBA), samples were placed in a boiling water bath (100 °C) for 45 min together with the tubes from the standard curve. After cooling, the absorbance was measured at 532 nm by spectrophotometry (Shimadzu Model UV-1800, Shimadzu, Japan). The standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) solution in 3.86% perchloric acid. Results were calculated as milligrams of MDA per 100 g of the sample.

2.5.2. Analysis of Protein Degradation and Protein Overall Digestibility. Basal feed, intraluminal material (digests), and animal tissues from each compartment from GIT were analyzed for moisture content and concentration of protein by the official Association of Official Agricultural Chemists (AOAC) methods.[22] The Kjeldahl

method was performed as previously described by other authors.[23] In addition to total nitrogen (TN), feed, and digests were analyzed for water-soluble nitrogen (WSN) content and nonprotein nitrogen (NPN) using the same Kjeldahl procedure. For the WSN, samples were homogenized twice with 5 volumes (w/v) of deionized water and centrifuged at 5000*g* and 4 °C for 10 min. Combined supernatants were filtered through Whatman No. 1 filter paper and subsequently subjected to the Kjeldahl method for nitrogen quantification.[22] For the quantification of NPN, an aliquot of the aforementioned filtrate was mixed with an equal volume of 20% trichloroacetic acid (TCA), allowed to stand at room temperature for 30 min, centrifuged at 5000*g* at 4 °C for 10 min, and then filtered through Whatman No. 4 filter paper. NPN was also quantified using the Kjeldahl method.[22] Total protein nitrogen (TPN) was calculated as follows: TPN (g) = WSN − NPN. Total dietary nitrogen (TDN) at each compartment of the GIT tract was calculated as follows: TDN (g) = (TPN − Ep) where Ep is the defined metabolic/endogenous nitrogen.[24,25] Ep refers to nitrogen-containing biomolecules (e.g., proteins and peptides) secreted at each stage of the GIT of an animal receiving a protein-free diet. Ep was calculated for each stage and subtracted to TPN at such stage. Total dietary protein (TDP) was calculated from TDN using a conversion factor of 6.25.

An estimation of the amount of TDP degraded in each compartment of the GIT tract was calculated as follows: TDP degraded at specific compartment (g) = (TDP_1 − TDP_2). TDP_1 is the total concentration of TDP in the immediately previous compartment and TDP_2 is the concentration of TDP in the compartment under study in which digestion was assumed finished (samples taken at the end of such stage). For further accuracy, the concentration of protein in each stage was calculated considering the moisture content of feeds and luminal contents at each stage (all protein data are shown as dry matter). For the calculation of protein degradation in the stomach, TDP_1 was considered TDP in the feeds, which corresponds to TN in the feed (×6.25), as Ep does not apply in this case for obvious reasons. The combination of TDP degraded at the stomach and at the small intestine was considered as digested protein (DP), while TDP degraded at both the cecum and the colon was considered fermented protein (FP).

An estimation of total true protein digestibility (TPD) (considering the entire GIT) was calculated according to the formula: True digestibility (%) = {[TNf − (FN − TEp)]/TNf} × 100, where TNf is total nitrogen from feeds (dietary nitrogen), FN is fecal nitrogen, and TEp is the total metabolic/endogenous nitrogen found in feces from a rat fed a protein-free diet.[25]

2.5.3. Fecal Microbiota. Microbiota from *Wistar* rats was analyzed from feces obtained at slaughter, as aforementioned. DNA was isolated from feces using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit (Thermo Fisher Scientific, MA) following the manufacturer's instructions and the KingFisher Flex Instrument (Thermo Fisher Scientific, Waltham, WA).

Genomic DNA was amplified using specific primers for V3 and V4 variable regions of the 16S rRNA gene. Amplification, sequencing, and basic analysis were performed using an Illumina MiSeq platform, using the MiSeq Reagent Kit v3 and 300b paired end. The analysis of the generated raw sequence data was carried out using QIIME2 v2021.4. Finally, the operational taxonomic units (OTUs) were classified by taxon using the SILVA database (release 138 QIIME) and trained by a scikit-learn classifier using the UNITE (release 8.3) database. Different α-diversity indices (i.e., dominance, taxa richness, individuals, Shannon index, Simpson index, and evenness) were calculated from phylum and genus OTUs' counts using the software package Past v4.09, and the results were expressed as $\log_2$.

2.5.4. Untargeted MS-Based Metabolomics. Metabolites were analyzed in the intraluminal colonic contents of *Wistar* rats. The extraction was carried out with both an aqueous and an organic solvent to get most of the metabolites. Briefly, 100 μL of homogenized colonic content was mixed with both 0.5 mL of cyclohexane and 0.5 mL of Milli-Q water. The mixing was homogenized in a mixer mill using small steel balls for 2 min at 30 Hz and subsequently centrifuged at 9000*g* and 4 °C for 15 min. Two phases were obtained (aqueous and organic phase) and separated into single *Eppendorf* tubes using 0.22 μm nylon filters. Additionally, 200 μL of acetonitrile HPLC quality was added to 50 μL of the aqueous phase to ensure a correct flux through the column. Samples were analyzed using a Dionex UltiMate 3000 RSLC system coupled with a Q-Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA). An Accucore C18 HPLC (150 × 2.1 mm^2 I.D., particle size 2.6 μm) column was used as a stationary phase for the analysis of the organic phase, while an Accucore HILIC (150 × 3 mm^2 I.D., particle size 2.6 μm) column was used as an aqueous phase (Thermo Fisher Scientific, San Jose, CA). The mobile phase was solvent water (eluent A) and acetonitrile (eluent B), both with 0.1% formic acid. The injection volume was 8 μL.

The gradient used for the organic phase separation was set as follows: 0−1 min isocratic 2% B, 1−14 min linear gradient 2−95% B, 14−16 min isocratic 95% B, 16−16.1 min linear gradient 95−2% B, 16.1−20 min isocratic 2% B; flow rate 400 μL/min; column temperature 45 °C; and total run time: 20 min. The gradient used for the aqueous phase separation was set as follows: 0−1 min isocratic 99% B, 1−3 min linear gradient 99−85% B, 9−10 min isocratic 5% B, 10−10.5 min linear gradient 5−99% B, 10.5−15 min isocratic 99% B; flow rate 500 μL/min; column temperature 35 °C; and total run time: 15 min. The organic phase was run under positive ionization mode, and the aqueous phase was run under both positive and negative modes.

To identify as many compounds as possible, a pool of all of the samples was run iteratively on MS^2 analysis to achieve the mass fragmentation spectra. Full-scan analysis was used for regular samples in a scan range of 53.4−800 m/z and 70000 fwhm. MS^2 analysis was performed for the top five data-dependent acquisitions. For both aqueous and organic LC-MS and LC-MS/MS analyses, a pool of all samples (quality control sample) was injected in every eight samples for the aligning of small shifts in retention times, mass accuracy, signal drift, and carryover, as well as normalizing peak areas if necessary. A positive identification was confirmed for discriminating metabolites by comparing MS data with those from available standard compounds. The equipment was calibrated weekly using both a Pierce LTQ Velos ESI Positive Ion Calibration Solution and a Pierce LTQ Velos ESI Negative Ion Calibration Solution (Thermo Fisher Scientific, San Jose, CA).

Data were analyzed using Compound Discoverer software (Thermo Fisher Scientific, San Jose, CA). Among the main settings used for aligning, identifying, and comparing, the metabolites found in every group had a maximum shift of 1 min and mass tolerance lower than 5 ppm.

2.6. Statistical Analysis. Analyses were performed in six animals per group, and each sample was technically analyzed twice. The distribution of raw data was determined by using the Shapiro−Wilk normality test. The statistical analysis of the differences among the different glycoxidative markers of the intraluminal contents and the tissues along the GIT from the two groups was carried out using a two-way ANOVA test and a Tukey test as *post hoc* analysis. The significance of differences among the protein digestibility markers and between the diversity indices was evaluated using Student-*t* tests. The data analyzed for tables and graphs by parametric tests are expressed as the mean ± standard error of the mean. Data not passing normality testing were analyzed using the Mann−Whitney U test and were expressed as the median [interquartile range (Q3 − Q1)] in the graphs. Statistical analysis was performed in SPSS version 27.0, and *p*-values lower than 0.05 were considered statistically significant. Fructose-responsive metabolites were assessed in the MetaboAnalyst (https://www.metaboanalyst.ca/), establishing standard deviation as a statistical filter for the 40% of the noninformative variables and the Pareto scaling for normalizing the raw data. Partial least-squares discriminant analysis (PLS-DA) as multivariant analysis was used, and the top 30 metabolites were ranked by the variable importance in projection (VIP) score from PLS-DA outcomes. Moreover, metabolite profile distinctions between the groups were evaluated by the Volcano plot as a one-factor statistical method to further analyze the impact of fructose on the colonic metabolome of *Wistar*

Table 1. Concentration of Markers of Glycoxidative Stress (Means ± Standard Deviation) in the Feed, Luminal Contents (Digests) at Each Stage of the Gastrointestinal Tract, and in the Feces of *Wistar* Rats (n = 6 Per Group) Fed *Ad Libitum* for 10 Weeks with a Control Base Diet and either Drinking Water (Control) or a 30% Fructose Water Solution (Fructose)

		α-AS[1]	γ-GS[2]	total PPC[3]	APOPs[4]	TBARS[5]
feed		$0.34^{f} \pm 0.09$	$0.15^{e} \pm 0.04$	$0.49^{f} \pm 0.26$	$210^{f} \pm 52$	$0.07^{d} \pm 0.01$
stomach	control	$0.55^{e} \pm 0.07$	$0.19^{de} \pm 0.02$	$0.74^{e} \pm 0.25$	$305^{de} \pm 63$	$0.12^{c} \pm 0.03$
	fructose	$0.94^{cd} \pm 0.13$	$0.35^{cd} \pm 0.06$	$1.29^{c} \pm 0.32$	$541^{c} \pm 48$	$0.11^{c} \pm 0.02$
jejunum	control	$0.24^{f} \pm 0.04$	$0.19d^{e} \pm 0.04$	$0.43^{f} \pm 0.12$	$340^{d} \pm 51$	$0.29^{a} \pm 0.06$
	fructose	$0.67^{de} \pm 0.12$	$0.39^{c} \pm 0.08$	$1.06^{de} \pm 0.28$	$784^{b} \pm 102$	$0.35^{a} \pm 0.07$
cecum	control	$1.81^{b} \pm 0.39$	$0.37^{c} \pm 0.08$	$2.18^{b} \pm 0.59$	$244^{ef} \pm 47$	$0.17^{b} \pm 0.02$
	fructose	$2.06^{b} \pm 0.29$	$0.58^{b} \pm 0.09$	$2.64^{b} \pm 0.35$	$511^{c} \pm 62$	$0.19^{b} \pm 0.03$
colon	control	$1.17^{c} \pm 0.25$	$0.28^{d} \pm 0.07$	$1.45^{c} \pm 0.38$	$366^{d} \pm 43$	$0.15^{bc} \pm 0.02$
	fructose	$3.25^{a} \pm 0.62$	$1.01^{a} \pm 0.18$	$4.26^{a} \pm 0.79$	$1025^{a} \pm 125$	$0.13^{c} \pm 0.02$
feces	control	$0.45^{ef} \pm 0.10$	$0.23^{d} \pm 0.08$	$0.68^{e} \pm 0.15$	$201^{f} \pm 42$	$0.11^{c} \pm 0.03$
	fructose	$0.86^{d} \pm 0.15$	$0.34^{cd} \pm 0.07$	$1.20^{cd} \pm 0.19$	$192^{f} \pm 36$	$0.13^{c} \pm 0.03$
p-value[6]	stage	***	**	***	**	*
	diet	**	*	***	**	ns
	S × D	ns	ns	ns	*	ns

[1]α-Aminoadipic semialdehyde. Results are expressed as nmol carbonyl/mg protein. [2]γ-Glutamic semialdehyde. Results are expressed as nmol carbonyl/mg protein. [3]Total primary protein carbonyls. Results are expressed as nmol carbonyl/mg of protein. [4]Advanced protein oxidation products. Results are expressed as arbitrary fluorescent units. [5]Thiobarbituric acid reactive substances. Results are expressed as mg MDA/100 g sample (feed, digests, feces). [6]Significance level in two-way ANOVA with the effects of the stage (S) (feed, GIT compartments, feces), diet (D) (control vs fructose), and the interaction (S × D). Means with different letters within the same column were significantly different in Tukey *post hoc* analysis ($p < 0.05$). ns: no significance, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

rats, which combines results from fold change (FC) analysis and t tests into one single study using a p-value threshold of <0.05 and a fold change threshold >2.

3. RESULTS

3.1. Effect of Dietary Fructose on Feed, Water, and Calories Consumption and Weights of Wistar Rats. Feed and fructose-supplemented water provided 15.31 and 5.02 kJ/g energy, respectively. Table S1 shows the median energy intake expressed as kJ/day provided by the feed, the fructose solution, and the sum of both to the experimental animals for 10 weeks. The fructose-supplemented group received significantly higher calorie intake from water consumption ($p < 0.001$). However, total energy intake per day was not significantly different. Moreover, there was no significant difference in the body-weight of the rats during the experiment (Table S2).

3.2. Effect of Dietary Fructose on the Extent of Protein and Lipid Glycoxidation that Occurred in the Luminal Content of the GIT during Digestion. *3.2.1. Carbonylation of Digests at Different Locations of GIT.* Table 1 shows the concentration of α-AS, γ-GS, and total primary protein carbonyls (sum of both α-AS and γ-GS) in the feed, digests at each stage of the GIT, and feces of the rats. Irrespective of the treatment, there were significant differences among the protein carbonylation in the feed, digests along the different gastrointestinal compartments, and in the feces ($p < 0.001$). Thus, the levels of α-AS in the digests at the stomach stage were significantly higher than those in the feed. γ-GS and total PPC showed the same trend. Overall luminal protein carbonylation increased up to 2-fold at the stomach from the experimental animals. However, the concentration of primary protein carbonyls showed a decrease in the luminal content at the jejunum stage (−30% than those in the stomach contents) ($p < 0.001$). Thereafter, the concentration of the protein glycoxidation markers displayed a progressive increase during the advance of the digest along the next stages of the GIT, the colon being the compartment where the highest concentration of carbonyls was found regardless of fructose treatment. The carbonylation level at this stage was more than 5-fold higher than that in feed. Interestingly, the concentration of both semialdehydes in the feces was 3-fold lower than in the colon stage.

Fructose supplementation had a significant effect on the concentration of both α-AS and γ-GS in the luminal contents at the different stages of the GIT and in the feces of the treated animals ($p < 0.01$ and $p < 0.05$, respectively). F rats showed significantly greater amounts of total PPC in the digests at all stages than their control counterparts ($p < 0.001$). At the stomach stage, the intraluminal levels of PPC in F rats were found to be nearly doubled than those found in the stomach of control animals. At the jejunum stage, both F and C groups showed a significant decrease in the amounts of luminal PPC ($p < 0.001$). Then, the carbonyl contents in the digests at the cecum and colon stages increased, but the results showed a different trend between the groups. The amount of carbonylated proteins in the digests in the cecum from F rats was lower than those in the colon, where a significant and intense protein carbonylation occurred. Colonic digests contained the highest concentration of PPC (4.26 nmol carbonyls/mg of protein), being more than 8-fold higher than that found in feeds ($p < 0.001$). Instead, the highest concentration of semialdehydes in the digests from the C group occurred in the cecum. The concentration of carbonyls in the feces from the F group was significantly lower than in the feces from C rats. The interaction between fructose supplementation and the effect of the different stages of GIT was not statistically significant, meaning that the effect of fructose is location-independent.

3.2.2. Formation of APOPs in Digests at Different Locations of GIT. In addition to the glycoxidation markers described above, Table 1 shows the evolution of the amounts of APOPs in the digests along the different GIT stages as markers of advanced protein glycation processes. The intensity of the fluorescence emitted by APOPs significantly showed 2.7-fold higher values from feed to digests at the jejunum stage

Table 2. Concentration of Markers of Glycoxidative Stress (Means ± Standard Deviation) in the Tissues from Each Compartment of the Gastrointestinal Tract from *Wistar* Rats (n = 6 Per Group) Fed *Ad Libitum* for 10 Weeks with a Control Base Diet and either Drinking Water (Control) or a 30% Fructose Water Solution (Fructose)

		α-AS[1]	γ-GS[2]	total PPC[3]	APOPs[4]	TBARS[5]
stomach	control	$0.38^{e} \pm 0.09$	$0.22^{c} \pm 0.02$	$0.61^{d} \pm 0.14$	$350^{f} \pm 22$	$0.26^{b} \pm 0.06$
	fructose	$0.69^{d} \pm 0.12$	$0.46^{b} \pm 0.05$	$1.15^{c} \pm 0.22$	$506^{d} \pm 31$	$0.29^{b} \pm 0.04$
jejunum	control	$1.29^{c} \pm 0.16$	$0.50^{a} \pm 0.07$	$1.80^{b} \pm 0.25$	$439^{e} \pm 30$	$0.38^{a} \pm 0.06$
	fructose	$1.38^{c} \pm 0.19$	$0.56^{a} \pm 0.06$	$1.95^{b} \pm 0.31$	$627^{c} \pm 44$	$0.41^{a} \pm 0.09$
colon	control	$1.99^{b} \pm 0.25$	$0.12^{d} \pm 0.03$	$2.03^{b} \pm 0.29$	$840^{b} \pm 87$	$0.36^{ab} \pm 0.07$
	fructose	$2.45^{a} \pm 0.29$	$0.15^{d} \pm 0.04$	$2.61^{a} \pm 0.32$	$1203^{a} \pm 99$	$0.41^{a} \pm 0.11$
p-value[6]	stage	***	***	**	***	*
	diet	***	**	**	***	ns
	S × D	*	**	*	ns	ns

[1]α-Aminoadipic semialdehyde. Results are expressed as nmol carbonyl/mg protein. [2]γ-Glutamic semialdehyde. Results are expressed as nmol carbonyl/mg protein. [3]Total primary protein carbonyls. Results are expressed as nmol carbonyl/mg of protein. [4]Advanced protein oxidation products. Results are expressed as arbitrary fluorescent units. [5]Thiobarbituric acid reactive substances. Results are expressed as mg MDA/100 g sample (feed, digests, feces). [6]Significance level in two-way ANOVA with the effects of the stage (S) (feed, GIT compartments, feces), diet (D) (control vs fructose), and the interaction (S × D). Means with different letters within the same column were significantly different in Tukey *post hoc* analysis ($p < 0.05$). ns: no significance, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

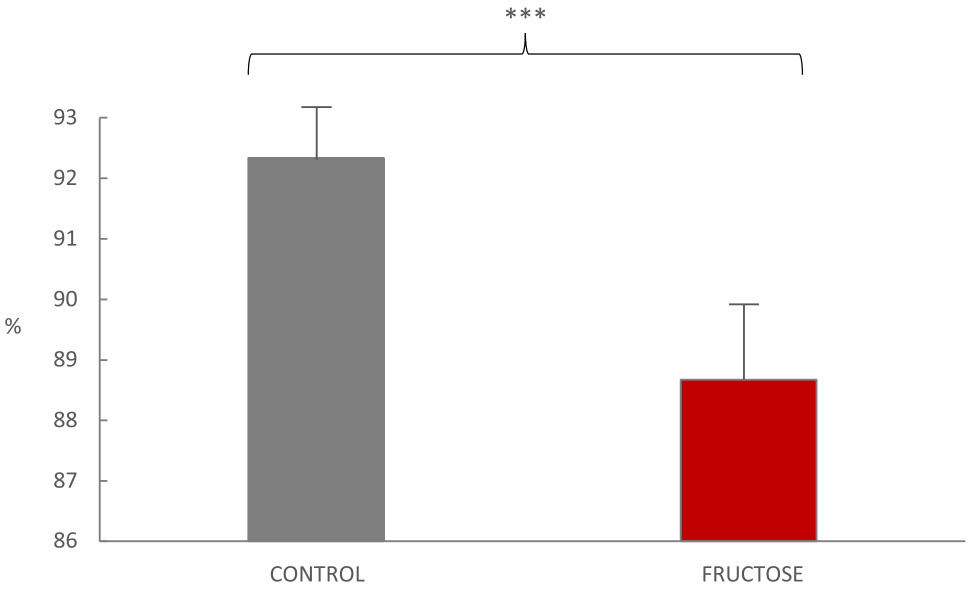

Figure 1. True protein digestibility[a] of a basal feed (~15% crude protein dry matter) in *Wistar rats* as affected by either drinking water (control) or a 30% fructose water solution for 10 weeks. [a]True protein digestibility (%) = {[TNf − (FN − TEp)]/TNf} × 100, where TNf is total nitrogen from feeds (dietary nitrogen), FN is fecal nitrogen, and TEp is the total metabolic/endogenous nitrogen found in feces from a rat fed a protein-free diet.[25] The pair of means with asterisks is significantly different in Student-t tests: ***$p < 0.001$.

($p < 0.01$). Then, the presence of these compounds reached the highest values in the intraluminal contents at the colon stage, while it diminished in the feces. Fructose treatment significantly enhanced the formation of APOPs in the luminal contents from the GIT ($p < 0.01$), except in feces. The colonic contents from F rats showed 1.5-fold higher values of fluorescent units due to the presence of APOPs than their C counterparts. Fructose enhanced the formation of APOPs at all digestion stages and samples except in the feces.

3.2.3. Lipid Oxidation in Digests at Different Locations of GIT. Table 1 also shows the extent of lipid oxidation expressed as amounts of TBARS (mg of MDA/100 g sample). Lipid oxidation significantly increased up to 4.5-fold in the jejunal contents from the rats after basal diet ingestion (i.e., mean values of 0.32 mg MDA/100 g sample) ($p < 0.05$). These highest mean values significantly decreased at the next stages of digestion until mean values of 0.12 mg MDA/100 g sample in the feces of the animals. Fructose treatment did not have any significant effects on the extent of lipid oxidation.

3.3. Effect of Dietary Fructose on Glycoxidative Stress in Tissues from GIT. Table 2 shows the concentration of individual carbonyls and total PPC in the tissues from each compartment of the GIT from *Wistar* rats. The levels of total PPC in the tissues significantly increased through the different GIT stages regardless of the treatment with fructose, reaching more than 2-fold higher PPC at the jejunum stage from the experimental animals than that found in the stomach tissue ($p < 0.01$). The fructose treatment significantly increased the amounts of semialdehydes in both, the stomach and jejunum tissues ($p < 0.01$). At the colonic stage, the concentration of the glycoxidative markers in the tissue increased significantly in animals subjected to fructose supplementation. Fructose intake significantly enhanced the formation of APOPs in all tissues of the GIT ($p < 0.01$). Moreover, the values of APOPs in the

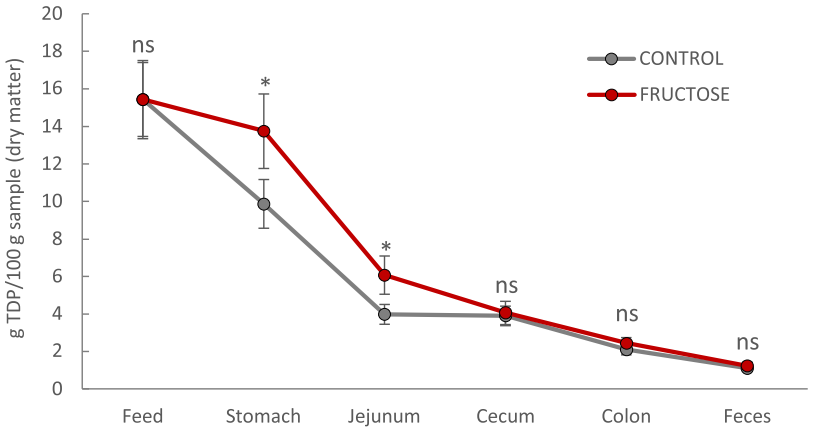

Figure 2. Evolution of the concentration of total dietary protein (TDP)[a] at the different stages of the *in vivo* digestion of basal feed (~15% crude protein dry matter) as affected by either drinking water (control) or a 30% fructose water solution for 10 weeks. [a]Total dietary protein (TDP, g/100 g digests) was calculated in feeds, luminal material of each compartment of the GIT, and feces as follows: TDP (g)= [(WSN − NPN) − Ep] × 6.25; WSN is water-soluble nitrogen, NPN is nonprotein nitrogen, and Ep is the metabolic/endogenous nitrogen.[24,25] Ep refers to nitrogen-containing biomolecules (i.e., proteins, peptides, etc.) secreted at each stage of the GIT of an animal receiving a protein-free diet. Results are presented in dry matter, and hence, the moisture of each sample was also taken into account.

colon from the F group significantly peaked at 1203 FU. Meanwhile, lipid oxidation showed some variations among GIT tissues, and fructose consumption did not have any significant effect on these values.

3.4. Effect of Dietary Fructose on Protein Digestion. *3.4.1. Protein Degradation during Digestion.* Figure 1 shows that the TPD of the basal diet provided to *Wistar* rats significantly decreased in rats exposed to fructose as compared to C rats (88.7% vs 92.3%, respectively; $p < 0.001$). To comprehend underlying mechanisms, an in-depth study of protein digestion was carried out. Figure 2 shows the evolution of TDP from the feed, during the different digestion stages at the GIT, and in the feces of experimental animals. TDP decreased as the digests advanced through the compartments of the GIT of the animals regardless of the fructose supplementation. However, the trend of dietary protein degradation was different when fructose was consumed by the rats. In fact, we analyzed the extent of protein degradation at each compartment as TDP degraded. Figure 3A shows the amount of degraded TDP at the different compartments of the GIT from C and F *Wistar* rats. Figure 3B shows the percentage of proteins that were degraded in the stomach and jejunum ("digested proteins"), and the percentage of proteins that were degraded at the cecum and colon stages ("fermented proteins"). Irrespective of the treatment, the highest rates of protein degradation were found at the initial stages of digestion. Yet, when fructose was supplied to animals, the digestion of TDP in the stomach was significantly reduced to less than half of the TDP digested in the stomach of C rats. Overall, 80% of dietary proteins were digested (stomach and jejunum) in GIT of C rats, while only 68% of dietary proteins was digested in rats drinking fructose ($p < 0.01$). Conversely, around 32% of TDP was fermented (cecum and colon) in rats drinking fructose, while a significantly lower protein percentage (20%) was fermented at the same stages in C animals ($p < 0.001$).

3.5. Effect of Dietary Fructose on Microbiota. To elucidate possible changes in the gut microbiome of *Wistar* rats after the high intake of fructose for 10 weeks, we analyzed the different α-diversity indices at the phylum and genus levels from the different OTU counts obtained. There were no significant differences between C and F rats in either the values of the diversity indices or the relative abundance of taxa at the phylum level (data are not shown). However, at the genus level, the microbiota of F rats contained significantly higher amounts of individuals than the microbiota of C rats ($p < 0.05$) (Table 3). In fact, specific genera were found only in the fecal microbiota from F rats.

Nevertheless, significant changes in the relative abundance of some microorganisms at the genus level were observed (Supporting Information). Although its different occurrence did not alter the α-diversity index, these changes may be remarkable and deserve attention. Thus, the microbiome of F rats was characterized by significantly higher amounts of *Christensenellaceae R-7 group* species, uncultured *Lachnospiraceae* spp., *Clostridia vadin* BB60 group spp. and uncultured *Ruminococcaceae* spp. Meanwhile, *Lactobacillus* spp., *Egerthellaceae DNF00809* spp., and *Bifidobacterium* spp. were significantly lower expressed in the F group than in their control counterparts. Species of the *Eubacterium nodatum* group from the *Anaerovoraceae* family and *Adlercreutzia* spp. were found only in the fructose group. *Desulfovibrio* spp. and genera of the family *Oscillospirales UCG-10* showed an increased trend in the F group, while *Streptococcus* spp. diminished ($0.05 < p < 0.1$).

Moreover, a range of species from selected genera proposed as fructose-sensitive and/or proteolytic was analyzed (Table S3). Long-term fructose intake significantly decreased the relative abundance of *Bifidobacterium animalis* ($p < 0.05$). In addition, *Alistipes shashii* ($p < 0.05$) occurred only in the microbiome of F rats. Moreover, some trends were remarkable in relation to the impact of fructose on the microbiota of F rats (p-values = 0.05), such as a lower relative abundance of *Lactobacillus grasseri* and an unclassified bacterium from genera *Streptococcus*, as well as the higher expression of an uncultured bacterium from genera *Marvinbryantia*.

3.6. Effect of Dietary Fructose on Colonic Metabolome from Wistar Rats. The untargeted metabolomic analysis revealed 2317 metabolites in the intraluminal contents of the colon from C and F *Wistar* rats. Compound Discoverer software paired the compounds name and/or formula with the calculated weights of the detected molecules using different databases (i.e., AKos, BioCyc, Chemspace, FooDB, Human

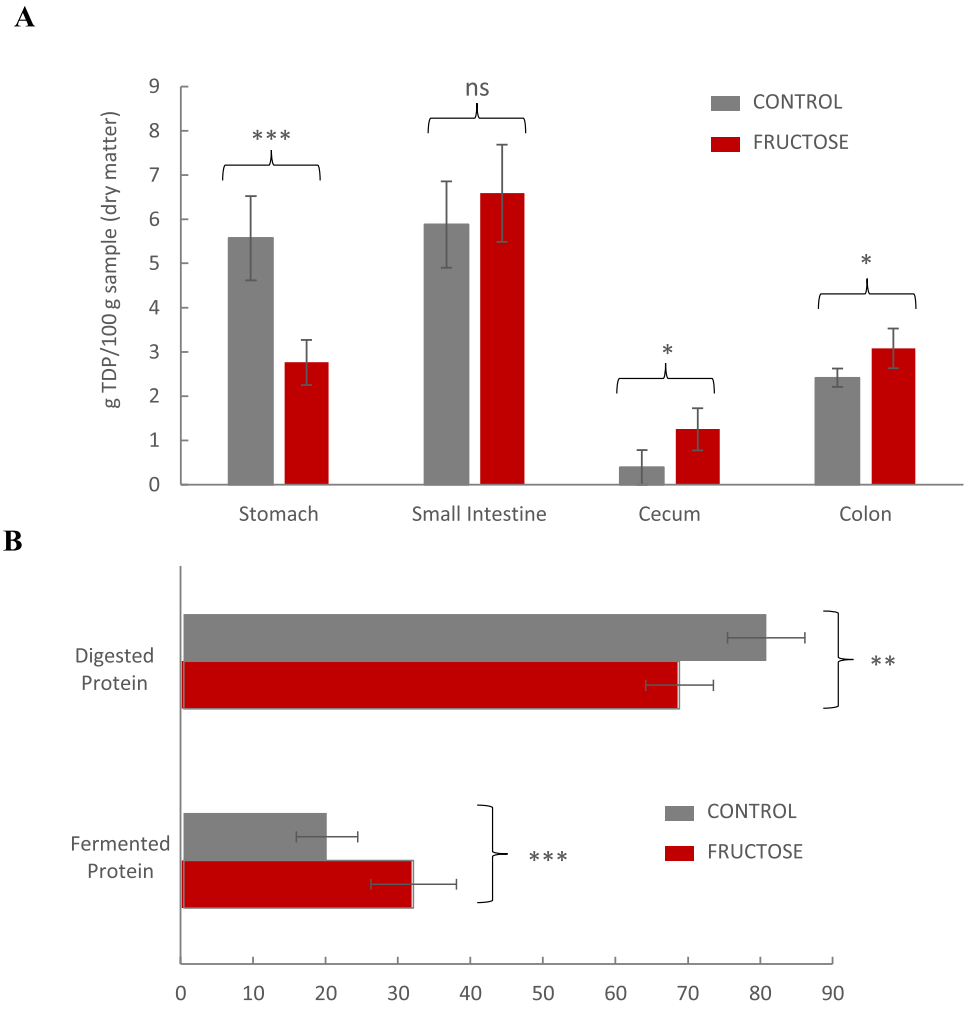

Figure 3. (A) Amount of total dietary protein (TDP) degraded[a] at the different compartments of the GIT of from *Wistar* rats ($n = 6$ per group) fed *ad libitum* for 10 weeks with a control base diet and either drinking water (control) or a 30% fructose water solution (fructose). (B) Percentage of TDP digested (degraded in stomach + small intestine) vs percentage of TDP fermented (degraded in cecum + colon) in control and fructose groups. [a]Degraded TDP at each specific compartment (g) was calculated as ($TDP_1 - TDP_2$); where TDP_1 is the total concentration of TDP in the immediately previous compartment and TDP_2 is the concentration of TDP in the compartment under study in which digestion was assumed finished (samples taken at the end of such stage). For further accuracy, the concentration of protein in each stage was calculated considering the moisture content of feeds and luminal contents at each stage (all protein data are shown as dry matter). For the calculation of protein degradation in the stomach, TDP_1 was considered TDP in the feeds, which corresponds to TP in the feed (TN × 6.25), as Ep does not apply in this case for obvious reasons. The pair of means with asterisks is significantly different in Student-*t* tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns: no significant differences.

Table 3. α-Diversity Index Values Expressed as Log_2-Means ± Standard Error of the Mean at the Genus Level from the Fecal Microbiome of *Wistar* Rats ($n = 6$ Per Group) Fed *Ad Libitum* for 10 Weeks with a Control Base Diet and Either Drinking Water (control) or a 30% Fructose Water Solution (Fructose)

	taxa richness	individuals	dominance	Simpson index	Shannon index	evenness
fructose	62.83 ± 1.80	27 917.00 ± 14 179.10	0.19 ± 0.01	0.81 ± 0.01	3.31 ± 0.09	0.16 ± 0.01
control	58.83 ± 2.32	22 663.83 ± 1680.78	0.21 ± 0.02	0.79 ± 0.02	3.18 ± 0.16	0.16 ± 0.01
p-value[a]	ns	*	ns	ns	ns	ns

[a]Significance level in the Student-*t* test with the effects of the diet (fructose and control). *$p < 0.05$ and ns: not significant.

Metabolome Database, KEGG, LipidMAPS, Mcule, Nature Chemical Biology, Nature Chemistry, NPAtlas, Toxin, Toxin-Target Database and Urine Metabolome Database). According to the routine calibration and optimization of the equipment, as well as our metabolite extraction method, the identification and characterization of the metabolites (Table S4) belong to level 2 of the identification levels proposed by the published metabolomics literature.[26]

Overall, 385 metabolites were only detected in the colonic contents of the C rats, while 520 were only found in the colonic digests of F rats. In order to analyze the results, the peak intensities of the metabolites were compared using Metaboanalyst software (https://www.metaboanalyst.ca/). According to the PLSD-DA plot, a different clustering of colonic contents was observed due to the fructose treatment (Figure 4). The VIP score is an important measure that

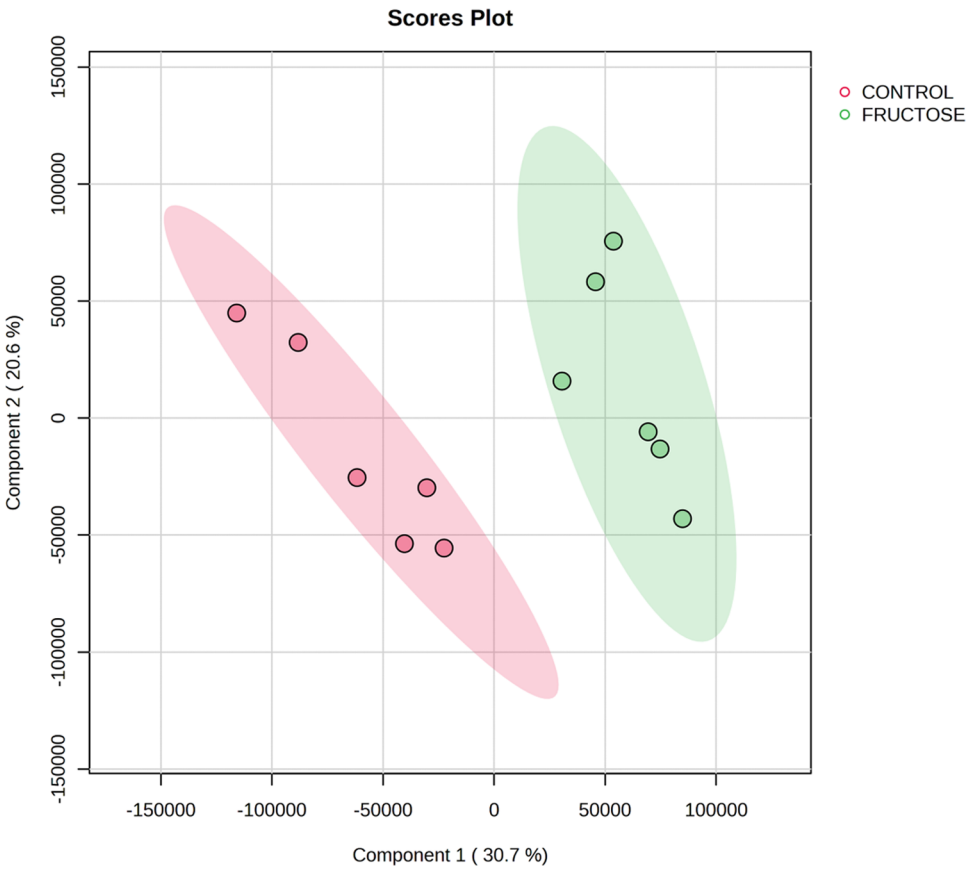

Figure 4. Score plots from the partial least-squares discriminant analysis multivariant analysis of the colonic contents from *Wistar* rats (n = 6 per group) fed *ad libitum* for 10 weeks with a control base diet and either drinking water (control) or a 30% fructose water solution (fructose).

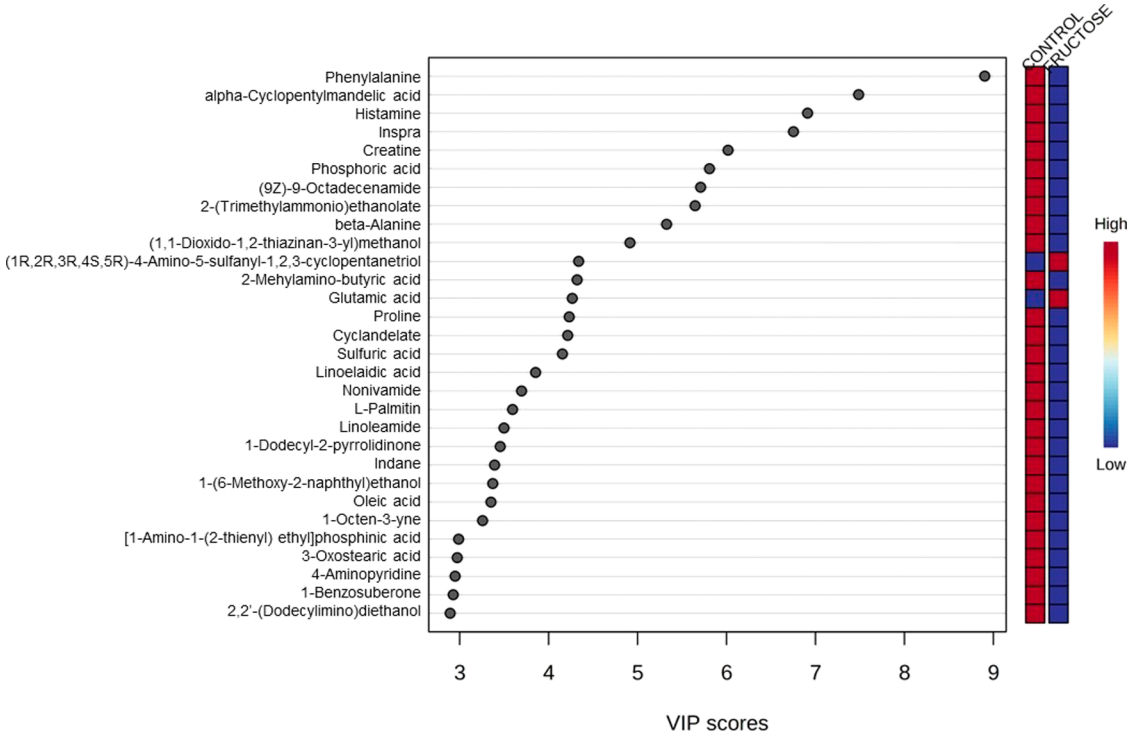

Figure 5. Variable importance in projection (VIP) score plot multivariant analysis outcomes from metabolomic results of the colonic contents from *Wistar* rats (n = 6 per group) fed *ad libitum* for 10 weeks with a control base diet and either drinking water (control) or a 30% fructose water solution (fructose).

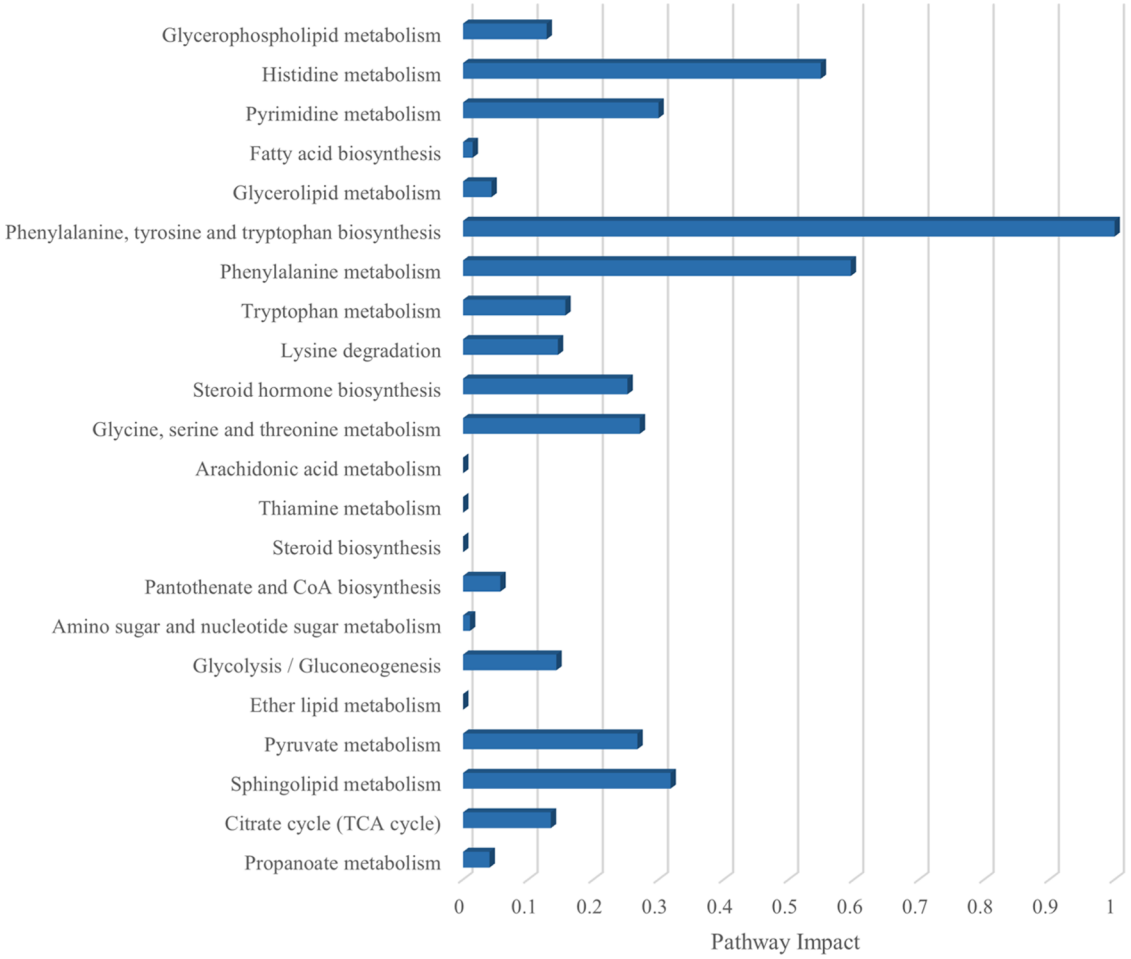

Figure 6. Categorization of the main pathways resulting from the pathway enrichment analysis ($p < 0.005$) and the pathway impact values according to the pathway topology analysis, highlighted from the metabolomics analysis of the data from the colonic contents from treated *Wistar* rats ($n = 6$ per group), fed *ad libitum* for 10 weeks with a control base diet and a 30% fructose water solution (fructose) regarding control water-consumer counterparts.

estimates the importance of each variable in the projection used in a PLS-DA model. Figure 5 shows the main loadings inferred by the analysis, with the relative concentrations of the corresponding metabolite in each group under study in the colored boxes on the right.

Fructose intake increased the concentration of 196 metabolites and decreased the concentration of 486 metabolites as compared to that of colonic digests from C rats. In particular, fructose promoted a higher abundance of some relevant metabolites such as β-D-glucose 6-phosphate (fold change: 7.56; *p*-value: 0.03), 2-aminobutanoic acid (fold change: 6.90; *p*-value: 0.001), cadaverine (fold change: 6.27; *p*-value: 0.006), prolylleucylglycine (fold change: 4.26; *p*-value: 0.002), serylglycine (fold change: 3.32; *p*-value <0.001), pyruvic acid (fold change: 2.67; *p*-value <0.001), lactic acid (fold change: 2.24; *p*-value <0.001), tryptophan (fold change: 1.10; *p*-value: 0.02), and 2-oxobutyric acid (fold change: 1.09; *p*-value <0.001), among several others (volcano, Supporting Information). On the other hand, fructose intake reduced the quantity of several metabolites, such as β-alanine (fold change: −5.57; *p*-value <0.001), spermidine (fold change: −3.70; *p*-value <0.001), hypotaurine (fold change: −3.24; *p*-value <0.001), acetic acid (fold change: −2.76; *p*-value <0.001), 2,6-diaminopimelic acid (fold change: −2.64; *p*-value <0.001), maleic acid (fold change: −2.61; *p*-value <0.001), glyceraldehyde (fold change: −2.54; *p*-value <0.001), γ-aminobutyric acid (GABA) (fold change: −2.21; *p*-value: 0.003), glycerol 3-phosphate (fold change: −1.87; *p*-value:0.02), dihydroxyphenylalanine (L-dopa) (fold change: −1.83; *p*-value: 0.02), and histamine (fold change: −1.63; *p*-value: 0.004), among others (volcano, Supporting Information).

Based on the categorical differential metabolites, a pathway enrichment analysis and KEGG topology analyses were performed in Metaboanalyst software (https://www.metaboanalyst.ca/) to evaluate metabolic changes induced by the long-term intake of fructose. The categorization of the results was carried out according to the *p*-values from the pathway enrichment analysis and the pathway impact values from the topology analysis. Thus, 22 metabolic pathways were significantly affected by the fructose treatment in the intraluminal colonic content of the rats. Figure 6 shows the significant pathways affected by the treatment after the enrichment analysis, including pathways involved in energy metabolism as glycolysis, citrate cycle, or pyruvate metabolism ($p < 0.001$, respectively) and pathways related to the metabolism of certain amino acids as histidine, phenylalanine, tryptophan, and lysine ($p < 0.05$).

4. DISCUSSION

To the best of our knowledge, this study provides the first assessment of the impact of sustained consumption (10 weeks) of fructose on the intraluminal (digests) and tissue oxidative stress from different compartments of the GIT from *Wistar* rats. Our results are novel in highlighting the molecular mechanisms behind the potential reactivity of fructose with dietary proteins and other components from GIT that could be the basis of the undesirable effects of the consumption of this reducing sugar on tissues and peripheral organs.

4.1. Glycoxidative Stress in the Lumen of GIT and Impaired Digestibility. Dietary protein digestion begins once it reaches the stomach. The gastric juices promote the unfolding of proteins, ensuring the recognition and action of gastric enzymes.[27] However, protein denaturation could also enhance the exposure of hydrophobic groups in proteins, which, along with protein oxidation, facilitates protein cross-linking and aggregation.[28] In fact, it has been documented that the pro-oxidative environment of the stomach promotes the oxidation of proteins in several *in vitro*[15,29,30] and *in vivo* studies,[31] which is in agreement with our results. The ability of reducing sugars to induce the onset of glycoxidative reactions in dietary proteins has been documented by a few *in vitro* studies in which the physiological conditions of the stomach were simulated.[15,32,33] The present *in vivo* study confirms that dietary fructose promotes the creation of a severe pro-oxidative environment in the stomach of *Wistar* rats, stimulating the oxidative damage of dietary proteins. Fructose has been profusely studied in relation to the mechanisms implicated in such glycoxidative stress. One of these mechanisms is the ability of fructose to generate RCS (i.e., glyoxal and methylglyoxal), either by products of its autoxidation ("Wolf pathway") or by its role in Maillard reactions ("fructosylation").[34] Moreover, fructose has long been described as much more reactive than glucose in Maillard reactions due to the stability of its open-chain form and its keto group.[34,35] The glycoxidation of proteins involves the reaction of susceptible protein residues with RCS.[6,8] RCS triggers the deamination of protein-bound alkaline amino acids, which leads to the formation of primary protein carbonyls, such as α-AS, derived from lysine, and γ-GS, derived from arginine and proline.[36] These semialdehydes represent the most abundant carbonyls formed during protein glycoxidation,[37] so both individual detection and quantification are relevant as expressions of the levels of glycoxidative stress. Accordingly, our results indicate that the intake of a high-fructose (30%) solution for 10 weeks significantly promotes the *in vivo* formation of PPC in the stomach contents of Wistar rats ($p < 0.001$). On the other hand, at the first steps of fructosylation, a covalent interaction between the free carbonyl group of open-chain fructose and the amino group of proteins could occur and generate Schiff bases, which would lead to the formation of Heyns products by several chains of reactions. It is believed that the Heyns products, RCS, and reactive oxygen species (ROS) formed during fructosylation are important precursors of non-enzymatic adducts of the proteins as APOPs (i.e., AGES). Each protein fructosylation reaction releases a superoxide radical, so fructose generates 100 times more ROS than glucose and promotes cell apoptosis and inflammation.[38] Since α-AS and γ-GS are also formed in proteins as the direct electrophilic attack of ROS,[39] it is impossible to state the extent to which RCS and/or ROS contributed to the carbonylation of dietary proteins. It is, yet, indisputable that fructose effectively contributes to creating a pro-oxidative environment in the stomach, as previously stated for glucose in an *in vitro* study.[15] Up to now, there was *in vitro* evidence of fructose inducing formation of APOPs at the first stages of the GIT.[32,33] This study confirms for the first time that such reactions also occur in an *in vivo* gastrointestinal system. Our results revealed the harmful reactivity of fructose with dietary proteins during *in vivo* gastric digestion and the lack of effect of glycoxidative reactions on dietary lipids. These results are in agreement with previous reports in which proteins seemed to be the most relevant target of oxidative reactions during both *in vitro* and *in vivo* digestion of various muscle foods.[31,40]

The increased protein glycoxidation caused by the intake of 30% of fructose in the stomach seemed to affect the digestion pattern of proteins, which would remain undigested in the lumen of the next stages of the GIT. Thus, this is reflected in the higher values of the glycoxidative stress markers analyzed in the digests at the jejunum stage of F-treated rats as compared to C ones. The small intestine has many more specific proteolytic enzymes than the stomach.[41] The resulting di- and tripeptides and single amino acids from enzymatic digestion can be absorbed into the bloodstream, as well as the carbonylated residues, and this could be the reason for the significant decrease in PPC in the jejunum digests from the F group. Likewise, the amount of TDP decreased in the jejunal contents, and the protein degradation reached the highest values at this intestinal compartment, as expected. Unlike what was found in the stomach, fructose administration had no effect on the degradation of dietary proteins in the small intestine ($p > 0.05$). It is worth highlighting that the degree of protein digestion in C rats was similar in the stomach and small intestine (5−6 g of TDP digested in each stage). The amount of TDP digested in the small intestine of rats treated with fructose was remarkably more abundant than that digested in the stomach (6.7 g vs 2.6 g). It is hence reasonable to hypothesize that the impaired digestion caused by fructose in the stomach was partially counteracted by more intense protein digestion in the small intestine. Yet, the total digested protein (stomach + small intestine) was significantly lower in animals exposed to dietary fructose. Severe protein glycoxidation impairs protein digestibility by modifying the amino acid composition (carbonylation) and reasonably altering the accessibility and recognition of proteolytic enzymes to the cleavage site.[42−44] These results confirm previous findings in which glucose-mediated protein carbonylation during simulated digestion of meat and dairy proteins led to an impaired digestibility of such proteins.[15,36] Therefore, the amount of undigested and presumably glycoxylated proteins reaching distant locations of the GIT was significantly higher in rats exposed to fructose.

The lack of degradation of glycoxylated proteins in the first compartments of the GIT could have facilitated their arrival to the cecum and colon, where they were eventually fermented by gut microbiota.[45] In fact, the depletion of TDP in the cecum/colon, attributed to the degradation of proteins by microbiota, was significantly higher in rats fed with fructose than in the C counterparts. The occurrence of oxidative and glycoxidative reactions at this stage is of particular clinical interest, given that most functional and organic disorders diagnosed in human GIT are located in the colon.[46] It is, therefore, highly meaningful that the concentration of all protein glycoxidation markers (PPC and APOPs) peaked in the colonic lumen and

tissue of rats provided with dietary fructose. In addition to the arrival to this stage of glycoxylated proteins from previous stages, there was a net increase of all protein oxidation markers in the colon. The remarkable buildup of PPC, glycoxylated proteins, and AGES in the intraluminal contents at the colon stage shows the relevance of this GIT compartment as a truly redox-active environment where both the oxidation of dietary components and microbiota interact.[47] The fact that fructose-exposed rats suffered more intense glycoxidative reactions at this stage may imply that fructose and/or their reactive degradation products reached this distant location of the GIT as well as nondigested glycoxylated proteins, which would promote the onset of further oxidative reactions in the colon. In this regard, a timely connection at this stage of redox reactions and inflammatory processes has been described since chronic oxidation would lead to proinflammatory pathways, and inflammation, itself, contributes to the onset of a pro-oxidative environment.[47] The role of dietary AGES in gut inflammation and gut microbial composition was deciphered.[48] While the occurrence of dietary fructose/RCS at this stage cannot be ruled out, the products of its protein glycosylation reactions may be implicated more likely in the promotion of luminal and tissue oxidative stress in the colon. It is common knowledge that the transformation of undigested compounds either by the host or by the microbiota increases the rate of oxidative stress and the formation of several metabolites in the luminal content of the GIT.[47] An increased pro-oxidative environment and a greater amount of undigested protein owing to a previously impaired digestibility would facilitate the microbiome degradation of this luminal material to the production of potentially toxic metabolites.[49] The identification of some of these microbial metabolites is of enormous scientific interest since it is reported that certain protein fermentation products in the colon can be proinflammatory and carcinogenic.[49,50] The highest uptake of nitrogen at these stages in rats exposed to fructose may have relevant pathophysiological consequences, given that most of that nitrogen compounds would have resulted from microbiota fermentation of at least partially oxidized proteins.

4.2. Glycoxidative Stress in Tissues of GIT. It is well documented that increased glycoxidative stress in the lumen of the gastrointestinal tract contributes to the damage of neighboring tissues.[51,52] It is therefore reasonable that the stomach tissue from rats provided with fructose had higher rates of protein glycoxidation markers (PPC and APOPs) than their C counterparts. Therefore, the onset of intraluminal glycoxidative stress in the stomach could have promoted *in situ* protein glycoxidation of the tissue. In addition to the potential uptake of oxidized species at this stage, the absorption of reactive fructose and RCS derived from its degradation could have promoted oxidative damage in proteins from the stomach tissue. Numerous gastroduodenal diseases are related to increased inflammatory processes derived from ROS attacks, such as peptic ulcer, gastritis, or gastric cancer.[53] More specifically, protein oxidation was emphasized as the most salient biochemical process in patients suffering from *Helicobacter pylori* chronic infection and gastric cancer.[54] Moreover, these authors displayed that the extent of lipid oxidation was not a reliable marker of the disease, even though it decreased in cancer patients as compared to healthy individuals. This is in line with the current results, in which lipid oxidation was negligible as compared to the oxidative damage to proteins. Carbonylation levels in mucosa from healthy individuals are around 1−2 nmol protein hydrazones/mg protein,[55] while above 2 nmol protein hydrazones/mg protein was reported in plasma from gastric cancer patients.[54] It is crucial to highlight that the aforementioned authors quantified total protein carbonyls using the routine spectrophotometric dinitrophenylhydrazine method, which is well known for overestimating the concentration of primary protein carbonyls in biological samples.[8] Taking into account that the sum of α-AS and γ-GS account for between 50 and 70% of protein hydrazones,[8,37] the concentration of PPC found in the stomach tissue of rats subjected to sustained consumption of fructose may be within the pathological range. The lack of information on specific protein carbonyls in pathological conditions affects the comprehension of the role of protein carbonylation in human diseases.[56]

The extent of protein glycoxidation in the jejunal tissue from F rats was higher than that in the previous compartment (stomach). The accretion of oxidation products, such as protein carbonyls in the epithelium of the intestinal mucosa, as a first stage of their intestinal uptake and bloodstream distribution to internal organs was hypothesized.[52] This, in fact, could explain the depletion of carbonylated proteins in the luminal content at the intestinal stage under study and, consequently, the increased carbonylation in the jejunal tissue. Additionally, fructose and related RCS may have been uptake and induce, *in situ*, carbonylation of tissue proteins at this location as well. Some authors have carried out *in vivo* experiments aiming to evaluate the levels of oxidative stress in the tissue of the small intestine by different markers when high amounts of fructose are ingested.[57] In line with the present results, the authors found increased concentrations of various markers of oxidative and nitroxidative stress in proteins from the small intestine of rodents that were exposed to a 30% fructose drinking water solution for 8 weeks.[57] Fructose-exposed mice suffered intestinal barrier dysfunction and endotoxemia along with liver fibrosis.[11] How fructose contributes to the disintegration of intestinal tight junction proteins, which may facilitate the subsequent uptake of intestinal toxins, was comprehensively illustrated in a previous study.[58] Further to the role of PPC in intestinal function and health, it is also involved in the formation of advanced glycation and oxidation products such as AGES/APOPs.[59] The involvement of PPC in such reactions could explain its depletion in the jejunal lumen and the increased amounts of APOPs at the same location, particularly in fructose-exposed rats ($p < 0.001$). Some authors reported that the formation of intestinal AGES from the reaction of dietary fructose with peptides and amino acids might be the triggering point of the inflammatory bowel response associated with high fructose intake.[32] Consistently, in our study, the jejunal tissue from rats supplemented with fructose showed higher amounts of APOPs than C rats ($p < 0.05$), which could be secondary to the uptake of luminal glycoxidation products or formed *in situ*, subsequent to the uptake of reactive carbonyls.

Diet-derived AGES has been demonstrated to interfere with many cell functions such as lipid synthesis, inflammation, antioxidant defenses, and mitochondrial metabolism due to its accretion in target tissues,[10] but this is the first study that analyzed the endogenous formation of AGES and its plausible accretion in the tissues from GIT stages in an *in vivo* experiment. Oral administrated fructose is mainly cleared by the small intestine, where it is converted into glucose and organic acid.[60] Hence, the small intestine exerts a great

influence on the consequent metabolic disorders associated with excessive fructose intake.[58] Intestinal metabolism of fructose is ATP-dependent, which could increase the protein carbonylation in the tissue at the stage by the increased secondary-ROS production.[3,60,61] When high amounts of fructose are ingested, changes in the energy homeostasis are manifested and oxidative stress and intestinal inflammatory response are induced, disturbing functions of both local tissues and the liver.[3] Fructose intestinal metabolism implies rapid generation and accumulation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, which are effective proglycation agents and precursors of RCS such as glyoxal and methylglyoxal, which, in turn, are precursors of more stable AGES.[10]

In the colonic tissue, the protein glycoxidation markers (PPC and APOPs) also reached the highest values, suggesting intense damage to the intestinal barrier due to the increased luminal glycoxidative stress plus the likely accretion of undesirable metabolites. Oxidative stress plays a key role in the development of IBD and cancer by the continuous exposure of the colonic cells to the intraluminal metabolic-derived free radicals.[17,62] A comparing study about the levels of protein hydrazones in human colonic tissues with different degrees of primary colorectal tumors (colorectal adenopolyps) with their normal/surrounding tissues was carried out and highlighted that damaged tissues contained around 70 nmol hydrazones/mg protein, while healthy neighboring tissues had between 10 and 15 nmol hydrazones/mg protein.[62] Assuming the previously mentioned equivalence factor between the sum of α-AS and γ-GS and protein hydrazones, the PPC levels of the intraluminal colonic contents from F-treated rats are close to the dangerous threshold values described by the authors in the precancerous states of CRC. The glycoxidative state in the colonic tissue was promoted by the harmful intraluminal environment. This is an important approach as it could directly link fructose consumption with colonic tissue damage.

4.3. Colon Microbiota, Metabolomics, and Potential Health Implications. The imbalance in gut microbiota may result in disruption of several metabolic mechanisms and immune functions, which might lead to several diseases, such as IBD, metabolic syndrome, diabetes, insulin resistance, obesity, cardiovascular diseases, and even cancer.[63] In order to further investigate the underlying chemistry of the processes occurring in the colon of the experimental animals, comprehensive analyses of the microbiota and metabolomics of colonic digests were performed. In our study, fructose promoted alterations in the gut microbiota profile of the *Wistar* rats. Several authors have previously related the fecal microbiota shift as a consequence of an increased fructose intake.[16,64] Nevertheless, the influence of a high-fructose diet on gut microbiota is still largely unknown. Most of the dietary fructose was metabolized in the small intestine.[60] Moreover, liquid formulations of fructose were more rapidly absorbed and gave greater induction of hepatic lipid accumulation compared to solid counterparts.[65] It is reasonable to hypothesize that indefinite (not analyzed in the present study) amounts of nonmetabolized fructose reached the colon of our treated animals under the experimental conditions (9 g of fructose/kg of live weight/day), and such fructose could have promoted shifts on the microbiota. Other studies in which fructose was found to reach the colon of mice registered an increase in amino acid metabolism genes in the microbiota of treated animals.[65] In addition, it is remarkable that our results reflected that the intake of high amounts of fructose for 10 weeks increased the metabolism and/or absorption rates of protein-related glycoxylated compounds at the colonic stage, as can be inferred by the different amounts of the markers between the colonic contents and the feces. Thus, the concentration of carbonyls in the feces from the fructose group (vs control) suggested that more than 70% of the carbonylated proteins were assimilated in the colonic stage (vs 50%). Moreover, greater metabolism and/or absorption of APOPs were observed when fructose was consumed, which might support the change in the colonic protein metabolism already suggested. The higher abundance of several metabolites involved in energy metabolism, such as β-D-glucose-6-phosphate, lactic acid, or pyruvic acid in the colonic contents of the fructose rats, might support the suggested higher metabolism in the colon of F animals. In fact, pathway enrichment analysis significantly enhanced changes in glycolysis, pyruvate, and citrate cycle pathways. Some authors previously described changes in the oxidative phosphorylation pathway in plasma from fructose-consumer human volunteers,[64] which might well be related to the intestinal events described above. However, comparisons between studies should be made with caution as the results from the aforementioned works were obtained with different experimental conditions, species, and diet formulations (solid vs liquid fructose).

The lower abundance of probiotic genera *Lactobacillus* and *Bifidobacterium* due to an enduring high-fructose intake has already been highlighted by other authors when evaluating the impact of fructose consumption on microbiota.[11,66] Increased intestinal permeability, liver inflammation, and/or fibrosis were attributed to fructose consumption when different rats and mouse strains were exposed to tap water vs 30% fructose in drinking water for 8 weeks *ad libitum*.[57] Other authors who considered the effect of oxidized protein intake on microbiota also reported a diminished abundance of *Lactobacillus* spp.[31,67] Furthermore, a decrease in *Bifidobacterium animalis* due to the presence of AGES in the colon was described in a review.[45] Overall, the fructose-related decrease of probiotic bacteria could be plausibly attributed to the buildup of *in vivo* oxidized proteins in the colon as a result of the consumption of the reducing sugar. Even though other genera described as beneficial gut bacteria, such as *Adlercrautzia*[63] or *A. shashii*,[68] were slightly expressed only in the group of fructose rats, the identification of *L. grasseri* and *Bifidobacterium animalis* as species affected by fructose-liquid diet is highly relevant from the perspective of probiotic supplementation research.

The shift in microbiota observed in F rats could explain the decreased amounts of several colonic metabolites in these rats, such as acetic acid. Acetic acid production was related to the occurrence of *Lactobacillus* spp. and *Bifidobacterium* spp. in the colon by some authors.[69] Other authors reported that some of the species from the genera *Lactobacillus* and *Streptococcus* are able to produce biogenic amines such as spermidine, described as an important compound for normal mucosa development.[69,70] Such a metabolite was found to be significantly decreased in the colonic metabolome of *Wistar* rats exposed to dietary fructose in our study. Moreover, the capacity of some gut microorganisms to synthesize neuroactive compounds such as neurotransmitters through the catabolism of several amino acids has been described.[71] Particularly, the authors related the gut synthesis of GABA, histamine, and serotonin with the microbial fermentation of glutamic acid, histidine, and

tryptophan by genera *Lactobacillus, Bifidobacterium*, and *Streptococcus*, among others.[71] Interestingly, in our study, the multivariant metabolomic analysis revealed that the increased amounts of tryptophan and glutamic acid in the intraluminal colonic contents of F rats were the main metabolites that explained the clustering of the samples (PLS-DA loadings, Supporting Information). Although the statistical analysis detected no changes in the abundance of histidine and serotonin between groups, our results suggest that the lower abundance of probiotic bacteria may be involved in diminishing the presence of some active compounds resulting from the degradation of amino acids, such as tryptophan, which were, in fact, increased in the colonic content of F rats. The impact of fructose on microbiota in control vs colitis-induced rats displayed that in both groups of animals, arginine and proline metabolism pathways were altered, with the expression of GABA diminished,[66] which is in agreement with our results. However, these authors used 12.5% g of fructose in a solid-diet formula, which makes it difficult to compare the results. It is worth noting that the abundance of histamine was related with energy homeostasis and neurological disorders.[71] Other authors described that histamine reduced the production of proinflammatory cytokines.[69] Plausible inflammation of the intestinal mucosa could explain the significantly increased amounts of lactic acid detected in the F group (fold change: 2.24), in agreement with other findings after the measurement of the levels of lactate in feces from patients with active ulcerative colitis.[72] Another relevant finding was the increased amount of cadaverine in the metabolomic profile of F rats (fold change: 6.27). Higher colonic levels of this polyamine, synthesized from lysine, have been linked by some authors to ulcerative colitis,[72] but the effect of cadaverine on the colonic cells remains unknown yet.

The potential implications of protein fermentation in the gut of humans, pigs, and poultry were reviewed and some of the outcomes derived from a defective metabolism of amino acids in both the gut and the microbiota were addressed.[70] These authors linked high expressions of sulfide-producing bacteria (i.e., *Desulfovibrio* spp., which showed an increase trend in our results) with IBD since this type of bacteria can reduce dietary sulfide and sulfate and sulfated polysaccharides from mucins, decreasing mucus barrier integrity in IBD.[70] The decreased amounts of cysteine (fold change: −3.26; p-value <0.001) observed in the intracolonic metabolome of treated rats might be related to the growth of the sulfate-reducing bacteria. Fructose has been proven to be associated with impaired mucus production by enterocytes.[66] The mechanisms remain unclear, but the decreased protein digestibility promoted by fructose intake could be responsible for the increased expression of genera *Desulfovibrio* at the colon stage, which, in turn, might be involved in the impairment of the mucosa along with the other changes described. Another study about the impact of protein oxidation on microbiota revealed an increased presence of *Desulfovibrio* spp. after the intake of oxidized meat proteins.[67]

Uncultured *Lachnospiraceae* spp. and unclassified *Marvynbryantia* bacteria were increased in the microbiota of F rats in our experiment. Accordingly, an increased abundance of genera of the *Lachnospiraceae* family in Sprague-Dawley (SD) rats exposed to different doses of fructose during 20 weeks was assessed.[73] The fructose dose that promoted the increase of *Lachnospira* spp. and *Marvynbryantia* spp. in that study is similar to that used in the present assay (10.5 g/kg/day). The intake of fructose also increased unclassified genera of the *Lachnospiraceae* family in a comparative study,[66] where the authors attributed the changes in microbiota to fructose intake rather than induced colitis, which is in agreement with our results. Interestingly, other authors that evaluated the effect of the intake of high amount of cured meat-derived proteins on the microbiota described an increased *Lachnospiraceae* spp.[31] The *Lachnospiraceae* family has been reported to be butyrate-producing bacteria that may protect the intestinal epithelium from inflammation.[70,74] Moreover, the *Marvynbryantia* and *Christensenelleceae R-7 groups*, also increased in the microbiome of our F rats, were associated in humans with a lower insulin index and lower BMI in human research.[75] The *Christensenelleceae R-7 group* was decreased in populations that consumed a high-fructose corn syrup-based diet.[76]

The microbiome of the F rats showed an increase in uncultured *Ruminococcaceae* spp. Several studies that made associations between increased *Ruminococcacea* with fructose-rich diets and liver disease (i.e., NAFLD) were reviewed.[77] On the other side, other authors described increased colonic *Ruminoccocaceae* related to the intake of oxidized proteins from cured meat consumption.[31] Anyway, members of the *Ruminococcaceae* family can expand as a consequence of a high availability of proteins.[78]

Likewise, it would be the first full assessment of the *in vivo* glycoxidative stress promoted by fructose during gastrointestinal digestion and its relevant impact on the intraluminal protein and amino acid metabolism, which may be related to immunity and proinflammatory functions.[66] The intake of 9 g of fructose/kg of live weight/day for 10 weeks strongly affects the fate of dietary proteins during digestion in *Wistar* rats. The glycoxidative environment promoted by the reducing sugar at the first stages of the GIT condition the whole intraluminal protein digestion. Glycoxidative markers are increased along the digestion, and the surrounding tissues are affected. At the colon stage, fructose and its promoted protein-degradation products (i.e., carbonyls and AGES) increase the glycoxidative environment and have an impact on the microbiota and the metabolomic fingerprint, boosting an amino acidic dysbiosis that could be the basis of the microbiota shift and the related mucosal inflammation and metabolic disorders. Thus, fructose intake decreases the expression of probiotic bacteria as well as the abundance of biogenic amines with neurotransmitter properties while enhancing the expression of sulfate-reducing bacteria and harmful metabolites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c04515.

Energy (kJ/day) provided by the feed and the supplemented water to the experimental animals during the assay (Table S1), weight evolution of the animals during the experiment (Table S2), list of species of microorganisms selected to statistical analysis based on the literature (Table S3), and described metabolite characterization (Table S4) (PDF)

Relative abundance of selected species of microorganisms (L6), volcano results with the changes in the abundance of colonic metabolites and statistical significance (Volcano), and PLS-DA loadings list from

the colonic metabolome with the component coordinates (PLS-DA loadings) (XLSX)

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M.E. contributed to funding acquisition, project administration, conceptualization, investigation, methodology, supervision, data analysis, validation, and writing—review and editing. G.S.-T. contributed to investigation, methodology, data analysis, validation, and writing—original draft. R.M. contributed to investigation, methodology, supervision, data analysis, validation, and writing—review and editing. J.R. contributed to supervision, data analysis, validation, and writing—review and editing. C.L. contributed to investigation, data analysis, validation, and writing—review and editing. All authors made critical revisions to the manuscript for key intellectual content and read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GIT, gastrointestinal tract; WHO, World Health Organization; HFCS, high-fructose corn syrup; MetS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; T2D, type 2 diabetes mellitus; RCS, reactive carbonyls species; AGES, advanced glycation end products; IBD, inflammatory bowel disease; CRC, colorectal cancer; C, control group; F, fructose group; α-AS, α-aminoadipic semialdehyde; γ-GS, γ-glutamic semialdehyde; PPC, primary protein carbonyls; APOPs, advanced protein oxidation products; FU, fluorescent units; TBARS, thiobarbituric reactive substances; MDA, malondialdehyde; BHT, butylated hydroxytoluene; TBA, thiobarbituric acid; TEP, 1,1,3,3-tetraethoxypropane; AOAC, Association of Official Agricultural Chemists; TN, total nitrogen; WSN, water-soluble nitrogen; NPN, nonprotein nitrogen; TCA, trichloroacetic acid; TPN, total protein nitrogen; TDN, total dietary nitrogen; Ep, endogenous nitrogen; TDP, total dietary protein; DP, digested proteins; FP, fermented proteins; PLS-DA, partial least-squares discriminant analysis; VIP, variable importance in projection; FC, fold change; TPD, true protein digestibility; OTUs, operational taxonomic units; ROS, reactive oxygen species; S, stage; D, diet

REFERENCES

(1) *Guideline: Sugars Intake for Adults and Children* WHO: 2018.

(2) Sangüesa, G.; Cascales, M.; Griñán, C.; Sánchez, R. M.; Roglans, N.; Pallàs, M.; Laguna, J. C.; Alegret, M. Impairment of Novel Object Recognition Memory and Brain Insulin Signaling in Fructose- but Not Glucose-Drinking Female Rats. *Mol. Neurobiol.* **2018**, *55* (8), 6984−6999.

(3) Herman, M. A.; Birnbaum, M. J. Molecular Aspects of Fructose Metabolism and Metabolic Disease. *Cell Metab.* **2021**, *33* (12), 2329−2354.

(4) Baynes, J. W. Role of Oxidative Stress in Development of Complications in Diabetes. *Diabetes* **1991**, *40* (4), 405−412.

(5) Yaribeygi, H.; Sathyapalan, T.; Atkin, S. L.; Sahebkar, A. Molecular Mechanisms Linking Oxidative Stress and Diabetes Mellitus. *Oxid. Med. Cell. Longevity* **2020**, *2020*. 8609213.

(6) Hecker, M.; Wagner, A. H. Role of Protein Carbonylation in Diabetes. *J. Inherit. Metab. Dis.* **2018**, *41* (1), 29−38.

(7) Almogbel, E.; Rasheed, N. Protein Mediated Oxidative Stress in Patients with Diabetes and Its Associated Neuropathy: Correlation with Protein Carbonylation and Disease Activity Markers. *J. Clin. Diagn. Res.* **2017**, *11* (2), BC21−BC25.

(8) Estévez, M.; Díaz-Velasco, S.; Martínez, R. Protein Carbonylation in Food and Nutrition: A Concise Update. *Amino Acids* **2022**, *54*, 559−573, DOI: 10.1007/s00726-021-03085-6.

(9) Luna, C.; Arjona, A.; Dueñas, C.; Estevez, M. Allysine and α-Aminoadipic Acid as Markers of the Glyco-Oxidative Damage to Human Serum Albumin under Pathological Glucose Concentrations. *Antioxidants* **2021**, *10* (3), 474.

(10) Aragno, M.; Mastrocola, R. Dietary Sugars and Endogenous Formation of Advanced Glycation Endproducts: Emerging Mechanisms of Disease. *Nutrients* **2017**, *9* (4), 385.

(11) Cho, Y. E.; Kim, D. K.; Seo, W.; Gao, B.; Yoo, S. H.; Song, B. J. Fructose Promotes Leaky Gut, Endotoxemia, and Liver Fibrosis through Ethanol-Inducible Cytochrome P450−2E1−Mediated Oxidative and Nitrative Stress. *Hepatology* **2021**, *73* (6), 2180−2195.

(12) Muriel, P.; López-Sánchez, P.; Ramos-Tovar, E. Fructose and the Liver. *Int. J. Mol. Sci.* **2021**, *22* 13. 6969.

(13) Estaras, M.; Ameur, F. Z.; Estévez, M.; Díaz-Velasco, S.; Gonzalez, A. The Lysine Derivative Aminoadipic Acid, a Biomarker of Protein Oxidation and Diabetes-Risk, Induces Production of Reactive Oxygen Species and Impairs Trypsin Secretion in Mouse Pancreatic Acinar Cells. *Food Chem. Toxicol.* **2020**, *145*. 111594.

(14) Cigliano, L.; Spagnuolo, M. S.; Crescenzo, R.; Cancelliere, R.; Iannotta, L.; Mazzoli, A.; Liverini, G.; Iossa, S. Short-Term Fructose Feeding Induces Inflammation and Oxidative Stress in the Hippocampus of Young and Adult Rats. *Mol. Neurobiol.* **2018**, *55* (4), 2869−2883.

(15) Sánchez-Terrón, G.; Morcuende, D.; Martínez, R.; Ruiz-Carrascal, J.; Estévez, M. Glucose Boosts Protein Oxidation/Nitration during Simulated Gastric Digestion of Myofibrillar Proteins by Creating a Severe pro-Oxidative Environment. *Food Chem.* **2022**, *397*, No. 133805, DOI: 10.1016/j.foodchem.2022.133805.
(16) Kawano, Y.; Edwards, M.; Huang, Y.; Bilate, A. M.; Araujo, L. P.; Tanoue, T.; Atarashi, K.; Ladinsky, M. S.; Reiner, S. L.; Wang, H. H.; Mucida, D.; Honda, K.; Ivanov, I. I. Microbiota Imbalance Induced by Dietary Sugar Disrupts Immune-Mediated Protection from Metabolic Syndrome. *Cell* **2022**, *185* (19), 3501−3519.
(17) Wang, Z.; Li, S.; Cao, Y.; Tian, X.; Zeng, R.; Liao, D. F.; Cao, D. Oxidative Stress and Carbonyl Lesions in Ulcerative Colitis and Associated Colorectal Cancer. *Oxid. Med. Cell. Longevity* **2016**, *2016*. 9875298.
(18) Lopes, A.; Vilela, T. C.; Taschetto, L.; Vuolo, F.; Petronilho, F.; Dal-Pizzol, F.; Streck, E. L.; Ferreira, G. C.; Schuck, P. F. Evaluation of the Effects of Fructose on Oxidative Stress and Inflammatory Parameters in Rat Brain. *Mol. Neurobiol.* **2014**, *50* (3), 1124−1130.
(19) Marriott, B. P.; Cole, N.; Lee, E. National Estimates of Dietary Fructose Intake Increased from 1977 to 2004 in the United States. *J. Nutr.* **2009**, *139* (6), 1228S−1235S.
(20) Utrera, M.; Morcuende, D.; Rodríguez-Carpena, J. G.; Estévez, M. Fluorescent HPLC for the Detection of Specific Protein Oxidation Carbonyls - α-Aminoadipic and γ-Glutamic Semialdehydes - in Meat Systems. *Meat Sci.* **2011**, *89* (4), 500−506.
(21) Ganhão, R.; Estévez, M.; Morcuende, D. Suitability of the TBA Method for Assessing Lipid Oxidation in a Meat System with Added Phenolic-Rich Materials. *Food Chem.* **2011**, *126* (2), 772−778.
(22) *Official Methods of Analysis* AOAC: 1990.
(23) Etheridge, R. D.; Pesti, G. M.; Foster, E. H. A Comparison of Nitrogen Values Obtained Utilizing the Kjeldahl Nitrogen and Dumas Combustion Methodologies (Leco CNS 2000) on Samples Typical of an Animal Nutrition Analytical Laboratory. *Anim. Feed Sci. Technol.* **1998**, *73* (1−2), 21−28.
(24) Curtis, K. J.; Kim, Y. S.; Perdomo, J. M.; Silk, D. B. A.; Whitehead, J. S. Protein Digestion and Absorption in the Rat. *J. Physiol.* **1978**, *274*, 409−419.
(25) McDonough, F. E.; Steinke, F. H.; Sarwar, G.; Eggum, B. O.; Bressani, R.; Huth, P. J.; Barbeau, W. E.; Mitchell, G. V.; Phillips, J. G. In Vivo Rat Assay for True Protein Digestibility: Collaborative Study. *J. AOAC Int.* **1990**, *73* (5), 801−805.
(26) Sumner, L. W.; Amberg, A.; Barrett, D.; Beale, M. H.; Beger, R.; Daykin, C. A.; Fan, T. W. M.; Fiehn, O.; Goodacre, R.; Griffin, J. L.; Hankemeier, T.; Hardy, N.; Harnly, J.; Higashi, R.; Kopka, J.; Lane, A. N.; Lindon, J. C.; Marriott, P.; Nicholls, A. W.; Reily, M. D.; Thaden, J. J.; Viant, M. R. Proposed Minimum Reporting Standards for Chemical Analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* **2007**, *3* (3), 211−221.
(27) Giromini, C.; Cheli, F.; Rebucci, R.; Baldi, A. Dairy Proteins and Bioactive Peptides: Modeling Digestion and the Intestinal Barrier. *J. Dairy Sci.* **2019**, *102* (2), 929−942.
(28) Lund, M. N.; Heinonen, M.; Baron, C. P.; Estévez, M. Protein Oxidation in Muscle Foods: A Review. *Mol. Nutr. Food Res.* **2011**, *55* (1), 83−95.
(29) Oueslati, K.; De La Pomélie, D.; Santé-Lhoutellier, V.; Gatellier, P. Impact of the Fenton Process in Meat Digestion as Assessed Using an in Vitro Gastro-Intestinal Model. *Food Chem.* **2016**, *209*, 43−49.
(30) Kanner, J.; Lapidot, T. The Stomach as a Bioreactor: Dietary Lipid Peroxidation in the Gastric Fluid and the Effects of Plant-Derived Antioxidants. *Free Radical Biol. Med.* **2001**, *31* (11), 1388−1395.
(31) Van Hecke, T.; Vossen, E.; Goethals, S.; Boon, N.; De Vrieze, J.; De Smet, S. In Vitro and in Vivo Digestion of Red Cured Cooked Meat: Oxidation, Intestinal Microbiota and Fecal Metabolites. *Food Res. Int.* **2021**, *142*, No. 110203.
(32) Bains, Y.; Gugliucci, A.; Caccavello, R. Advanced Glycation Endproducts Form during Ovalbumin Digestion in the Presence of Fructose: Inhibition by Chlorogenic Acid. *Fitoterapia* **2017**, *120*, 1−5.
(33) Martinez-Saez, N.; Fernandez-Gomez, B.; Cai, W.; Uribarri, J.; del Castillo, M. D. In Vitro Formation of Maillard Reaction Products during Simulated Digestion of Meal-Resembling Systems. *Food Res. Int.* **2019**, *118*, 72−80.
(34) Semchyshyn, H. M. Fructation in Vivo: Detrimental and Protective Effects of Fructose. *BioMed Res. Int.* **2013**, *2013*. 343914.
(35) Gugliucci, A. Formation of Fructose-Mediated Advanced Glycation End Products and Their Roles in Metabolic and Inflammatory Diseases. *Adv. Nutr.* **2017**, *8* (1), 54−62.
(36) Luna, C.; Estévez, M. Formation of Allysine in β-Lactoglobulin and Myofibrillar Proteins by Glyoxal and Methylglyoxal: Impact on Water-Holding Capacity and in Vitro Digestibility. *Food Chem.* **2019**, *271*, 87−93.
(37) Akagawa, M.; Sasaki, D.; Kurota, Y.; Suyama, K. Formation of α-Aminoadipic and γ-Glutamic Semialdehydes in Proteins by the Maillard Reaction. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 129−134.
(38) Jiang, H.; Lin, Q.; Ma, L.; Luo, S.; Jiang, X.; Fang, J.; Lu, Z. Fructose and Fructose Kinase in Cancer and Other Pathologies. *J. Genet. Genomics* **2021**, *48* (7), 531−539.
(39) Luna, C.; Estévez, M. Oxidative Damage to Food and Human Serum Proteins: Radical-Mediated Oxidation vs. Glyco-Oxidation. *Food Chem.* **2018**, *267*, 111−118.
(40) Van Hecke, T.; Basso, V.; De Smet, S. Lipid and Protein Oxidation during in Vitro Gastrointestinal Digestion of Pork under Helicobacter Pylori Gastritis Conditions. *J. Agric. Food Chem.* **2018**, *66* (49), 13000−13010.
(41) Duerksen, D. *Protein Digestion and Absorption*, 2nd ed.; Elsevier Inc., 2019.
(42) He, J.; Zhou, G.; Bai, Y.; Wang, C.; Zhu, S.; Xu, X.; Li, C. The Effect of Meat Processing Methods on Changes in Disulfide Bonding and Alteration of Protein Structures: Impact on Protein Digestion Products. *RSC Adv.* **2018**, *8* (31), 17595−17605.
(43) Li, L.; Liu, Y.; Zou, X.; He, J.; Xu, X.; Zhou, G.; Li, C. In Vitro Protein Digestibility of Pork Products Is Affected by the Method of Processing. *Food Res. Int.* **2017**, *92*, 88−94.
(44) Sante-Lhoutellier, V.; Aubry, L.; Gatellier, P. Effect of Oxidation on in Vitro Digestibility of Skeletal Muscle Myofibrillar Proteins. *J. Agric. Food Chem.* **2007**, *55* (13), 5343−5348.
(45) Snelson, M.; Coughlan, M. T. Dietary Advanced Glycation End Products: Digestion, Metabolism and Modulation of Gut Microbial Ecology. *Nutrients* **2019**, *11* 2. 215.
(46) Ber, Y.; García-Lopez, S.; Gargallo-Puyuelo, C. J.; Gomollón, F. Small and Large Intestine (II): Inflammatory Bowel Disease, Short Bowel Syndrome, and Malignant Tumors of the Digestive Tract. *Nutrients* **2021**, *13*, 2325.
(47) Tian, T.; Wang, Z.; Zhang, J. Pathomechanisms of Oxidative Stress in Inflammatory Bowel Disease and Potential Antioxidant Therapies. *Oxid. Med. Cell. Longevity* **2017**, *2017*. 4535194.
(48) van Dongen, K. C. W.; Linkens, A. M. A.; Wetzels, S. M. W.; Wouters, K.; Vanmierlo, T.; van de Waarenburg, M. P. H.; Scheijen, J. L. J. M.; de Vos, W. M.; Belzer, C.; Schalkwijk, C. G. Dietary Advanced Glycation Endproducts (AGEs) Increase Their Concentration in Plasma and Tissues, Result in Inflammation and Modulate Gut Microbial Composition in Mice; Evidence for Reversibility. *Food Res. Int.* **2021**, *147*, No. 110547.
(49) Rodríguez-Romero, J. J.; Durán-Castañeda, A. C.; Cárdenas-Castro, A. P.; Sánchez-Burgos, J. A.; Zamora-Gasga, V. M.; Sáyago-Ayerdi, G. S. What We Know about Protein Gut Metabolites: Implications and Insights for Human Health and Diseases. *Food Chem. X* **2022**, *13*, No. 100195, DOI: 10.1016/j.fochx.2021.100195.
(50) Estévez, M.; Xiong, Y. Intake of Oxidized Proteins and Amino Acids and Causative Oxidative Stress and Disease: Recent Scientific Evidences and Hypotheses. *J. Food Sci.* **2019**, *84* (3), 387−396.
(51) Soladoye, O. P.; Juárez, M. L.; Aalhus, J. L.; Shand, P.; Estévez, M. Protein Oxidation in Processed Meat: Mechanisms and Potential

Implications on Human Health. *Compr. Rev. Food Sci. Food Saf.* **2015**, *14* (2), 106−122.
(52) Estévez, M.; Luna, C. Dietary Protein Oxidation: A Silent Threat to Human Health? *Crit. Rev. Food Sci. Nutr.* **2017**, *57* (17), 3781−3793.
(53) Bhattacharyya, A.; Chattopadhyay, R.; Mitra, S.; Crowe, S. E. Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. *Physiol. Rev.* **2014**, *94* (2), 329−354.
(54) Ma, Y.; Zhang, L.; Rong, S.; Qu, H.; Zhang, Y.; Chang, D.; Pan, H.; Wang, W. Relation between Gastric Cancer and Protein Oxidation, DNA Damage, and Lipid Peroxidation. *Oxid. Med. Cell. Longevity* **2013**, *2013*. 543760.
(55) Li, C. Q.; Pignatelli, B.; Ohshima, H. Increased Oxidative and Nitrative Stress in Human Stomach Associated with CagA+ Helicobacter Pylori Infection and Inflammation. *Dig. Dis. Sci.* **2001**, *46* (4), 836−844.
(56) Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R. Protein Carbonyl Groups as Biomarkers of Oxidative Stress. *Clin. Chim. Acta* **2003**, *329* (1−2), 23−38.
(57) Cho, Y. E.; Kim, D. K.; Seo, W.; Gao, B.; Yoo, S. H.; Song, B. J. Fructose Promotes Leaky Gut, Endotoxemia, and Liver Fibrosis Through Ethanol-Inducible Cytochrome P450−2E1−Mediated Oxidative and Nitrative Stress. *Hepatology* **2021**, *73* (6), 2180−2195.
(58) Guo, P.; Wang, H.; Ji, L.; Song, P.; Ma, X. Impacts of Fructose on Intestinal Barrier Function, Inflammation and Microbiota in a Piglet Model. *Nutrients* **2021**, *13* (10), 3515.
(59) Suyama, K.; Akagawa, M.; Sasaki, T. Oxidative Deamination of Lysine Residue in Plasma Protein from Diabetic Rat: α-Dicarbonyl-Mediated Mechanism. *Int. Congr. Ser.* **2002**, *1245*, 243−248.
(60) Jang, C.; Hui, S.; Lu, W.; Cowan, A. J.; Morscher, R. J.; Lee, G.; Liu, W.; Tesz, G. J.; Birnbaum, M. J.; Rabinowitz, J. D. The Small Intestine Converts Dietary Fructose into Glucose and Organic Acids. *Cell Metab.* **2018**, *27* (2), 351−361.
(61) Zhang, D. M.; Jiao, R. Q.; Kong, L. D. High Dietary Fructose: Direct or Indirect Dangerous Factors Disturbing Tissue and Organ Functions. *Nutrients* **2017**, *9* (4), No. 335, DOI: 10.3390/nu9040335.
(62) Mehrabi, S.; Wallace, L.; Cohen, S.; Yao, X.; Aikhionbare, F. O. Differential Measurements of Oxidatively Modified Proteins in Colorectal Adenopolyps. *Int. J. Clin. Med.* **2015**, *06* (4), 288−299.
(63) Olovo, C. V.; Huang, X.; Zheng, X.; Xu, M. Faecal Microbial Biomarkers in Early Diagnosis of Colorectal Cancer. *J. Cell. Mol. Med.* **2021**, *25* (23), 10783−10797.
(64) Beisner, J.; Gonzalez-granda, A.; Basrai, M.; et al. Fructose-Induced Intestinal Microbiota Shift. *Nutrients* **2020**, *12*, No. 3444, DOI: 10.3390/nu12113444.
(65) Mastrocola, R.; Ferrocino, I.; Liberto, E.; Chiazza, F.; Cento, A. S.; Collotta, D.; Querio, G.; Nigro, D.; Bitonto, V.; Cutrin, J. C.; Rantsiou, K.; Durante, M.; Masini, E.; Aragno, M.; Cordero, C.; Cocolin, L.; Collino, M. Fructose Liquid and Solid Formulations Differently Affect Gut Integrity, Microbiota Composition and Related Liver Toxicity: A Comparative in Vivo Study. *J. Nutr. Biochem.* **2018**, *55*, 185−199.
(66) Song, G.; Gan, Q.; Qi, W.; Wang, Y.; Xu, M.; Li, Y. Fructose Stimulated Colonic Arginine and Proline Metabolism Dysbiosis, Altered Microbiota and Aggravated Intestinal Barrier Dysfunction in DSS-Induced Colitis Rats. *Nutrients* **2023**, *15*, 782.
(67) Yin, Y.; Cai, J.; Zhou, L.; Xing, L.; Zhang, W. Dietary Oxidized Beef Protein Alters Gut Microbiota and Induces Colonic Inflammatory Damage in C57BL/6 Mice. *Front. Nutr.* **2022**, *9* (3), No. 980204, DOI: 10.3389/fnut.2022.980204.
(68) Parker, B. J.; Wearsch, P. A.; Veloo, A. C. M.; Rodriguez-Palacios, A. The Genus Alistipes: Gut Bacteria with Emerging Implications to Inflammation, Cancer, and Mental Health. *Front. Immunol.* **2020**, *11*, 906.
(69) Oliphant, K.; Allen-Vercoe, E. Macronutrient Metabolism by the Human Gut Microbiome: Major Fermentation By-Products and Their Impact on Host Health. *Microbiome* **2019**, *7* (1), 91.
(70) Gilbert, M. S.; Ijssennagger, N.; Kies, A. K.; van Mil, S. W. C. Protein Fermentation in the Gut; Implications for Intestinal Dysfunction in Humans, Pigs, and Poultry. *Am. J. Physiol. − Gastrointest. Liver Physiol.* **2018**, *315* (2), G159−G170.
(71) Portune, K. J.; Beaumont, M.; Davila, A. M.; Tomé, D.; Blachier, F.; Sanz, Y. Gut Microbiota Role in Dietary Protein Metabolism and Health-Related Outcomes: The Two Sides of the Coin. *Trends Food Sci. Technol.* **2016**, *57*, 213−232.
(72) Le Gall, G.; Noor, S. O.; Ridgway, K.; Scovell, L.; Jamieson, C.; Johnson, I. T.; Colquhoun, I. J.; Kemsley, E. K.; Narbad, A. Metabolomics of Fecal Extracts Detects Altered Metabolic Activity of Gut Microbiota in Ulcerative Colitis and Irritable Bowel Syndrome. *J. Proteome Res.* **2011**, *10* (9), 4208−4218.
(73) Wang, Y.; Qi, W.; Song, G.; Pang, S.; Peng, Z.; Li, Y.; Wang, P. High-Fructose Diet Increases Inflammatory Cytokines and Alters Gut Microbiota Composition in Rats. *Mediators Inflamm.* **2020**, *2020*. 6672636.
(74) Mancabelli, L.; Milani, C.; Lugli, G. A.; Turroni, F.; Cocconi, D.; van Sinderen, D.; Ventura, M. Identification of Universal Gut Microbial Biomarkers of Common Human Intestinal Diseases by Meta-Analysis. *FEMS Microbiol. Ecol.* **2017**, *93* (12), fix153.
(75) Chen, Z.; Radjabzadeh, D.; Chen, L.; Kurilshikov, A.; Kavousi, M.; Ahmadizar, F.; Ikram, M. A.; Uitterlinden, A. G.; Zhernakova, A.; Fu, J.; Kraaij, R.; Voortman, T. Association of Insulin Resistance and Type 2 Diabetes with Gut Microbial Diversity: A Microbiome-Wide Analysis from Population Studies. *JAMA Netw. Open* **2021**, *4* (7), e2118811.
(76) Wang, X.; Zhu, L.; Li, X.; Wang, X.; Hao, R.; Li, J. Effects of High Fructose Corn Syrup on Intestinal Microbiota Structure and Obesity in Mice. *npj Sci. Food* **2022**, *6* (1), 17.
(77) Lambertz, J.; Weiskirchen, S.; Landert, S.; Weiskirchen, R. Fructose: A Dietary Sugar in Crosstalk with Microbiota Contributing to the Development and Progression of Non-Alcoholic Liver Disease. *Front. Immunol.* **2017**, *8*, No. 1159, DOI: 10.3389/fimmu.2017.01159.
(78) Amaretti, A.; Gozzoli, C.; Simone, M.; Raimondi, S.; Righini, L.; Pérez-Brocal, V.; García-López, R.; Moya, A.; Rossi, M. Profiling of Protein Degraders in Cultures of Human Gut Microbiota. *Front. Microbiol.* **2019**, *10*, 2614.

Capítulo III

Pomegranate Supplementation Alleviates the Dyslipidemia and the Onset of Non-Alcoholic Fatty Liver Disease Caused by Chronic Dietary Fructose in Wistar Rats

La suplementación con granada disminuye la dislipidemia y el comienzo de la enfermedad de hígado no graso causados por la ingesta crónica de fructosa dietética en ratas Wista

Pomegranate Supplementation Alleviates the Dyslipidemia and the Onset of Non-Alcoholic Fatty Liver Disease Caused by Chronic Dietary Fructose in Wistar Rats

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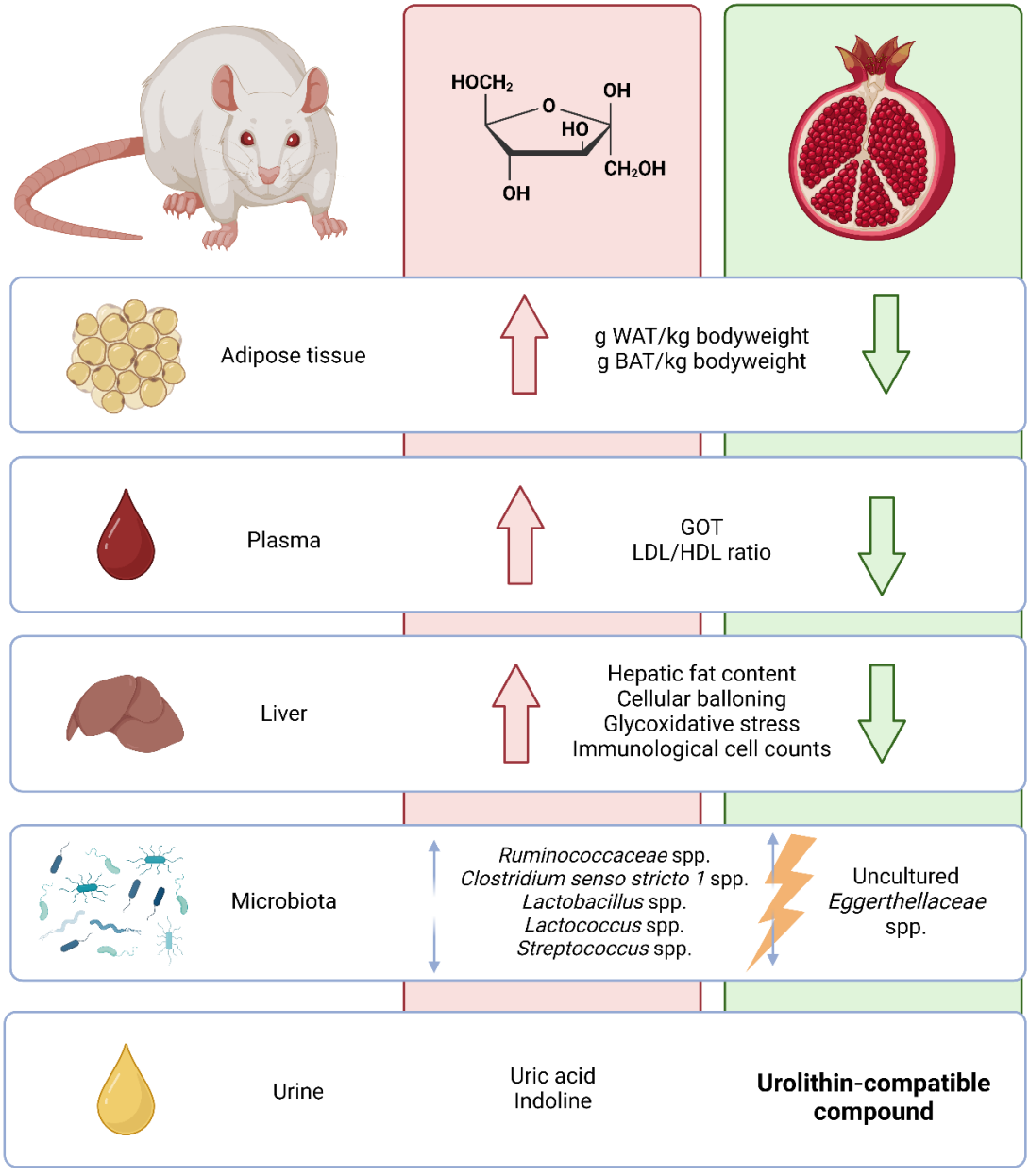

Figura 4.2. Resumen gráfico del Capítulo III. Elaboración propia.

Pomegranate Supplementation Alleviates the Dyslipidemia and the Onset of Non-Alcoholic Fatty Liver Disease Caused by Chronic Dietary Fructose in Wistar Rats

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; MetS, metabolic syndrome; P, punicalagin-rich commercial pomegranate dietary supplement; WHO, World Health Organization; T2D, type 2 diabetes *mellitus*; GIT, gastrointestinal tract; GLUT5, facilitative glucose transporter 5; GLUT2, facilitative glucose transporter 2; PU, punicalagin; EA, ellagic acid; U, urolithin; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; WAT, white adipose tissue; BAT, brown adipose tissue; α-AS, α-aminoadipic semialdehyde; γ-GS, γ-glutamic semialdehyde; PPC, primary protein carbonyls; AGEs, advanced glycation end-products; TCA, trichloroacetic acid; TP, total protein; ALB, albumin; GLB, globulins; CREAT, creatinine; ALP, alkaline phosphatase; AST/GOT, aspartate transaminase/glutamic oxaloacetic transaminase; TG, triglycerides; GTS-α, α-glutathione S-transferase; SDH, sorbitol dehydrogenase; GIP, glucose-dependent insulinotropic peptide; GPL-1, glucagon-like peptide 1; IL-6, interleukin; TNF-α, tumor necrosis alpha; IQR, interquartile range.

Keywords: dyslipidemia, fatty liver disease, fructose, metabolomic, punicalagin

ABSTRACT

Scope: Non-alcoholic fatty liver disease (NAFLD), obesity and related chronic diseases remain one of the major non-communicable diseases with high mortality rates worldwide. While dietary sugars are known to be responsible for insulin resistance and metabolic syndrome (MetS), the underlying pathophysiological effects of sustained fructose consumption requires further elucidation. The present study aimed to elucidate both the molecular mechanisms involved in the pathophysiology associated with fructose intake and the effect of a punicalagin-rich commercial pomegranate dietary supplement (P) used as nutritional strategy to alleviate the fructose-induced metabolic impairments.

Methods and results: Nineteen *Wistar* rats fed on a basal commercial feed were supplemented with either 30% (w/v) fructose in drinking water (F; n=7) or 30% (w/v) fructose solution plus 0.2% (w/v) P (F+P; n=6) for 10 weeks. Results were compared to a control group fed on the basal diet and provided with drinking water (C; n=6). Body weights and energy intake were weekly registered. Fat depots, liver tissues, microbiota and blood markers were analyzed after euthanasia of the animals, after which in addition the urine profile were assessed by means of untargeted MS-based metabolomic.

Conclusions: Promising results were obtained regarding the effect of P on the impaired metabolism of F rats

1. INTRODUCTION

Fructose consumption has increased strongly in the last decades, despite the efforts of governments and health societies to alert population on the health risks linked to sustained intake of simple sugars. The World Health Organization (WHO) recommends consuming less than 5% of total calories ingested in the form of reducing sugars.[1] Extensive literature supports the connection between fructose intake and several metabolic impairments associated with metabolic syndrome (MetS), such as obesity, adiposity, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), insulin resistance, type 2 diabetes mellitus (T2DM), gout, hyperuricemia and cardiometabolic diseases, likely related to disturbances of liver metabolism.[2]

Impaired hepatic energy metabolism and lipid deposition might be responsible for the negative outputs related to high fructose consumption. Excessive stimulation of glycolysis and gluconeogenesis pathways together with decreased activity of fatty acid oxidation pathways appear to be the basis of these impairments.[3] However, it is known that many pathophysiological effects of sustained sugar consumption are associated with events occurring at the gastrointestinal (GIT) level.[4] Our previous *in vivo* study illustrated the deleterious effects of dietary fructose on the glycoxidative stress in both, the intraluminal contents and GIT tissues, on impaired protein digestibility, as well as its negative impact on the microbiota and the intraluminal colonic metabolome.[5] The glycoxidative reactions (or pro-glycoxidative status) promoted by excessive fructose consumption could also potentially adversely contribute to fructose-associated metabolic impairments, but other factors are involved. At 0.5 g/kg body mass, fructose is transported into epithelial cells through the facilitative glucose transporter 5 (GLUT5) and metabolized in the small intestine into other species (glucose, lactate, and/or glycerate), which are subsequently released into the portal blood to the liver. Higher levels of dietary fructose (> 1 g/kg body mass) are known to not only induce overexpression of GLUT5,[3] but also the translocation of basolateral facilitative glucose transporter 2 (GLUT2) to the apical membrane, optimizing fructose intestinal uptake.[6] Despite this, high intake of fructose could overwhelm the intestinal clearance mechanisms and would pass to later GIT stages, as well as would reach the liver via portal circulation.[2] In the liver, GLUT5 is not expressed at high levels, and GLUT2 is likely the major membrane transporter.[3,7]

Phenolic-rich dietary supplements are commonly marketed along with health claims of protecting

against numerous health disorders and enhance health status. Yet, the molecular mechanisms of their potential bioactivities as well as the actual effectiveness of some of these supplements requires further elucidation. Pomegranate is rich in a variety of phenolic compounds and its consumption is related to assorted health benefits including anti-obesity, antidiabetic and hepatoprotective effects.[8,9] Punicalagin (PU) is a component of ellagitannins family naturally present in pomegranate, together with ellagic acid (EA), which makes punicalagin a component of great importance from the point of health, and the interaction between the compound and the gut microbiota seems to be the key to its beneficial effects.[10,11] EA is a bioactive compound with antioxidant properties which is further biotransformed into urolithins (U), with these latter microbial metabolites having a remarkable positive impact on human health.[12] Several studies linked PU supplementation with improvements in metabolic pathologies as T2DM through their capability to interact with intestinal GLUT2.[13] Other authors related the inhibition of intestinal GLUT5 with several plant extracts like green tea or chamomile,[2] so this encouraged us to gain further insight into the unknown impact of PU on the metabolism of fructose during an *in vivo* experiment and the consequences in terms of liver health.

Hence, this study aims to decipher the adverse effects of sustained fructose consumption on lipid metabolism and depots and liver function and health. Additionally, we aimed to unveil the mechanisms of the potential hepatoprotective effects of a PU-rich commercial pomegranate dietary supplement (P).

2. MATERIALS AND METHODS

2.1. Chemicals

*Sigma Chemicals (*Sigma Aldrich*,* Stheinheim, Germany), *Fisher* (Fisher Scientific S.L., Madrid, Spain) and *Panreac* (Panreac Química, S.A., Barcelona, Spain) provided all reagents, chemicals, and standard compounds. Ultrapure water was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Animals, feeds, and other materials

Nineteen male *Wistar* breed rats of the *Rattus novergicus* species were used in our experiment according to Spanish legal requirements (RD 53/2013), bioethics committee of University of

Extremadura (137-2020) and approval of the Board of Extremadura (EXP20200904). The design and performance of the experiment, including animal manipulation and euthanasia, were carried out by licensed veterinarians with all requirements by legal authority (Dirección General de Sanidad Animal, Junta de Extremadura, Spain). The rats were supplied by the Animal Experimentation Station from the University of Extremadura (Caceres, Spain). At the beginning of the assay, they were 6-7 weeks old and weighed 186 g in average. During the entire study period, the same rodent basal feed used was the "*Teklad Global Diet 2014*", supplied by *ENVIGO* (Madison, WI, USA), with a crude protein content of 14.3%. The PU-rich pomegranate supplement (Granatum PLUS) was provided by "Antioxidantes Naturales del Mediterráneo S.L." (Murcia, Spain). The pomegranate powder had 183 mg of punicalagin per pill (670 mg of pomegranate extract). A more detailed analysis of the supplement was described in previous works.[10]

2.3. Experimental design

During a 1-week adaptation period, the rats were individually identified by means of a perforation code in the auditory pavilion and maintained in ventilated cages, with water and feed *ad libitum*, under controlled climatic conditions (20-22ºC °C temperature, 40-50% humidity and 12-12 hours light/dark cycle).

Once the adaptation period finished, animals were randomly allocated into one of these three experimental groups: i) control group (C), which received the basal feed and drinking water during the entire assay (n=6); ii) fructose group (F), in which drinking water was replaced by 30% (w/v) fructose solution (n=7); and iii) fructose and P group (F+P), in which drinking water was replaced by 30% (w/v) fructose solution along with 0.2% (w/v) pomegranate supplement (n=6).

The experiment was conducted for 10 weeks. Animals were checked daily to ensure safety and well-being. During the treatment, food and water/solutions consumption were gravimetrically monitored every time they were filled in order to calculate the energy intake. This procedure was performed depending on the demand of the animals (every two or three days, approximately). Body weights were register weekly.

Dosage information: On average, rats from F group consumed 9 g fructose/kg live bodyweight/day. Based on the amounts of PU in P, and the quantities of fructose-solution with P consumed by the rats, the animals ingested an average of 18.71±7.60 mg PU/kg bodyweight/day.

2.4. Slaughter, necropsy and sampling

Wistar rats were euthanized at the end of the experimental period at an approximate age of 16-17 weeks-old and an average weight of 437 grams. Euthanasia was performed by exsanguination via cardiac puncture. Previously, the animals were anaesthetized using 5% inhaled isoflurane. All the blood was collected in tubes with EDTA and subsequently stored at -80ºC. Fatty tissues were taken, and the fat depots of the animals was weighed according to type and location. The subcutaneous adipose tissue (SAT) as well as the visceral adipose tissue (VAT) was gently removed and individually weighed. VAT was dissected from different locations in the abdomen of the rats (retroperitoneal, perirenal, gonadal and inguinal). The sum of the weight of both depots was considered as white adipose tissue (WAT).[14] Moreover, the fat depots from interscapular region were also removed and weighed as brown adipose tissue (BAT). The liver of the animals was readily dissected from corpses, dispensed in a suitable container, and stored immediately at -80 °C. A portion of tissue was preserved in 5% formalin for microscopic analyses. The urine was aseptically collected through puncture in the bladder and properly stored. Feces from the rectum were aseptically dispensed in Eppendorf tubes and stored at -80ºC until analyses were performed.

2.5. Analytical procedures

2.5.1. Assessment of glycoxidative stress in liver tissue

2.5.1.1. Protein carbonylation

The detection and quantification of α-aminoadipic (α-AS) and γ-glutamic semialdehydes (γ-GS) were assessed through HPLC technologies in order to evaluate the accretion of protein carbonyls in the liver as previously were described.[15] Thus, 500 mg of tissue were homogenized with 0.5 mL of PBS solution and the remaining steps of the procedure were exact to those reported in the above-mentioned study. Results from the quantification of α-AS and γ-GS were expressed individually and as total Primary Protein Carbonyls (PPC) (nmol carbonyl/mg protein).

2.5.1.2. Pentosidine

Pentosidine is a specific marker of advanced glycation endproducts (AGEs). The compound was also assessed using the specific liquid chromatographic procedure described above with slight modifications.[15,16] Tissue homogenates were treated with 10% cold trichloroacetic acid (TCA) to removed proteins and ethanol-ethyl-acetate (1:1; v/v) to remove lipids. Purified extracts were

filtered through a 0.45 μm pore size hydrophilic polypropylene GH Polypro (GHP) syringe filters (Pall Corporation, Port Washington, NY, USA). HPLC analysis and conditions were described somewhere else [15]. Standard of the compound was used to compare retention time and to identify the pentosidine peak, which was manually integrated from fluoresce detector chromatograms. Pentosidine was expressed as fluorescence units.

2.5.2. Analysis of biochemical profile of plasma

The stored plasma was sent under suitable conditions to the Internal Medicine Laboratory of the Veterinary Clinic Hospital on the Faculty of Veterinary (University of Extremadura) to obtain a complete biochemical profile of the samples. Specifically, total proteins content (TP), albumin (ALB), globulins (GLB), creatinine (CREAT), urea, phosphorous, alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase/ glutamic oxaloacetic transaminase (AST/GOT), triglycerides (TG), total cholesterol, LDL and HDL were determined. The samples were analyzed in an Saturno 100 VetCrony ® automatic blood chemistry analyzer (Crony Instruments, Rome, Italy).

2.5.3. Fecal Microbiota

DNA was isolated from feces obtained at slaughter as aforementioned and the microbiota from *Wistar* rats was analyzed following the procedure described previously by our group.[5]

2.5.4. Microscopy analysis of liver tissues

Sections from formalin-fixed liver samples from treated and control rats were microscopically assessment by an experienced veterinary pathologist unaware of the animal treatment groups. Paraffin-embedded specimens were deparaffinized in xylene and rehydrate with successive washes in an increased gradient of ethanol concentrations. Then, they were stained with Hematoxylin 80% (LabKem) and Eosin Yellowish (Panreac), and the slides were examined with a Leica DFC-280 light microscope. The Leica Q Win Plus Image Analysis System (Leica Micros Imaging Solutions Ltd, Cambridge, UK) was used for morphometric analysis. Occurrence of pathophysiological observations like necrosis, apoptosis, dilation of biliary canaliculi, or inflammatory cell infiltration were measured in ten different fields for each section at x20 augments (surfaces of observed fields: 20 x 38663 μm^2).

On the other hand, deparaffinized and rehydrated hepatic sections were incubated with CD45

Antibody, anti-rat REAfinity™ APC (Miltenyi Biotec) and Hoechst 33258 (Thermo Fisher Scientific), once the process to expose antigens were carried out. The APC- and Hoechst-treated samples were excited at 405nm and 635nm respectively, and the images were taken with a Fluview 1000, Olympus-Evident confocal microscope.

2.5.5. Hepatic fat content

Total lipids were extracted from 1 g of liver using a mixture of chloroform/methanol (1:2), according to the method described previously.[17]

2.5.6. Untargeted MS-based metabolomics

Metabolites from urine of *Wistar* rats were extracted and analyzed. Briefly, 150 µL of urine were mixed with 200 µL of methanol 100%, in order to precipitate undesirable compounds. The mixing was homogenized in a vortex and subsequently centrifuged at 9000 G and 4ºC for 10 minutes. The supernatants were placed in new *Eppendorf* tubes and dried using a centrifugal vacuum concentrator (Gyrozen, Daejeon, Korea). The residues were reconstituted with 75 µL of methanol 70% and centrifuged. The supernatants were placed into singled *Eppendorf* tubes using 0.22 µm nylon-filters. Samples were analyzed using a Dionex UltiMate 3000 RSLC system coupled with a Q Exactive High Resolution Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The specific conditions for the analysis and data treatment are similar to those detailed in a previous publication.[5]

2.5.7. Detection of urolithin-related compounds by HPLC-DAD assay

A liquid chromatographic assessment of the urine samples was performed based on the guidelines described by other authors with small modifications, in order to detect the presence of urolithin-derived compounds.[18] The analytical procedure for extraction and purification of urine samples was performed as in the previous section. Injection volume was 8mL for each sample. The identification and quantification of the expected compounds was performed in a Shimadzu Prominence HPLC (Shimadzu Corp., Japan) equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, an SIL-20A autosampler and a SPD-M20A Diode Array Detector. The columns used were a reversed-phase Agilent Poroshell 120 SB-C18 column (150 × 4.6 mm, particle size 2.7 µm) and a guard column (10 × 4.6 mm) with the identical material. Solutions of 0.2% (v/v) formic acid (A) and acetonitrile with 0.2% (v/v) formic acid (B) were used

as eluents. The separations were obtained using the following gradient: 0 min, 7% B; 20 min, 35% B; 35 min, 65% B; 40 min, 70% B, and this percentage was maintained for 4 min and then came back to the initial conditions. The flow rate was maintained at 0.5 mL/min and the temperature of the column was kept at 25 °C. The spectra were recorded in the range of 200–750 nm and the chromatograms were obtained at 280, 332 and 356 nm. Urolithins-related compounds were identified using their UV spectral properties and whenever possible by comparison with authentic standards. Quantification was based on external calibration curves with a linear range over 0.1-100 µg/mL for Urolithin A (URO-A) (Sigma-Aldrich, St. Louis, USA) and 0.2-200 µg/mL for Urolithin B (URO-B) (Sigma-Aldrich, St. Louis, USA).

2.6. Statistical analysis

The statistical analysis of raw data was carried out applied parametric (i.e., ANOVA test and Tukey post hoc test) and non-parametric test (i.e., Kruskal-Wallis's test and multiple comparisons post hoc test) based on the normality and homoscedasticity of the data using the R statistical software (R 4.2.3) and SPSS version 27.0. Box plots and bar chart were generated using ggplot2.[19] *p* values less than 0.05 was considered statistically significant. Both F- and F+P-responsive metabolites were assessed in the MetaboAnalyst (https://www.metaboanalyst.ca/). Principal Component Analysis (PCA) as multivariant analysis were used. Moreover, metabolite profile distinctions between the groups were evaluated by the ANOVA test as one-factor statistical method to further analyze the impact of the treatments on the urine metabolome of *Wistar* rats.

3. RESULTS

3.1. Caloric intake, body weights and fat depots

Table 1 shows the energy provided by the diet to the different groups of experimental animals expressed as the median [interquartile range (IQR)] of kJ/kg body weight registered. The energy consumed by C was supplied only by the feed (96.58 kJ/kg body weight) and was statistically similar to that consumed by F and F+P through the solid feed. However, drinking fructose-rich solutions provided extra energy supply to animals from F and F+P (41.54 and 45.47 kJ/kg body weight respectively) as compared to animals from C, which drank water. For this reason, there was significant differences among the total energy intake of the experimental animals during the assay ($p<0.05$), with F and F+P being the groups having the most total energy intake (137.08 kJ/kg body

weight and 129.28 kJ/body weight, respectively). The occurrence of P in fructose solution did not affect the solution intake and hence, both group of animals had equivalent total energy supply during the assay.

TABLE 1. Energy provided by the feed and the fructose supplemented waters expressed as median [IQR] values in the experimental consumer groups fed with basal diet and either drinking water (C), 30% (w/v) fructose solution (F), or 0.2% (w/v) P in a 30% (w/v) fructose solution (F+P).

	Feed energy (kJ/day)	Fructose-solution energy (kJ/day)	Total energy intake (kJ/day)
C	96.58 [94.20]	0.00b [0.00]	96.58b [94.20]
F	87.41 [81.63]	41.54a [29.96]	137.08a [88.40]
F+P	78.31 [75.75]	45.47a [31.90]	129.28a [76.15]
p value[a]	ns	***	*

[a] Significance level in Kruskal-Wallis' test with the effect of diet (C, F, F+P). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. ns: no significant. Medians with different letters within the same column were significantly different in the multiple comparisons' *post hoc* analysis with the Bonferroni correction applied ($p<0.05$).

Unexpectedly, this higher energy input did not appear to significantly influence the weight of the different groups of animals during the experiment, which overall increased similarly from 230 to 423 g approximately (mean weights) (Figure 1).

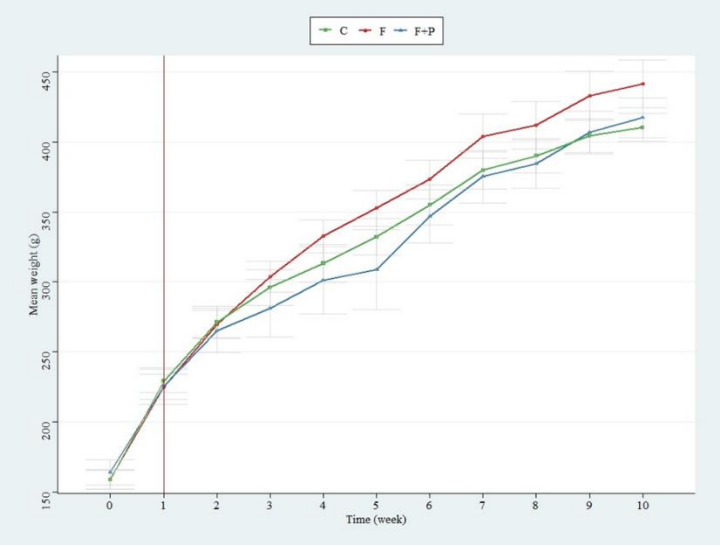

FIGURE 1. Evolution of the mean weights during the assay of the different experimental animals fed with basal diet and either drinking water (C), 30% (w/v) fructose solution (F), or 0.2% (w/v) P

in 30% (w/v) fructose solution (F+P). On the other hand, Figure 2 shows the weights of fat depots from experimental animals expressed as g/kg body weight. The BAT from C was equivalent to 1.81 g/kg body weight at the end of the experiment, while their WAT, expressed as the sum of both SAT and VAT, was 80.93 g/kg body weight. The consumption of fructose had a marked and significant lipogenic effect on all the fatty tissues analyzed, which were almost 2-fold significantly higher than those of their control counterparts ($p<0.001$). However, this increase was more pronounced in the VAT (27.13 g/kg body weight increased; $p<0.001$) than in the SAT (20.11 g/kg body weight increased; $p<0.001$). Meanwhile, the fatty depots from F+P were significantly similar to those of their control counterparts. Hence, PU supplement counteracted the pro-adipose effect of fructose in *Wistar* rats.

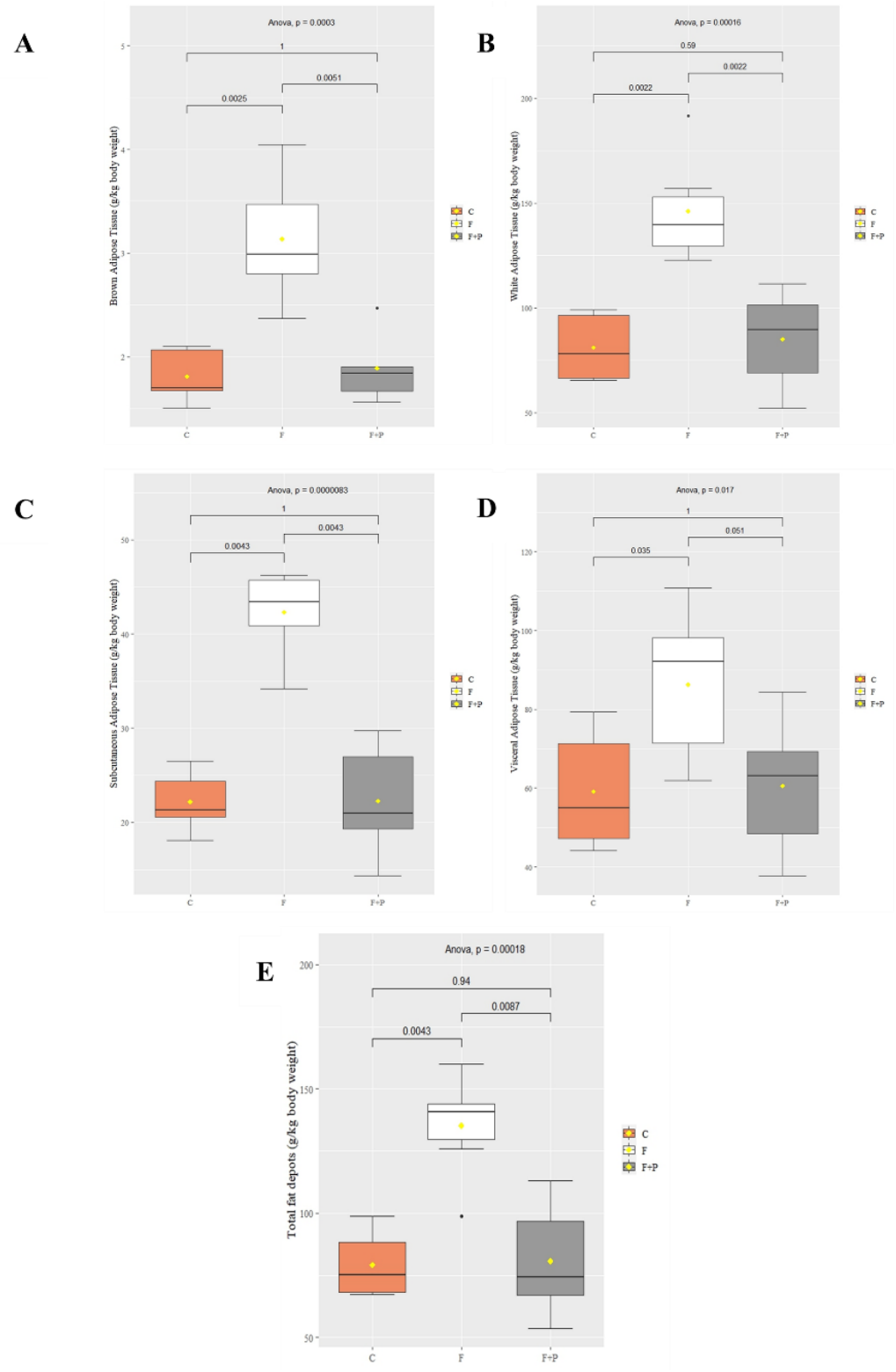

FIGURE 2. Mean weight of the different fat depots analyzed.

3.2. Plasma markers

Table 2 shows the results of the biochemical analyses made on the plasma from experimental rats fed with basal diet and either drinking water (C), 30% fructose solution (F) or 0.2% P in a 30% fructose solution (F+P). The consumption of fructose promoted significant changes in some of the analyzed markers in contrast to C, such as an increase of the concentration of albumin (ALB) (2.59 g/dL *vs.* 2.34 g/dL in C), phosphorous (3.80 mg/dL *vs.* 3.30 mg/dL in C) and aspartate transaminase (AST) (48.96 U/L *vs.* 25.46 U/L in C).

The intake of P together with fructose also influenced the concentration of some biochemical markers. Thus, the supplementation with P promoted increased amounts of total proteins (TP) ($p<0.01$), ALB ($p<0.05$) and globulins (GLOB) ($p<0.001$), phosphorous ($p<0.01$), cholesterol ($p<0.001$) and HDL ($p<0.01$) in the plasma of F+P rats as compared to that of C and F counterparts. There was no significant difference on the concentration of GOT between F+P and C. However, the levels of the liver injury marker were significantly lower in the plasma of F+P rats than in those treated with fructose ($p<0.001$).

TABLE 2. Statistical analysis of biochemical results from plasma of experimental rats fed with basal diet and either drinking water (C), 30% (w/v) fructose solution (F), or 30% (w/v) fructose solution with 0.2% P (F+P) expressed as mean±standard deviation.

Parameter[a]	C	F	F+P	*p* value[b]
TP (g/dL)	3.55b±0.40	3.93b±0.84	5.31a±1.03	**
ALB (g/dL)	2.34c±0.22	2.59b±0.44	3.03a±0.41	*
GLB	1.21b±0.26	1.34b±0.42	2.06a±0.28	***
CREAT	0.43±0.05	0.47±0.10	0.54±0.10	ns
Urea (mg/dL)	21.78±3.13	19.44±4.02	22.58±3.05	ns
Phosphorous (mg/dL)	3.30b±0.56	3.80ab±0.88	4.76a±0.82	**
ALP (U/L)	3.83±0.75	3.43±1.27	3.29±1.11	ns
ALT (U/L)	13.67±2.16	18.86±6.79	16.86±2.12	ns
GOT (U/L)	25.46b±3.67	48.96a±11.08	31.00b±5.10	***
TG (mg/dL)	106.83±28.34	143.14±10.43	162.39±62.09	ns
Cholesterol (mg/dL)	76.00b±11.80	67.43b±5.35	90.00a±9.17	***
LDL (mg/dL)	8.33±3.08	11.54±3.17	8.54±2.24	ns
HDL (mg/dL)	68.90b±7.54	69.61b±9.88	84.40a±4.90	**
ALB/GLB ratio	1.99b±0.37	2.03a±0.41	1.49b±0.22	*
LDL/HDL ratio	0.12b±0.04	0.17a±0.06	0.10c±0.02	*
TG/HDL ratio	1.55±0.39	2.10±0.37	1.91±0.70	ns

[a] Total protein content (TP); albumin (ALB); globulins (GLB), creatinine (CREAT), alkaline phosphatase (ALP); alanine transaminase (ALT); aspartate transaminase (GOT), triglycerides (TG).

[b] Significance level in ANOVA test with the effects of diet (C, F and F+P). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: no significant. Means with different letters within the same row were significantly different in Tukey *post hoc* analysis ($p<0.05$).

3.3. Liver histopathology, hepatic fat content, and glycoxidative stress

Diagnosis evaluation of liver tissues is largely based on a thorough examination of sections stained with hematoxylin and eosin (H&E). Figure 3 shows the most representatively hepatic images from H&E staining. Figures 3A and 3B, correspond to C and F livers respectively. As compared to the former, the latter shows multiple and larger size vesicle cells (namely cellular ballooning), while livers from F+P rats (3C) shows a hepatic tissue with hardly any vesicle cells. The images also show the hepatic fat content of each group of animals, expressed as g fat/100g liver tissue (%). The hepatic fat content of C rats was 1.80%, while the amount of lipid depots in rats exposed to drinking fructose raised up to 4.28% ($p<0.001$). On the other hand, the hepatic fat content of F+P rats was 1.61%,

with these values being statistically similar to the liver fat deposit in C rats.

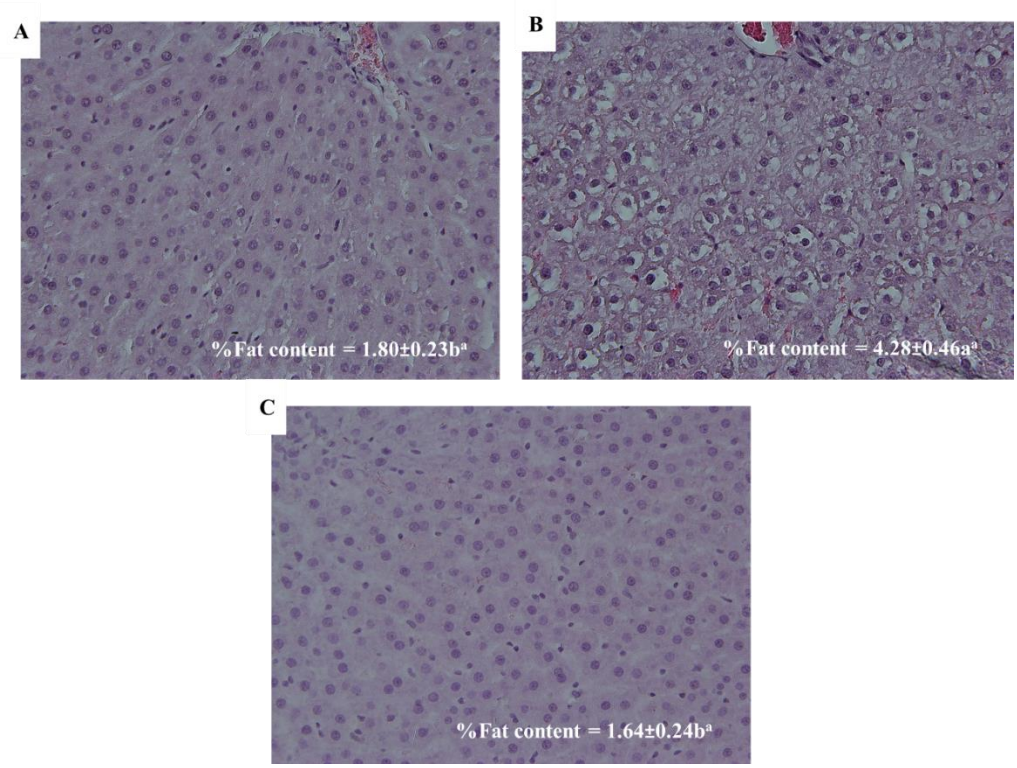

FIGURE 3. H&E-stained histological images and hepatic fat contents expressed as g fat/100 g liver of livers from the experimental rats: (A) Control group, (B) Fructose group, and (C) Fructose + P group.

[a] Significance level in ANOVA test with the effects of diet (C, F and F+P) on the liver's fat concentration = $p<0.001$. Means with different letters were significantly different in multiple comparisons' post-hoc analysis ($p<0.05$).

In order to evaluate additional pathophysiological features in the microscopic images, those were analyzed for the occurrence of apoptosis, necrosis, intrahepatic biliary dilation, infiltration and edema. Figure 4 shows the percentage of each event occurring in the liver cells of each group. Fructose intake significantly increased hepatic cells apoptosis, necrosis, intrahepatic biliary dilation and infiltration as compared to liver from C rats ($p<0.001$). Meanwhile, in livers from F+P rats, fructose significant promoted higher hepatic cells necrosis, infiltration and edema as compared to the livers from C counterparts ($p<0.001$). However, the presence of P in the drinking fructose-solution decreased hepatic cells apoptosis and dilation than those occurred in liver from C rats. Compared to F, F+P significantly showed lower events of hepatic cells apoptosis, necrosis, dilation, and infiltration, but instead the occurrence of cells edema in F+P were higher than in F ($p<0.001$).

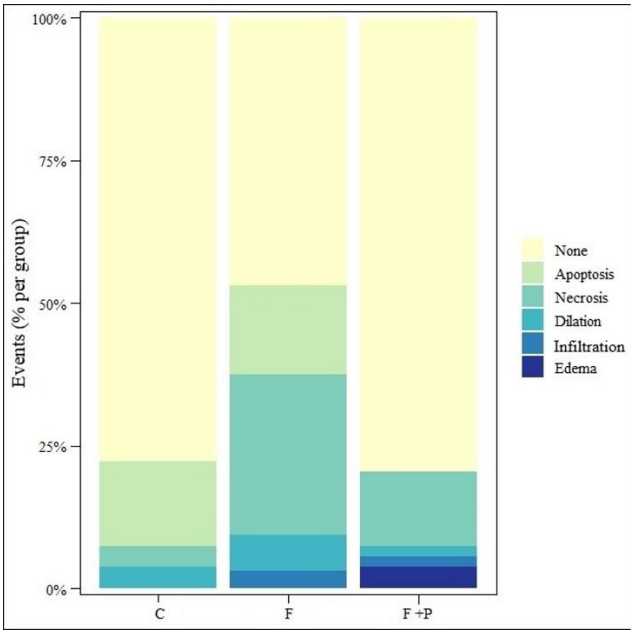

FIGURE 4. Percentage of qualitative events in the livers of each group of rats based on the diet (C, F and F+P).

Figure 5 shows the most representative images from CD45 staining of the hepatic tissues, which promote a blue fluorescence from immunoglobulins, and a red one from CD45+ cells. Figure 5A shows a hepatic region from C rats, while Figure 5B and 5C corresponding to a hepatic section from F and F+P respectively. The images also show the immunological cell counts results from each group of animals (i.e., CD45 cells, lymphocytes and macrophages counts), and displayed that the intake of fructose increased significantly the amounts of inflammatory and immunological cells (Figure 5B) compared to C and F+P.

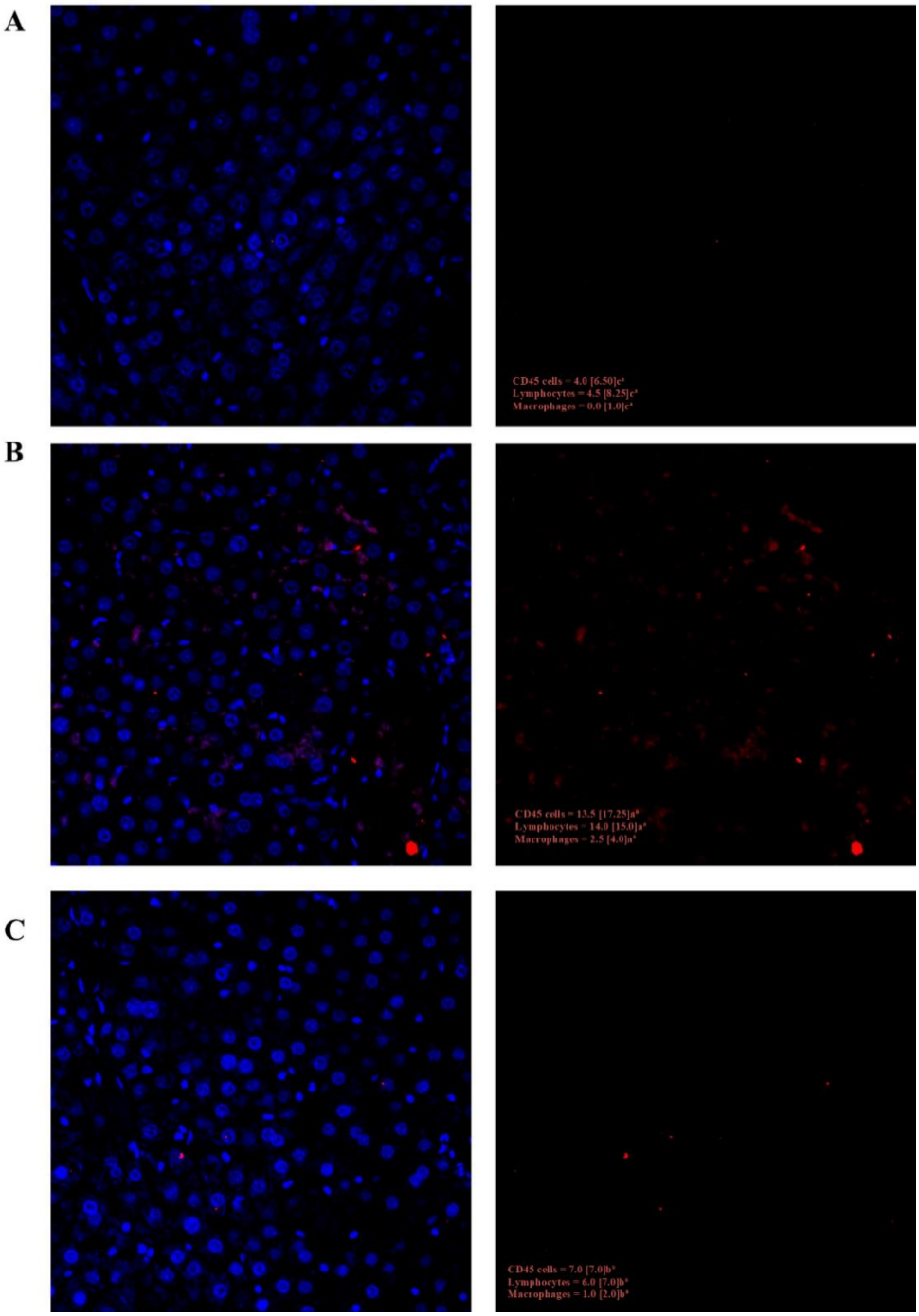

FIGURE 5. CD45-stained cells from livers of experimental rats and cell counts (CD45 cells, lymphocytes, and macrophages): (A) Control group, (B) Fructose group, and (C) Fructose + P group.

[a] Significance level in Kruskal-Wallis test with the effects of diet (C, F and F+P) on the liver's immunological cells counts = $p<0.01$. Medians with different letters were significantly different in multiple comparisons' post-hoc analysis ($p<0.05$).

In addition to the histological assessment, the pathophysiological damage of drinking fructose to liver of *Wistar* rats was also evaluated by the detection and quantification of markers of glyco-oxidative stress. Table 3 shows the concentration of α-AS and γ-GS as total primary protein carbonyls (PPC) and pentosidine, in the liver of the experimental rats. The hepatic levels of α-AS of C were 0.63 nmol carbonyl/mg protein. The intake of fructose and fructose with P promoted significant lower levels of α-AS in the liver of the rats than those of their control counterparts (p<0.01). The hepatic concentration of γ-GS was 0.08 nmol carbonyl/mg protein in C. When fructose solution was ingested, the amounts of the carbonyl were higher than those found in the livers from C rats (p<0.001). However, the supplementation with P in rats drinking fructose promoted a significantly lower production of the semialdehyde in the liver F+P rats than in those from C and F (p<0.01). Considering the PPC as an expression of the sum of both semialdehydes, the results showed that when the rats consumed the fructose solution, there was no significant change in the concentration of PPC between livers respective of the dietary treatment. However, P promoted a significant decrease in the amounts of PPC in the liver from F+P rats as compared to both C and F rats (p<0.001).

Pentosidine is a marker of advanced protein glycation processes. Table 3 reveals that in the livers from C the levels of pentosidine reached 0.61 fluorescent units. Fructose treatment significantly promoted the highest hepatic formation of pentosidine (2.57 fluorescent units) (p<0.001). In the liver from F+P rats, the concentration of pentosidine also suffered a significant increase compared to C, but the levels of pentosidine in F+P livers were lower than those from their F counterparts.

TABLE 3. Concentration of glycoxidative stress markers (mean ± standard deviation) in the liver of *Wistar* rats fed *ad-libitum* for 10 weeks with a control base diet and either drinking water (C), 30% (w/v) fructose water solution (F) or 0.2% (w/v) P in 30% fructose solution (F+P).

	α-AS[a] (nmol carbonyl/mg protein)	γ-GS[b] (nmol carbonyl/mg protein)	PPC[c] (nmol carbonyl/mg protein)	Pentosidine (fluorescence units)
C	0.63a±0.12	0.08b±0.01	0.71a±0.13	0.61c±0.11
F	0.48b±0.07	0.10a±0.02	0.58a±0.07	2.57a±0.40
F+P	0.37b±0.14	0.04c±0.02	0.42b±0.15	1.30b±0.40
p value[e]	**	***	***	***

[a] α-Amino adipic semialdehyde. [b] γ-Glutamic semialdehyde. [c] Total primary protein carbonyls. [e] Significance level in one-way ANOVA test with the effects of diet (C, F and F+P). *: p<0.05; **: p<0.01; ***: p<0.001; ns: no significant. Means with different letters within the same column were significantly different in Tukey post-hoc analysis (p<0.05).

3.4. Fecal microbiota

The fecal microbiome from experimental rats were analyzed to assess the extent to which PU supplementation could revert (or worsen) the already known impact of dietary fructose on the microbiota of *Wistar* rats.[5] Table 4 shows the significant changes in the relative abundance of some microorganisms that were detected at the genus level among the different groups of rats. Compared to C, the microbiome of F was characterized by the significant increase of uncultured *Lachnospiraceae* species and *Adlercreutzia* spp. Instead, the intake of P significantly modified the microbiome of F+P more than the consumption of the fructose solution alone (F), respective to their control counterparts. Thus, the microbiome of F+P (*vs.* C) was characterized by the increase of *Lachnospiraceae NK4A136 group* spp. ($p<0.05$), unclassified *Eggerthellaceae* spp. ($p<0.05$), *Oscillibacter* spp. ($p<0.05$), *Ruminococcaceae* spp. ($p<0.05$), uncultured *Lachnospiraceae* ($p<0.05$), *Barnesiella* spp. ($p<0.05$), *[Eubacterium] ruminantium group* species from *Lachnospiraceae* family ($p<0.001$), *Clostridia vadin BB60 group* spp. ($p<0.01$), *Butyricimonas* spp. ($p<0.05$), *Colidextribacter* spp. ($p<0.05$), uncultured *Ruminococcaceae* ($p<0.01$), uncultured *Flavobacteriaceae* ($p<0.05$) and *Peptococcus* spp. ($p<0.01$). Likewise, P in F-treated rats significantly promoted the decrease of some microorganisms occurred in C, such as *Lactobacillus* spp. ($p<0.01$), uncultured *Oscillospiraceae* spp. ($p<0.05$), *Streptococcus* spp. ($p<0.05$) and *Lactococcus* spp. ($p<0.05$).

Finally, species from the genus *Ruminococcaceae* spp. ($p<0.01$), *[Eubacterium] ruminantium group* species from *Lachnospiraceae* family ($p<0.001$), *Peptococcus* spp. ($p<0.05$) and *Bacillus* spp. ($p<0.01$) showed a significant increase of their relative abundance in the microbiome of F+P as compared to F. Other species such as *Oscillospiraceae UCG-005 group* spp., *Clostridium sensu stricto 1* spp., *Marvinbryantia* spp. and uncultured genus of Coriobacteriales species showed a significant decrease in the microbiota of F+P rats than in the F counterparts ($p<0.05$ respectively).

TABLE 4. Significantly different relative abundances of OTUs at genus level from rats fed with basal diet and either drinking water (C), 30% (w/v) fructose solution (F), or 0.2% (w/v) P in a 30% (w/v) fructose solution (F+P) expressed as color scale that represent the median relative abundance (MRA) of each OTU.

	C (MRA)[a]	F (MRA)[a]	F+P (MRA)[a]	*p* value[b]
Lactobacillus				**
Oscillospiraceae UCG-005				*
Lachnospiraceae NK4A136 group				*
Unclassified *Eggerthellaceae*				*
Oscillibacter				*
Ruminococcaceae				*
Clostridium sensu stricto 1				*
Uncultured *Lachnospiraceae*				**
Barnesiella				*
Uncultured *Oscillospiraceae*				*
[Eubacterium] ruminantium group				***
Clostridia vadinBB60 group				**
Butyricimonas				*
Marvinbryantia				*
Streptococcus				*
Uncultured genus of Coriobacteriales				*
Colidextribacter				*
Lactococcus				*
Uncultured *Ruminococcaceae*				**
Uncultured *Flavobacteriaceae*				*
Peptococcus				*
Bacillus				**
Adlercreutzia				*

[a] Median relative abundance (MRA) (not showed data) with different intensity of brown colour within the same row were significantly different in multiple comparisons post hoc analysis ($p<0.05$): saturated brown: highest MRA; degraded brown: lower MRA; white: no significant group in pair-comparisons.

[b] Significance level in Kruskal-Wallis' test with the effects of water supplement in almost two of the groups: C, F and F+P. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: no significant.

3.5. Non-targeted metabolomics of urine

The Compound Discoverer software paired compounds name and/or formula with the calculated weights of the detected molecules using different databases (i.e., AKos, BioCyc, Chemspace, FooDB, Human Metabolome Database, KEGG, LipidMAPS, Mcule, Nature Chemical Biology, Nature Chemistry, NPAtlas, Toxin, Toxin-Target Database and Urine Metabolome Database). According to the rutinary calibration and optimization of the equipment, as well as our metabolite's extraction method, the identification and characterization (Table S1 of supporting information) of the metabolites belongs to the level 2 of the identification levels proposed by the published metabolomics literature.[20]

In order to analyze the results, the peak intensities of the hepatic metabolites were compared using the Metaboanalyst software (https://www.metaboanalyst.ca/). More than two thousand (2052) metabolites were revealed by the untargeted metabolomic analysis of urine from C, F and F+P *Wistar* rats. The PCA analysis (Figure 6A), clearly positioned urine metabolome of F+P rats on a discriminating cluster where urine metabolomes from C and F were found to be located.

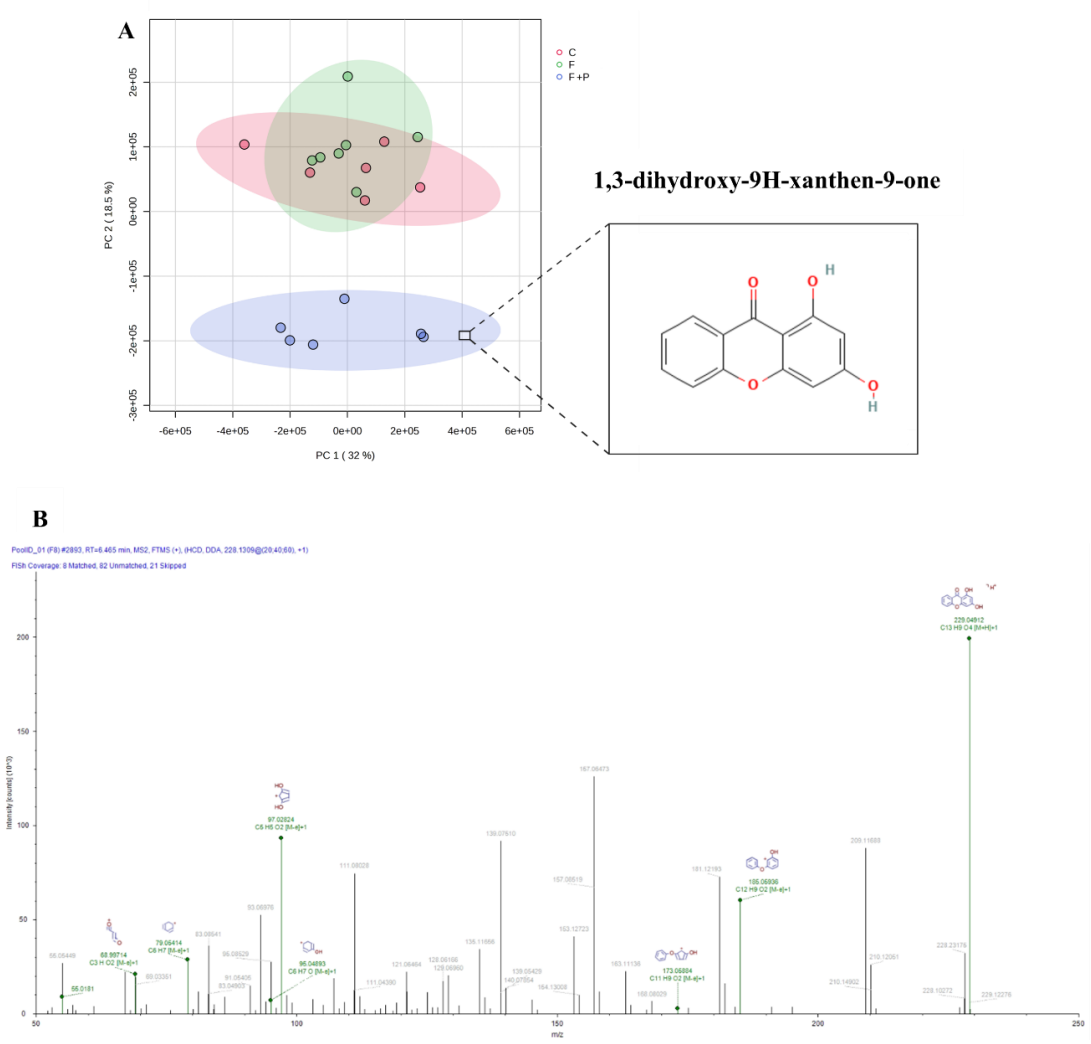

FIGURE 6. Score plot from PCA analysis and one of the main metabolites responsible for the clustering (A), and ion fragmentation pattern of the metabolite (C).

The one-factor ANOVA statistical analysis revealed 214 affected metabolites by the different supplementations in the urine from C, F and F+P animals (ANOVA from supporting information (2)). Compared to C, the urine metabolome of F showed increased amounts of benzamide (p<0.001), indoline (p<0.01) and norleucine (p<0.001), among other metabolites. Meanwhile, some metabolites from F urine, as biotin (p<0.001), coumarin (p<0.001) and tryptophol (p<0.01), were significantly decreased respective to C.

The supplementation with P in F-treated *Wistar* rats promoted significant higher amounts in urine of 1,3-dihydroxy-9H-xanthen-9-one (10^3-fold higher) (p<0.001) and uric acid (p<0.01) than in urine from C rats. On the other hand, some metabolites showed lower quantities in the urine from F+P

than their control counterparts, such as biotin or coumarin ($p<0.001$ respectively).

Likewise, the urine metabolome of F+P (*vs.* F) highlighted higher abundance of 1,3-dihydroxy-9H-xanthen-9-one ($p<0.001$), dihydrouridine ($p<0.01$) and tryptophol ($p<0.01$), among others. Meanwhile, the presence of P in F-treated rats led to the decrease of some urine metabolites found in F, i.e., benzamide ($p<0.001$), indoline ($p<0.01$), norleucine ($p<0.001$) and uric acid ($p<0.01$).

4. DISCUSSION

4.1. Fructose intake, dyslipidosis, obesity and liver steatosis

There is extensive literature that relates an excessive fructose consumption with the onset of several dysmetabolic features related to lipid and energy metabolism dysfunctions, such as obesity, hypertension, hypertriglyceridemia, T2DM, insulin resistance, hyperuricemia, visceral adiposity or NAFLD, which are included in the spectrum of pathological conditions ascribed to the MetS.[3,21,22] Intestinal and hepatic fructolysis could *per se* promote increased triose-phosphates pools in the liver, common to both glycolytic and gluconeogenesis processes, as well as to pathways related to lipid metabolism.[3] This metabolic pathway appears to be the most remarkable molecular mechanism by which fructose promote disturbances on energetic metabolism. Moreover, the pass of fructose through the gastrointestinal tract and disturbance caused to the microbiota, could have also contributed to the above-mentioned pathophysiological effects, while this latter microbiome-mediated mechanism may be much scarcely understood.

The impaired lipid metabolism associated to fructose consumption could have triggered an increase of adiposity. The distribution of fat between the different locations seems to be more important than the total adipose tissues for the risk of developing obesity-associated diseases.[23] The role of WAT is to store excess dietary fat in the form of triglycerides (TGs) and to release free fatty acids (FFAs) in times of starvation or energy demand. As a source of energy substrate, WAT responds to variations in the nutritional status and energy demand of the body.[24] WAT is found in both SAT and VAT. While SAT is associated with improved or preserved insulin sensitivity, mitigated risk of developing type II diabetes and other metabolic derangements, VAT is associated with an increased risk of insulin resistance and dyslipidemias. It is an independent risk factor for T2DM, hypertension and all-cause mortality [14]. Meanwhile, BAT, as thermogenic adipose tissue that dissipate energy as heat, is associated with improvements in circulating triglycerides and insulin sensitivity, among

others beneficial effects.[25]

Based on a previous review,[14] we grouped the fat tissues from the experimental animals into WAT, which englobed SAT and VAT, and BAT depending on their body location. Even though several assays related fructose supplementation with increased body weight,[26–28] our results suggested that the high fructose intake did not affect differently the body weight of the animals at the end of the experiment (Figure 1), in line with other researchers.[29,30] However, the results showed a significant increase of all fat depots, apparently due to the high fructose intake (Figure 2), which also increased the total energy consumption of F (Table 1).

Similar studies concluded that fructose consumption increased different fat depots, despite the differences in the experimental designs of the supplementation (12% and 20% of fructose, respectively) and the analyzed fat tissues (BAT was not included).[29,31] The knowledge about the impact of fructose intake on BAT is scarce. The increase of the BAT weight showed in our experiment could be related to beneficial outputs, but the increase of BAT did not necessarily implied an increase of its activity, which is just what several researchers highlighted in their works.[32,33] The authors described that fructose intake could impair the potential of the tissue on glucose uptake and thermogenesis, which was related to fructose-induced metabolism.

Ectopic lipid accumulation is promoted by nutritional imbalances.[34] Altered lipid metabolism might also implied increased amounts of infiltrated fat in the liver. Early studies in this field established the existence of a form of non-alcohol related chronic hepatitis characterized by fatty infiltration on the liver with inflammation, cellular injury evident by inflammation, cellular ballooning and fibrosis.[35] Our H&E-stained histopathological images allowed us to see that, certainly, the hepatic tissues of F had higher amounts and bigger vesicle cells than C, as well as higher amounts of hepatic fat content, as markers of lipidosis (Figure 3B). Our results are in line with what is already described in the literature.[2,26,30,33,36] Some of the authors related >5% of the hepatic fat content analyzed by H&E stain as expression of NAFLD with high fructose consumption, due to the activation of lipogenic pathway and the absence of a regulatory mechanism of fructose metabolism, as fructolysis is an insulin-independent process.[33] Moreover, they suggested that the activation of lipogenic pathways are time and dose dependent, but others factors could explain why other researchers did not see the lipogenic effect in their experiment,[37] who related the lack of the expected results with a degree of stress experienced by the animals. Thus, although the hepatic fat

content of the livers from F was lower than 5%, our results suggest an increasing fat deposition on the livers of rats that were supplemented with 30% fructose in drinking water for 10 weeks. It is important to note that fatty livers, as a benign state of liver disease, are more vulnerable to injury from some causes increasing the probably to progress to later stages of the disease.[30]

Circulating TG, LDL and total cholesterol in blood stream are increased by intestinal and hepatic fructolysis as an expression of altered lipid metabolism, which will eventually lead to insulin resistance and hyperglycemia.[26,38] Surprisingly, our results revealed a non-significant increased trend of the levels of the markers in the plasma from F compared to C. However, when the concentration of HDL was considered in LDL/HDL ratio, the marker reached the significantly highest levels in the plasma from F, in agreement with the knowledge of the role of fructose on the overproduction of hepatic LDL *vs.* the underproduction of HDL.[38]

4.2. Plasma markers of fructose damage

Regarding plasma markers, the two main proteins in the blood are albumin and globulin. Albumins are the most abundant proteins in the blood plasma and transport substances like hormones, fatty acid and drugs, being in humans the primary transport of fatty acids, bilirubin and other plasma compounds.[39] Moreover, albumin plays a role in the maintenance of redox state of the blood. Meanwhile, globulin proteins are made by the immune system and indicate the state of the immune function and the severity of any inflammation. The biochemical profile of plasma from F rats showed increased amounts of albumins and non-significant changes in the concentration of GLOB, so the ALB/GLOB ratio were statistically higher in the plasma from F than those from C (Table 2). The raise on the plasma protein levels could be related to its role on fatty acid transport, as the other results of the experiment highlighted an impaired hepatic lipid metabolism on F rats. Likewise, high albumin levels could be caused by a physiological response related to an antioxidant defense. Moreover, these results are in line with those of other authors who analyzed several serum markers of *Wistar* albino rats after two different supplementations of fructose in drinking water, and concluded that the intake of fructose promoted increased amounts of albumin in the animals' serum.[40] Plasma ALB/GLOB ratio is used to clinically diagnose impairments of liver and kidney functions, and decreased amounts of ALB is associated with inflammation and advanced states of liver injury.[41] So, we would conclude that despite of the changes of the marker's concentration, there was not an indicative of a consolidated induced severe liver damage but might be an indicative

of impaired lipid metabolism, increased glycoxidative stress and an early stage of lipid steatosis. In fact, the liver damage caused by fructose consumption was clearly noted. The most remarkable change in the plasma of F rats was the increase of the concentration of GOT (also known as AST), which is used in combination with other enzymes like ALT to monitoring the course of liver disorders. The increased levels of GOT in the plasma of F rats in the biochemical analysis distinctively illustrates a liver damage which is manifested as release of liver enzymes Table 2).[42]

4.3. Immunological response to fructose consumption

Fructose has been profusely studied in relation to the mechanisms involved in glycoxidative stress for its ability to generate both reactive oxygen species (ROS) and reactive carbonyl species (RCS) during metabolism. *In vivo* experiments of high fructose consumption have reported AGEs accumulation in different tissues in association with peripheral insulin resistance and lipid metabolism alterations.[43]

In a previous work, our group revealed for the first time an increased accumulation of advanced protein oxidation products (APOPs) on gastrointestinal tissues after fructose digestion for a plausible previous *in situ* glycation of proteins in the lumen.[5] In the current one we found that the consumption of high amounts of fructose also increased the accretion of pentosidine on hepatic tissue (Table 3), in line with other assays that revealed a hepatic increase of AGEs as expression of raise on glycoxidative stress in this organ.[38,44] The role of PPC as precursors of AGEs could explain the lack of significant changes in the concentration of these marker in the livers from F rats compared to the C counterparts. The clearance of fructose in the liver would promote glycoxidative stress, which may contribute to intracellular damage to DNA and proinflammatory responses. Formation of AGEs can trigger inflammatory pathways through the activation of some signaling pathways independently of caloric intake and weight gain.[45]

It has been demonstrated that fructose-induced metabolic impairments are closely related to inflammation, characterized by increased inflammation signaling activation in organs such as the liver.[46] The development of NAFLD in mice chronically fed a fructose diet was associated with increased endotoxin levels in portal blood for a decrease in tight junction proteins in the upper part of small intestine, which would have activated inflammatory responses.[30,47] In line with this, the microscopic observation of the liver from experimental animals showed significantly higher

infiltration of immunological cells (CD45+, macrophages, and lymphocytes) in F animals. Consistently, additional pathological microscopic signs of liver damage such of apoptosis, necrosis, edema, microtubule distension, etc. were found more often in liver from F rats than in C rats (Figure 4), illustrating the connection between fructose intake, glyco-oxidative stress, AGEs accumulation, liver damage and inflammation.

4.4. Positive outputs from punicalagin-rich commercial pomegranate-extract consumption

To our knowledge, the present study highlights for the first time the potential beneficial effects of a pomegranate commercial extract supplementation on the pathophysiological impairments associated with high fructose consumption. Considered as functional food, pomegranate (*Punica granatum*) is one of the oldest fruits involved in a growing number of studies that analyze the bioactive properties of pomegranate polyphenols, such as PU and ellagic acid EA, that have been extensive studied in the last years for their functional activities.[8–10,48]

Moreover, pomegranate is used in the prevention and treatment of MetS in recent decades, and the effect of pomegranate on lipid metabolism related to atherosclerosis, T2DM and NAFLD, as well as on oxidative status, was described in detail in an interesting review.[49] This paper brings attention on PU and EA as the main actors involved in the bioactivities displayed by this fruit.

Thus, the consumption of P in a fructose solution appears to decrease the adipogenic effect of high fructose intake, significantly diminishing the mean weight of the adipose tissues from F+P compared to those from F, i.e., BAT, WAT, SAT, VAT and total fat depots (Figure 2). Moreover, the images from H&E showed an improvement F+P hepatic tissues (Figure 3C), which in fact contained lower content of fat than its F counterpart. Moreover, the biochemical markers ALB/GLOB, LDL/HDL, or GOT was also stabilized near to control values in the plasma from F+P.

Some studies have shown that pomegranate extract contains large amounts of bioactive compounds that exhibit strong antioxidant and anti-inflammatory properties.[49,50] In fact, the hepatic markers of glycoxidative stress showed decreased amounts of both PPC and pentosidine after the supplementation of fructose solution with P (Table 3). Moreover, the diminished counts displayed by hepatic immunological CD45+ cells, as well as macrophages and lymphocytes, apparently for P consumption (Figure 5B), appears to support the beneficial effect of PU on glycoxidative stress and immunological response described. These results are in line with other authors that showed an

antioxidant effect and diminished amounts of human serum cytokines after the consumption of ellagic acid-rich pomegranate fruit extract tablets.[9]

The present results suggest that PU contained in P exerted a hepatoprotective effect. Based on the literature that related other antioxidants compounds with an impaired function of both GLUT5 and GLUT2,[2,13,26] we believe that PU-rich extract used in the experiment might interfere with the intestinal clearance and uptake and consequently hepatic fructose clearance of fructose, diminishing the negative effects attributed to its intestinal and hepatic metabolism.

However, these would mean that more abundance of fructose could reach the colon. In fact, when we analyzed the microbiota of F+P compared to C and F, the results suggested an increase of the abundance of microorganisms related to the negative effects of high fructose consumption, as *Ruminococcaceae* spp., uncultured *Lachnospiraceae* spp. or *Clostridium senso stricto 1* spp., as well as diminished amounts of some beneficial bacteria such as *Lactobacillus* spp., *Lactococcus* spp. or *Streptococcus* spp.[5,51,52] Nevertheless, the beneficial effect of the main polyphenols of pomegranate, i.e., PU and EA, are attributed precisely to their interaction with the microbiota, involving species of microorganisms from the genus *Gordonibacter* or *Eggerthellaceae* as responsible for conversion of the polyphenols into urolithins.[11,53] The occurrence of increased amounts of unclassified *Eggerthellaceae* spp. displayed by the microbiome of F+P rats suggested that in addition to the plausible hepatoprotective effect of PU by its interaction with GLUTs, its interaction with the microbiota might be behind the improvement of the impaired metabolic markers promoted by the fructose consumption. Urolithins could have a huge positive impact on health as an antioxidant and as a gut barrier function enhancer,[12,54] which effectively could be improving in our study, highlighted by the decrease of glycoxidative stress markers in the liver and the diminished inflammatory and immunological responses (Table 3 and Figures 6B, respectively).

In addition, the urine metabolome of F+P showed some changes in the abundance of several metabolites that could be indicators of both the presence and effect of urolithins. It is well known that upon urolithins production, they are accumulate in plasma and urine as glucuronide conjugates.[18]

With the purpose of identifying any potential pomegranate/PU-derived metabolites in the biological samples of P-treated animals, we identified 1,3-dihydroxy-9H-xanthen-9-one, as one highly

discriminating candidate between urine metabolomics from F+P, F and C. In urine of the former group of animals, this metabolite was found in 10^3-fold higher abundance than in the urine metabolome of F and C. This metabolite has a urolithin-compatible chemical structure and a plausible similar bioactivity based on the present results. This xanthone is, to our knowledge, the first time to be identified in the urine of a mammal provided with pomegranate.

With a growing interest in this metabolite, which shares formula and molecular weight with urolithin A, we reported the full characterization of the compound driven by the Compound Discoverer software (Table S1 of supporting information). The ion fragmentation pattern is showed in Figure 6B. Moreover, based on the UV spectrum generated in a HPLC-DAD assay, we found close similarities between an urolithin-compatible compound and a positively identified urolithin-A (URO-A) in the urine from F+P rats, even though the ion fragmentation pattern displayed by the metabolomic assay was not identical to that reported to URO-A.[18] Significant differences were found when compared the 3 experimental group of animals since the urolithin candidate were not even present in the urine from C and F groups (Figure S1 of supporting information).

Moreover, the metabolomic analysis revealed lower abundance of uric acid in the urine from F+P compared to F. Fructose promote uric acid overproduction,[22] but these impairment might be alleviate in the present study for the antihyperuricemic properties attributed to urolithins.[55] On the other hand, fructose, which is implicated in the glycoxidation of proteins that could reach the colon, was found to be responsible of the formation of undesirable microbial metabolites in the colonic environment.[5] One of the most concerning issues is related to the altered metabolism of tryptophan, which was showed in a higher abundancy in the colonic metabolome of fructose-consumer rats compared to a control group.[5] Tryptophan microbial metabolism results in different noxious indolic metabolites.[56] The urine metabolome of F rats showed higher amounts of indoline than their F+P counterparts, which exhibited the lowest amounts of the compound. Instead, this last one group exhibited higher amounts of tryptophol than F, with this tryptophan-related metabolite being regarded as a compound with antioxidant properties and was not present in the urine metabolome of F.[57]

The current experiment is part of a larger investigation on the metabolic impairments caused by fructose consumption and the plausible counteracting effects of a punicalagin-rich pomegranate commercial supplement. Excessive intake of fructose promoted the increase of fat depots, hepatic

fat content and glycoxidative stress, as well as inflammatory and immunological response in *Wistar* rats that consumed 30% fructose in drinking water for 10 weeks. Following the recommendation to reduce sugar intake to less than 5% of the total energy intake seems to be reasonable.[1] Yet, certain dietary components such as the punicalagin-rich pomegranate supplement tested in the present study, could be a suitable supportive complement to pharmacological treatments aimed to control obesity, liver steatosis and MetS. This pomegranate supplement has been shown to have a hepatoprotective effect on *Wistar* rats challenged with fructose. Several of the physiological mechanisms may involve interaction with intestinal transport of fructose and modification of the microbiota that may result in the formation of bioactive compounds such as urolithin-A and other urolithin-type compounds that should require further attention as the current knowledge on their formation and bioactivities is unknown.

Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

E., M. Funding acquisition, project administration, conceptualization, investigation, methodology, supervision, data analysis, validation and writing─review and editing.

S.-T., G.: Investigation, methodology, data analysis, validation and writing─original draft.

M., R.: Investigation, methodology, supervision, data analysis, validation and writing─review and editing.

M., D.: Supervision, data analysis, validation and writing─review and editing.

C., V.: Investigation, methodology, data analysis, validation and writing─rewiew and editing.

All authors made critical revisions to the manuscript for key intellectual content and read and approved the final manuscript.

Supporting information

Supporting information is available from the Wiley Online Library or from the author.

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References

[1] WHO, *World Heal. Organ*. **2018**, 57, 1716–1722.

[2] B. Merino, C. M. Fernández-Díaz, I. Cózar-Castellano, G. Perdomo, *Nutrients* **2020**, 12, 1–35.

[3] M. A. Herman, M. J. Birnbaum, *Cell Metab*. **2021**, 33, 2329–2354.

[4] C. Jang, S. Hui, W. Lu, A. J. Cowan, R. J. Morscher, G. Lee, W. Liu, G. J. Tesz, M. J. Birnbaum, J. D. Rabinowitz, *Cell Metab*. **2018**, 27, 351-361.e3.

[5] G. Sánchez-Terrón, R. Martínez, J. Ruiz, C. Luna, M. Estévez, **2023**, DOI 10.1021/acs.jafc.3c04515.

[6] S. Iametti, F. Bonomi, M. Di Nunzio, *Int. J. Mol. Sci*. **2022**, 23, DOI 10.3390/ijms232214355.

[7] S. A. Hannou, D. E. Haslam, N. M. McKeown, M. A. Herman, *J. Clin. Invest*. **2018**, 128, 545–555.

[8] M. Noori, B. Jafari, A. Hekmatdoost, *J. Sci. Food Agric*. **2017**, 97, 2327–2332.

[9] S. Jafarirad, R. Goodarzi, N. Mohammadtaghvaei, M. Dastoorpoor, P. Alavinejad, *Diabetes Metab. Syndr. Clin. Res. Rev*. **2023**, 17, 102693.

[10] V. Caballero, M. Estévez, F. A. Tomás-Barberán, D. Morcuende, I. Martín, J. Delgado, *J. Agric. Food Chem*. **2022**, 70, 16273–16285.

[11] Y. Yin, R. Martínez, W. Zang, M. Estévez, **2023**, DOI https://doi.org/10.1080/10408398.2023.2219763.

[12] F. A. Tomás-Barberán, A. González-Sarrías, R. García-Villalba, M. A. Núñez-Sánchez, M. V. Selma, M. T. García-Conesa, J. C. Espín, *Mol. Nutr. Food Res.* **2017**, 61, DOI 10.1002/mnfr.201500901.

[13] C. Olvera-Sandoval, H. E. Fabela-Illescas, E. Fernández-Martínez, M. A. Ortiz-Rodríguez, R. Cariño-Cortés, J. A. Ariza-Ortega, J. C. Hernández-González, D. Olivo, C. Valadez-Vega, H. Belefant-Miller, G. Betanzos-Cabrera, *Antioxidants* **2022**, 11, 1–13.

[14] D. E. Chusyd, D. Wang, D. M. Huffman, T. R. Nagy, *Front. Nutr.* **2016**, 3, DOI 10.3389/fnut.2016.00010.

[15] M. Utrera, D. Morcuende, J. G. Rodríguez-Carpena, M. Estévez, *Meat Sci.* **2011**, 89, 500–506.

[16] M. J. Sánchez-Quintero, J. Delgado, D. Medina-Vera, V. M. Becerra-Muñoz, M. I. Queipo-Ortuño, M. Estévez, I. Plaza-Andrades, J. Rodríguez-Capitán, P. L. Sánchez, M. G. Crespo-Leiro, M. F. Jiménez-Navarro, F. J. Pavón-Morón, *Nutrients* **2022,** 14, 4650.

[17] W. J. Bligh, E.G. and Dyer, Can. *J. Biochem. Physiol*. **1959**, 37.

[18] R. García-Villalba, J. C. Espín, F. A. Tomás-Barberán, *J. Chromatogr. A* **2016**, 1428, 162–175.

[19] H. Wickham, *Elegant Graphics for Data Analysis: Ggplot2*, **2008**.

[20] L. W. Sumner, A. Amberg, D. Barrett, M. H. Beale, R. Beger, C. A. Daykin, T. W. M. Fan, O. Fiehn, R. Goodacre, J. L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. N. Lane, J. C. Lindon, P. Marriott, A. W. Nicholls, M. D. Reily, J. J. Thaden, M. R. Viant, *Metabolomics* **2007**, 3, 211–221.

[21] S. Softic, K. L. Stanhope, J. Boucher, S. Divanovic, M. A. Lanaspa, R. J. Johnson, C. R. Kahn, *Crit. Rev. Clin. Lab. Sci*. **2020**, 57, 308–322.

[22] A. Miller, K. Adeli, *Curr. Opin. Gastroenterol*. **2008**, 24, 204–209.

[23] B. Bjørndal, L. Burri, V. Staalesen, J. Skorve, R. K. Berge, *J. Obes*. 2**011**, DOI 10.1155/2011/490650.

[24] A. Wronska, Z. Kmiec, *Acta Physiol*. **2012**, 205, 194–208.

[25] T. Becher, S. Palanisamy, D. J. Kramer, M. Eljalby, S. J. Marx, A. G. Wibmer, S. D. Butler, C. S. Jiang, R. Vaughan, H. Schöder, A. Mark, P. Cohen, *Nat. Med*. **2021**, 27, 58–65.

[26] S. Mary Varghese, J. Thomas, **2019**, DOI 10.5772/intechopen.82200.

[27] M. E. Bocarsly, E. S. Powell, N. M. Avena, B. G. Hoebel, *Pharmacol. Biochem. Behav.* **2010**, 97, 101–106.

[28] G. A. Bray, *Curr. Opin. Lipidol*. **2010**, 21, 51–57.

[29] H. Jürgens, W. Haass, T. R. Castañeda, A. Schürmann, C. Koebnick, F. Dombrowski, B. Otto, A. R. Nawrocki, P. E. Scherer, J. Spranger, M. Ristow, H. G. Joost, P. J. Havel, M. H. Tschöp, *Obes. Res.* **2005**, 13, 1145–1156.

[30] C. Sellmann, J. Priebs, M. Landmann, C. Degen, A. J. Engstler, C. J. Jin, S. Gärttner, A. Spruss, O. Huber, I. Bergheim, *J. Nutr. Biochem*. **2015**, 26, 1183–1192.

[31] R. V. Sandeva, S. M. Mihaylova, G. N. Sandeva, K. Y. Trifonova, R. D. Popova-Katsarova, *J. Biomed. Clin. Res*. **2015**, 8, 5–8.

[32] G. Richard, D. P. Blondin, S. A. Syed, L. Rossi, M. E. Fontes, M. Fortin, S. Phoenix, F. Frisch, S. Dubreuil, B. Guérin, É. E. Turcotte, M. Lepage, M. G. Surette, J. D. Schertzer, G. R. Steinberg, K. M. Morrison, A. C. Carpentier, *Cell Reports Med.* **2022**, 3, DOI 10.1016/j.xcrm.2022.100742.

[33] M. J. Meneses, I. Sousa-Lima, I. Jarak, J. F. Raposo, M. G. Alves, M. P. Macedo, *Front. Endocrinol. (Lausanne).* **2022**, 13, 1–16.

[34] A. Bosy-Westphal, W. Braun, V. Albrecht, M. J. Müller, *Eur. J. Clin. Nutr*. **2019**, 73, 209–214.

[35] J. Ludwig, T. R. Viggiano, D. B. McGill, B. J. Oh, *Mayo Clin. Proc*. **1980**, 55, 434—438.

[36] S. Zhao, C. Jang, J. Liu, K. Uehara, M. Gilbert, L. Izzo, X. Zeng, S. Trefely, S. Fernandez, A. Carrer, K. D. Miller, Z. T. Schug, N. W. Snyder, T. P. Gade, P. M. Titchenell, J. D. Rabinowitz, K. E. Wellen, *Nature* **2020**, 579, 586–591.

[37] M. Janevski, S. Ratnayake, S. Siljanovski, M. A. McGlynn, D. Cameron-Smith, P. Lewandowski, *Food Funct*. **2012**, 3, 141–149.

[38] C. C. Hsieh, C. C. Liao, Y. C. Liao, L. S. Hwang, L. Y. Wu, S. C. Hsieh, *J. Food Drug Anal*. **2016**, 24, 754–761.

[39] M. Paar, V. H. Fengler, D. J. Rosenberg, A. Krebs, R. E. Stauber, K. Oettl, M. Hammel, *Commun. Biol*. **2021**, 4, 1–9.

[40] R. Rajesh, V. Sreekala, Natl. *J. Physiol. Pharm. Pharmacol*. **20**20, 11, 1.

[41] J. A. Thompson, K. Krajnak, R. A. Johnston, M. L. Kashon, W. McKinney, J. S. Fedan, *Toxicol. Reports* **2022**, 9, 12–21.

[42] E. G. Giannini, R. Testa, V. Savarino, *C. Can. Med. Assoc. J.* **2005**, 172, 367–379.

[43] M. Aragno, R. Mastrocola, *Nutrients* **2017**, 9, 1–16.

[44] R. Mastrocola, D. Nigro, F. Chiazza, C. Medana, F. Dal Bello, G. Boccuzzi, M. Collino, M. Aragno, *Free Radic. Biol. Med*. **2016**, 91, 224–235.

[45] P. Muriel, P. López-sánchez, E. Ramos-tovar, *Int. J. Mol. Sci*. **2021**, 22, DOI 10.3390/ijms22136969.

[46] D.-M. Zhang, R.-Q. Jiao, L.-D. Kong, *Nutrients* **2017**, 9.

[47] G. Kanuri, R. Ladurner, J. Skibovskaya, A. Spruss, A. Königsrainer, S. C. Bischoff, I. Bergheim, *Liver Int*. **2015**, 35, 562–568.

[48] E. Yanpar, M. Yildirim, M. Akkapulu, U. Değirmenci, S. Könen Adigüzel, S. Yalin, A. E. Yalin, *J. Res. Pharm*. **2021**, 25, 600–608.

[49] C. Hou, W. Zhang, J. Li, L. Du, O. Lv, S. Zhao, J. Li, *Mol. Nutr. Food Res*. **2019**, 63, 1–12.

[50] A. Mandal, D. Bhatia, A. Bishayee, *Nutrients* **2017**, 9, 1–13.

[51] J. Lambertz, S. Weiskirchen, S. Landert, R. Weiskirchen, *Front. Immunol*. **2017**, 8, DOI 10.3389/fimmu.2017.01159.

[52] Y. E. Cho, D. K. Kim, W. Seo, B. Gao, S. H. Yoo, B. J. Song, *Hepatology* **2021**, 73, 2180–2195.

[53] M. W. Zhang, S. M. Cui, B. Y. Mao, Q. X. Zhang, J. X. Zhao, H. Zhang, X. Tang, W. Chen, *Crit. Rev. Food Sci. Nutr*. **2023**, 63, 6900–6922.

[54] R. Singh, S. Chandrashekharappa, S. R. Bodduluri, B. V. Baby, B. Hegde, N. G. Kotla, A. A. Hiwale, T. Saiyed, P. Patel, M. Vijay-Kumar, M. G. I. Langille, G. M. Douglas, X. Cheng, E. C. Rouchka, S. J. Waigel, G. W. Dryden, H. Alatassi, H. G. Zhang, B. Haribabu, P. K. Vemula, V. R. Jala, *Nat. Commun*. **2019**, 10, DOI 10.1038/s41467-018-07859-7.

[55] S. I. Adachi, K. Sasaki, S. Kondo, W. Komatsu, F. Yoshizawa, H. Isoda, K. Yagasaki,

Molecules **2020**, 25, 1–15.

[56] J. de J. Rodríguez-Romero, A. C. Durán-Castañeda, A. P. Cárdenas-Castro, J. A. Sánchez-Burgos, V. M. Zamora-Gasga, G. S. Sáyago-Ayerdi, *Food Chem. X* **2022**, 13, DOI 10.1016/j.fochx.2021.100195.

[57] M. Schirmer, S. P. Smeekens, H. Vlamakis, M. Jaeger, M. Oosting, E. A. Franzosa, T. Jansen, L. Jacobs, M. J. Bonder, A. Kurilshikov, J. Fu, L. A. B. Joosten, A. Zhernakova, C. Huttenhower, C. Wijmenga, M. G. Netea, R. J. Xavier, *Cell* **2016**, 167, 1125-1136.e8.

Capítulo IV

Molecular Mechanisms Underlying the Protective Effects of Pomegranate Supplementation against Fructose-Induced Liver Damage: The Mitochondria in the Eye of the Storm

Mecanismos moleculares que subyacen el efecto protector de la suplementación con granada sobre el daño hepático inducido por fructosa: la mitocondria en el ojo de la tormenta

Molecular Mechanisms Underlying the Protective Effects of Pomegranate Supplementation against Fructose-Induced Liver Damage: The Mitochondria in the Eye of the Storm

Guadalupe Sánchez-Terrón, Remigio Martínez, Josué Delgado, Javier Molina, & Mario Estévez

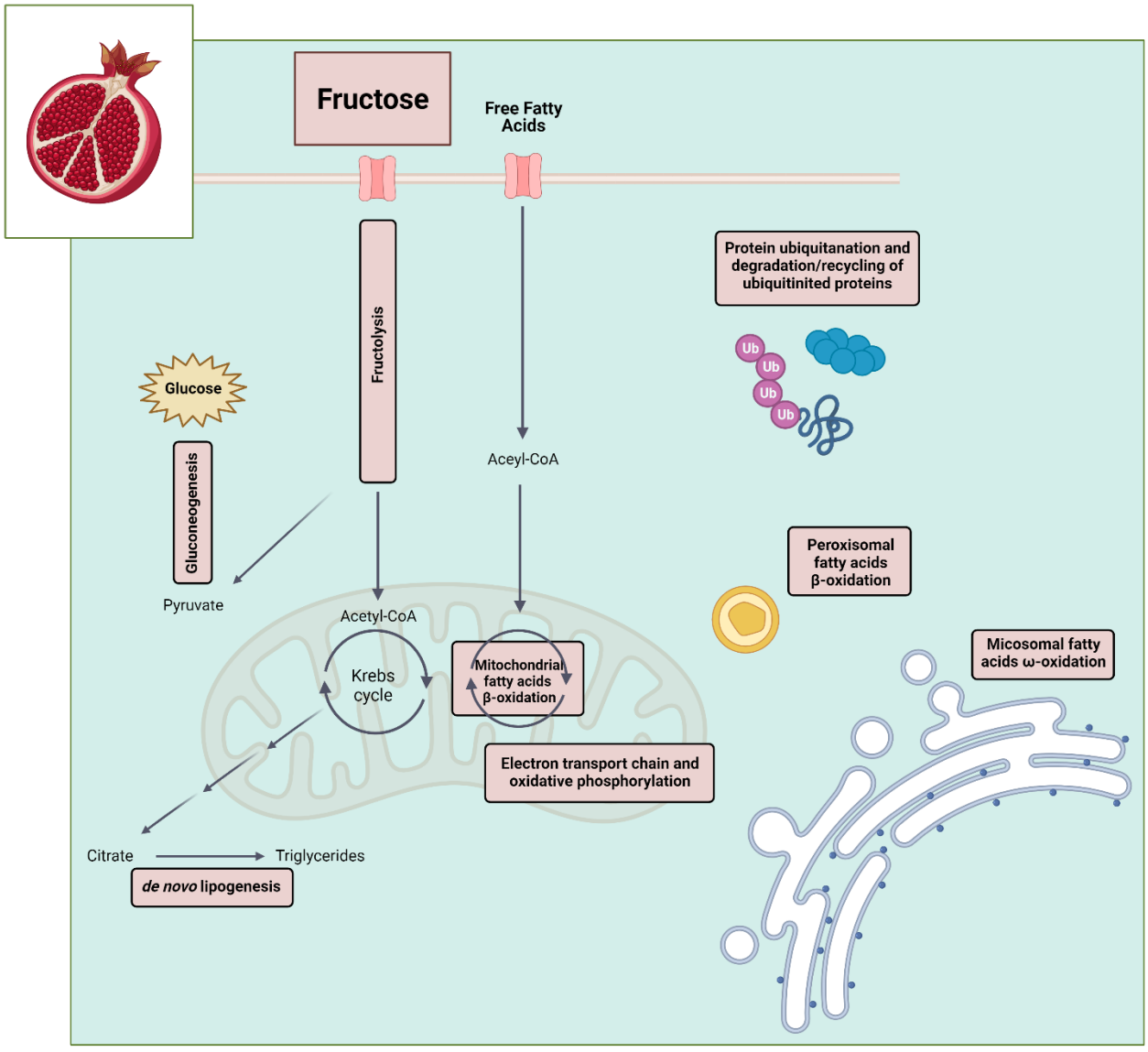

Figura *4.3.* Resumen gráfico del Capítulo II. Elaboración propia.

Molecular Mechanisms Underlying the Protective Effects of Pomegranate Supplementation against Fructose-Induced Liver Damage: The Mitochondria in the Eye of the Storm

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ABSTRACT

Despite evidence of a direct association between fructose consumption and the onset and progression of non-alcoholic fatty liver disease (NAFLD), the molecular mechanisms underlying this relationship are not fully defined. Given the high prevalence of this metabolic impairment, along with other conditions grouped under the metabolic syndrome (MetS), unravelling these mechanisms is of great importance for adopting potential nutritional strategies. Thus, an evaluation of the impact of sustained (10 weeks) and high fructose consumption (9 g fructose/kg live body weight/day) on the liver physiology of *Wistar* rats was made by studying the proteome of hepatocytes. Moreover, the effectiveness of a dietary punicalagin (PU)-rich pomegranate supplement (P) at counteracting fructose-induced liver injury was also assessed (18.71 mg/PU/kg live body weight/day). With the aim of unveiling the mechanisms underlying these effects, an untargeted proteomic analysis of the livers from nineteen *Wistar* rats fed on a basal commercial feed and supplemented with either drinking water (C), 30% (w/v) fructose in drinking water (F) or 30% (w/v) fructose solution plus 0.2% (w/v) P (F+P) was assessed. Proteomic analysis indicates that fructose administration severely affected peroxisomal and mitochondrial β-oxidation of fatty acids while promoting the hallmark features of fatty liver and MetS, namely, increased gluconeogenesis and *de novo* lipogenesis. Moreover, fructose intake caused a significant depletion of key proteins involved in the mitochondrial electron chain which could facilitate severe mitochondrial disturbance and oxidative stress. The P extract counteracted the aforementioned pathophysiological mechanisms by improving mitochondrial homeostasis and strengthening endogenous antioxidant mechanisms. These protective molecular mechanisms were manifested in lower oxidation rates and alleviated liver injury.

Keywords: fructose, liver, NAFLD, proteomics, punicalagin, pomegranate.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; MetS, metabolic syndrome; PU, punicalagin; P, punicalagin-rich commercial pomegranate dietary supplement; T2D, type 2 diabetes *mellitus*; GIT, gastrointestinal tract.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the studied manifestations of the metabolic syndrome (MetS), which involves a broad of metabolic impairments such as obesity, type 2 diabetes mellitus (T2DM) or dyslipidaemia, among others (Dharmalingam & Yamasandhi, 2018). The onset of fatty liver has been closely related for decades to obesity and excessive consumption of fat, but in recent years researchers linked the onset and progression of the disease with an excessive intake of dietetic fructose for their capacity to promote some of the mentioned MetS-related physiological impairments (Jensen et al., 2018).

The social context around increasing fructose consumption has triggered the current interest in this reducing sugar and its potential noxious pathophysiological effects. Fructose is clearance in the small intestine as it passes through the gastrointestinal tract (GIT) (Jang et al., 2018). When high amounts of fructose are consumed (~ 9 g fructose/kg live body weight/day), the capacity of the intestinal metabolic mechanisms appears to be overwhelmed, and certain amounts of fructose would reach both the liver and the colon, promoting a wide range of impairments that our group has tried to define in previous works and is summarized in Figure 1 (Sánchez-Terrón et al., 2023, *submitted*). Both, intestinal and hepatic fructolysis share the same metabolic routes, which start with the phosphorylation of fructose in fructose-1-phosphate (F1P), which is catalysed by a ketohexokinase (KHK), also known as fructokinase. KHK-mediated fructose phosphorylation appeared to be ATP-dependent and is essential for fructose-induced metabolic diseases (Herman & Birnbaum, 2021). F1P is then cleaved to dihydroxyacetone phosphate (DHAP) and glyceraldehyde by aldolase B (ALDOB). Glyceraldehyde requires an additional step to enter the glycolytic/gluconeogenesis carbon pools, a phosphorylation by triokinase (TKFC) to form glyceraldehyde 3-phosphate (G3P). DHAP and G3P are recognized pools that serve as substrates for several metabolic pathways related to energy generation and would be the main responsible for the effects of fructose on lipid homeostasis. In fact, fructose promotes *de novo* lipogenesis (DNL) in the liver and increased circulating triglyceride (TG), which are recognized as the main contributors to fat accumulation associated with obesity, NAFLD, and insulin resistance (Hannou et al., 2018; Lambert et al., 2014; Merino et al., 2020). Moreover, the excessive consumption of fructose appears to enhance an abnormal glucose flux through F1P as a signalling molecule of several molecular mechanisms.

Inhibited fatty acid oxidation is described as another driver of insulin resistance and NAFLD since changes in several enzymes triggered by excessive fructose consumption would lead to diminished antioxidant mechanisms and hence, higher susceptibility to oxidative stress and degeneration (Herman & Birnbaum, 2021; Merino et al., 2020).

Punicalagin (PU) is a pomegranate-derived antioxidant compound from the ellagitannins family which shows a remarkable beneficial activity particularly when specific microbiota species biotransform this polyphenol into the bioactive urolithins (Yin et al., 2023). The dietary supplementation with pomegranate has been long associated with health benefits, since the compounds naturally present in the product (*i.e.*, ellagic acid) could reduce inflammatory processes, act as hepatoprotective compounds, prevent carcinogenesis, and alleviate diabetes and oxidative stress, in fact, considered a potential promotor of multiple diseases such as the previously mentioned (Adachi et al., 2020; Mandal et al., 2017; Olvera-Sandoval et al., 2022; Yanpar et al., 2021). Yet, in the last few years, the discovery of biotransformation pathways of PU and ellagic acid into urolithins has increased the interest in these species as modulators of oxidative stress and their therapeutic effects (Caballero et al., 2022; Tow et al., 2022). In a previous study, we reported the anti-obesity and hepatoprotective effects of a PU-rich pomegranate supplement in *Wistar* rats subjected to a sustained consumption of fructose (Sánchez-Terrón et al., *submitted*). Unlike control rats, animals treated with the pomegranate supplement excreted urolithins, which reveals that such microbial metabolites were being formed, absorbed, and distributed in rats with such beneficial health effects. The mechanisms involved in the beneficial effects of punicalagin, and their microbial metabolites are not well defined yet, but several studies related their beneficial effects with both cellular detoxification and DNA repair mechanisms, as well as anti-inflammatory activity (Mandal et al., 2017; Tomás-Barberán et al., 2017; Zahin et al., 2014). The hepatoprotective effect of urolithins has also been reported in the literature (Tow et al., 2022), but the underlying molecular mechanisms of this protection require a more profound investigation.

Our goal in this research is to decipher the molecular mechanisms implicated in the ability of a PU-rich pomegranate supplement (P) to counteract the liver damage caused by a sustained consumption of fructose in *Wistar* rats. To fulfil this objective, we applied label-free MS-based proteomics to liver samples from *Wistar* rats supplemented with either drinking water (C), 30% fructose solution (F), or 0.2% P on a 30% fructose solution (F+P) for 10 weeks.

MATERIALS AND METHODS

Chemicals

All reagents, chemicals, and standard compounds were obtained from *Sigma Chemicals (*Sigma Aldrich*,* Stheinheim, Germany), *Fisher* (Fisher Scientific S.L., Madrid, Spain), and *Panreac* (Panreac Química, S.A., Barcelona, Spain). Ultrapure water was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

In vivo experimental assay

All the issues related to the animals, feed, and experimental design, as well as slaughter, necropsy, and sampling have been approved by the competent authorities according to Spanish legal requirements (RD 53/2013), bioethics committee of University of Extremadura (137-2020) and by the Board of Extremadura (EXP20200904). Briefly, nineteen male *Wistar* breed rats of the *Rattus novegicus* species were randomly allocated into one of these three experimental groups after a 1-week adaptation period: i) control group (C), which were fed basal feed and drinking water during the assay (n=6), ii) fructose group (F), whose drinking water was replaced by 30% (w/v) fructose solution (n=7); and iii) fructose and P group (F+P), in which drinking water was replaced by 30% (w/v) fructose solution with 0.2% (w/v) pomegranate supplement with 183 mg of punicalagin (PU) per pill (n=6) (Caballero et al., 2022).

The body weight of the animals was registered weekly, and the energy intake during the assay were calculated based on the gravimetrically measured consumption of food and water/solutions. At the end of the 10-week experiment, animals were euthanized. Blood collection and fatty tissues were properly sampled and stored according to the proposed experimental assays. The liver of the animals was readily dissected and dispensed in a suitable container and stored immediately at -80ºC until the proteomic analysis. On average, F rats consumed 9 g fructose/kg live body weight/day. Moreover, P animals ingested an average of 18.71 mg/PU/kg live body weight/day.

Label-free MS-based proteomics

Firstly, 500 µl of lysis buffer pH 7.5 [Tris-HCl 100 mM (Sigma Aldrich, USA), NaCl 50 mM, EDTA 50 mM (Sigma Aldrich), glycerol 10% (v/v) (Fisher Scientific), phenylmethylsulfonyl fluoride (PMSF) 1 mM (Sigma Aldrich) and 1 µg/ml of pepstatin A (Sigma Aldrich)] were added to 100 mg

of liver tissues before homogenization in a mixer mill (Retsch MM400) during 6 minutes at 30 Hz. Lysates were sonicated with a probe sonication in a Branson Sonifier^{TM} 250 (Emerson, Spain) and incubated in ice for at least 1 hour. Then, we centrifugated the lysates to remove unnecessary cell debris. Protein concentration was measured by spectrophotometry using a Nanodrop 2000c Spectophotometer and Nanodrop 2000 software (USA). Aliquots with 50 µg of proteins were run in an SDS-PAGE to be excised and in gel digested as previously described by Díaz-Velasco et al., (2022). The bands were subjected to a process of dithiothreitol-mediated reduction (Promega, USA) and alkylation with iodoacetoamide (Promega). Then, the trimmed bands were digested with sequencing-grade trypsin (Promega) and ProteaseMAX surfactant (Promega) for 1 hour at 50ºC. One µl of formic acid was added to stop the proteolysis and supernatant was removed from each sample after sonication in a water bath and placed into new screw-capped Eppendorf tubs for drying in a vacuum concentrator.

A total of 1 µg from each digest were analysed using a Q-Exactive Plus (Thermo Scientific, Germany) coupled to a Dionex Ultimate 3000 RSLCnano (Thermo Scientific), after previous reconstitution of the dried pellets with loading buffer (98% milliQ water, 2% acetonitrile, 0.05% trifluoroacetic acid). Data was collected using a Top15 method for MS/MS scans (Delgado et al., 2019). Label-free comparative proteome abundance and data analysis were performed using MaxQuant software (version 1.6.0.15.0) with Perseus (v. 1.6.14.0) applied to organize the data and conduct statistical analysis (Cox & Mann, 2008). Database searching was performed against *Rattus norvegicus* protein database downloaded from Uniprot (https://www.uniprot.org/). The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The label-free quantitative algorithm (LFQ) was used to generate normalised spectral intensities and infer relative protein abundance (Luber et al., 2010). Proteins that matched to a contaminant database or the reverse database were removed, and proteins were only retained in the final analysis if they were detected in at least three replicates from at least one treatment. Quantitative analysis was performed using a *t*-test to compare treatments with the control. *p* values less than 0.05 was considered statistically significant. Qualitative analysis was also performed to detect proteins found in at least two replicates of a particular treatment but undetectable in the compared treatment. Box plots were generated using ggplot2 from R statistical software (R 4.2.3.) (Wickham, 2008).

RESULTS AND DISCUSSION

Effect of high fructose consumption on the hepatic proteome of Wistar rats

A total of 1430 proteins were identified by LFQ algorithm in the liver tissues of C and F rats. Quantitative (p<0.05) and qualitative (only detected in one condition) changes in protein abundance were identified (Table S1 of supporting information). Thus, 370 proteins were significantly affected by fructose administration as compared to the C group of animals, among which, 115 were found in higher quantity in the liver of F rats, while 19 were only found in the livers of the same animals. Conversely, 255 proteins were detected in lower abundance in the livers of F rats, while 25 were only found in the livers of C rats. All these proteins were identified as discriminating proteins. In the following subsections, discriminating proteins involved in key metabolic pathways of liver function are grouped and discussed in detail (Table 1).

Boosted fructolysis and promoted gluconeogenesis

Table 1 shows the changes in the abundance of the most relevant discriminating hepatic proteins involved in fructolysis that were affected by fructose supplementation. KHK (Fold change: 1.38) is the main protein responsible for the hepatic phosphorylation of fructose to fructose-1-phosphate (F1P), and its abundance in the liver from F rats significantly increased, as expected. The increasing abundance of this hepatic enzyme would be a straightforward manifestation of higher amounts of fructose reaching the liver. In a previous study, we reported increased fructose delivery to the colon of *Wistar* rats supplemented with 30% fructose in drinking water (Sánchez-Terrón et al., 2023). Taken together, these results strongly suggest that dietary fructose concentration appeared to exceed the physiological clearance capacity of the intestine, which is in agreement with other authors who reported a similar effect (Jang et al., 2018).

Whereas KHK is constitutively active, hepatic glucokinase (GCK), the leading hexokinase in hepatocytes, is sequestered in the nucleus in an inhibited state by glucokinase regulatory protein (GCKR; Fold change: 1.34), limiting the net hepatic glucose uptake and hepatic glucose clearance (Herman & Birnbaum, 2021). The abundance of GCKR in the liver of F rats was increased when high amounts of fructose were consumed. Moreover, glycogen phosphorylase (PYGL; Fold change: 1.27), which was also found in higher quantities in the hepatic proteome of F rats as compared to

that from the C counterparts, inhibits glycogen synthesis and remains stable in the interaction between GCK and GCKR, maintaining GCK in an inhibitory state. PYGL is essential to disrupt this interaction and enhance both glucose uptake and glycogen synthesis. However, these results are not in agreement with those reported in the comprehensive review carried out by Herman & Birnbaum (2021), who, based on original works, noted that F1P would inhibit the expression of both GCKR and PYGL and would promote glycogen synthesis and glucose uptake. It is worth highlighting that articles revised by the aforementioned authors inferred the results from basic spectrophotometric analysis of certain enzymes which would explain the divergence with the present results. It is, in fact, reasonable to hypothesize that in a situation of massive fructose uptake, liver mechanisms implicated in the uptake of glucose would be inactivated.

The next step of fructolysis involves the cleavage of the KHK-phosphorylated F1P to dihydroxyacetone phosphate (DHAP) and glyceraldehyde by fructose-biphosphate aldolase B (ALDOB; Fold change 1.23). The abundance of ALDOB was likewise increased in the hepatic proteome of F rats, which is consistent with the extensive body of literature that has attributed this effect to excessive fructose intake (Hannou et al., 2018; Herman & Samuel, 2016; Merino et al., 2020). A plausible higher dissociation rate of F1P by ALDOB could explain why the inhibitory effect of F1P on GCKR and PYGL reported by Herman & Birnbaum (2021) seemed not to occur in our experiment.

Glyceraldehyde requires an additional phosphorylation to be converted into glyceraldehyde 3-phosphate (G3P), which together with DHAP enter the glycolytic/gluconeogenic carbon pools. This additional phosphorylation is carried out by the protein triokinase/FMN cyclase (TFKC; Fold change: 1.61), which quantity was also increased in the livers of *Wistar* rats exposed to F. TFKC stimulates the phosphorylation of G3P to produce pyruvate and acetyl CoA and this metabolic feature has been linked to lipid dysregulation (Muriel et al., 2021). It is worth highlighting that F animals from the present study showed dyslipidaemia and predisposition to both obesity and fatty liver (Sánchez-Terrón et al., *submitted*). The comparative proteomic analysis of liver tissues also revealed an increase in the abundance of pyruvate kinase (PKLR, Fold change: 1.94) because of the fructose supplementation, which confirms that the key downstream enzymatic machinery involved in fructolysis seemed to be upregulated in the F rats.

Fructolysis, glycolysis, and gluconeogenesis utilize common enzymes, and an elevated abundance of certain proteins among them could potentially lead to a misinterpretation of the metabolic pathways altered by fructose consumption. For instance, several proteins that showed increased amounts in the hepatic proteome of F rats, such as glucose 6-phosphate isomerase (GPI; Fold change: 1.69), glyceraldehyde-3-phosphate dehydrogenase (GADPH; Fold change: 1.21), or alpha-enolase (ENO1; Fold change: 1.30) may mean that despite the increased fructolysis, hepatic glycolysis was being also promoted in F rats. However, the higher amounts of both hepatic pyruvate carboxylase (PC; Fold change: 1.34) and fructose-1,6-bisphosphate 1 (FBP1; Fold change: 1.14), which are also required for fructose metabolism and tolerance (Bai et al., 2023), could be related to increased rates of gluconeogenesis in the liver cells of F rats. These results are consistent with other authors who reported increased rates of liver gluconeogenesis in experimental animals exposed to elevated dietary fructose, such as Hsieh et al. (2016) or Rajasekar & Anuradha (2007), who exposed both Sprague-Dawley (SD) and *Wistar* rats to a 60% fructose solid supplementation for 16 and 4 weeks respectively.

De novo lipogenesis (DNL)

DNL is an extensively studied process associated with high fructose intake since fructolysis promotes increased pyruvate pools in a similar manner to glycolysis, which would ultimately act as a precursor of *de novo* synthesis of fatty acids in the liver (Softic et al., 2016; Ter Horst & Serlie, 2017). Indeed, our previous results revealed increased hepatic fat content and fat depots in rats exposed to 30% fructose in drinking water for 10 weeks (Figure 1).

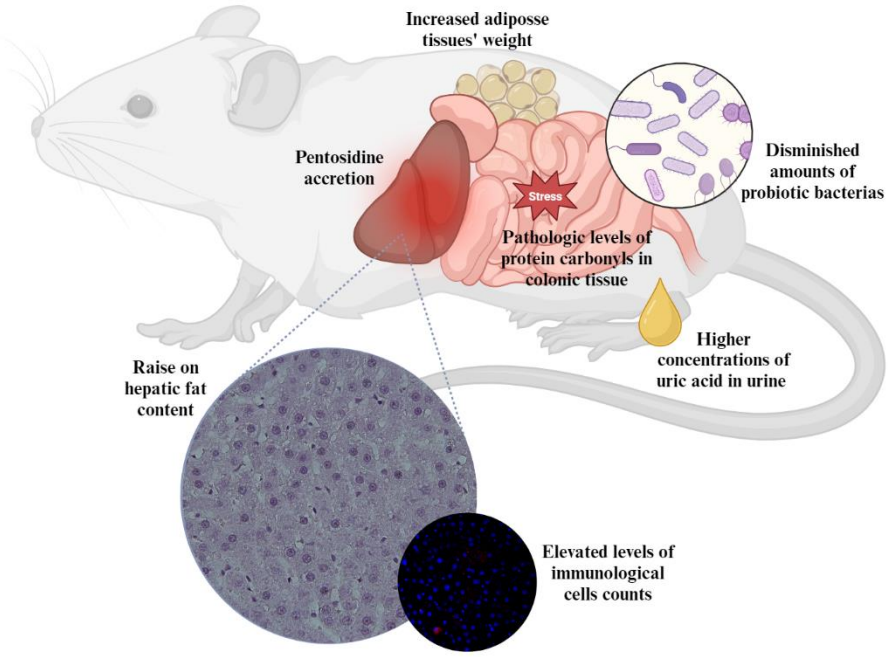

FIGURE 1. Overview of the most relevant results highlighted by our group in previous studies (Sánchez-Terrón et al., 2023 and Sánchez-Terrón et al., submitted).

Pyruvate is converted into acetyl-CoA, serving as a substrate for ATP production within the mitochondria. This metabolic pathway also leads to the production of citrate, which is subsequently utilised in the cytosolic synthesis of lipids. The enzyme responsible for pyruvate cleavage is the pyruvate dehydrogenase complex, composed of several subunits, which were found to be increased in the hepatic proteome of F rats. The specific components and subunits increased were the subunit beta and alpha of the mitochondrial pyruvate dehydrogenase E1 (PDHB; Fold change: 1.37; and PDHA1; Fold change: 1.19, respectively), as well as the mitochondrial dihydrolipoyllysine-residue acetyltransferase (component of pyruvate dehydrogenase complex) (DLAT; Fold change: 1.81). Likewise, the mitochondrial dihydrolipoyl dehydrogenase (DLD; Fold change: 1.09), which also showed elevated amounts in our hepatic results after fructose supplementation, is another protein required for the complete reaction of the above-mentioned complex. These results are in accordance with those reported by Janssens et al. (2017) and Hsieh et al. (2016), who observed an elevation in the expression of units from pyruvate dehydrogenase complex in the livers of rats subjected to high fructose consumption.

Moreover, the concentration of ATP citrate synthase (ACLY; Fold change: 1.77), a key enzyme connecting carbohydrate and lipid metabolism by converting mitochondrial citrate to cytosolic acetyl-CoA for fatty acid and cholesterol biosynthesis (Muriel et al., 2021), was significantly elevated in F rats as compared to C-counterparts. While ACLY is regarded as a crucial enzyme in hepatic fatty acid synthesis, the promotion of DNL due to increased fructose intake has been described as an ACLY-independent process (Zhao et al., 2020). However, in line with our results, other authors have shown increased expression of the enzyme following 30% (w/v) fructose supplementation for 10 weeks (Softic et al., 2017). Moreover, the same authors demonstrated that the expression of the protein decreased when using KHK-knockout mice, along with the expression of FASN (Softic et al., 2017). The second step in DNL involves the carboxylation of acetyl-CoA to generate malonyl-CoA, which is used by the protein fatty acid synthase (FASN; Fold change: 2.96) to generate palmitate. FASN exhibited one of the most significant increases in abundance in the livers of F rats. Whereas the connection between fructose consumption and the stimulation of enzymes involved in DNL has been already reported (Hsieh et al., 2016; Mastrocola et al., 2018), this study originally provides semi-quantitative results of the increase in abundance of this pro-adipogenic enzyme in livers from *Wistar* rats exposed to a chronic consumption of fructose. This finding provides relevant insight into the mechanisms behind the liver lipidosis observed in these rats and described in a previous study (Sánchez-Terrón et al., *submitted*). On the same line, the overabundance of proteins associated with both energy and lipid metabolism strengthens the association between fructose consumption and accelerated metabolism in hepatocytes. This, in turn, may result in an increase in oxidative stress and liver damage, as previously reported (Figure 1).

Peroxisomal, mitochondrial and microsomal fatty acid oxidation

Despite the well-established understanding that a decreased hepatic fatty acid oxidation process would contribute to hepatic dyslipidaemia in the context of excessive fructose intake (Herman & Birnbaum, 2021; Muriel et al., 2021), there remains a lack of knowledge about the underlying mechanisms governing this impairment.

There are several cellular organelles responsible for fatty acid β-oxidation. Thus, mitochondria catalyse the oxidation of long, medium, and short-chain fatty acids such as palmitate, oleate or linoleate, whereas peroxisomes are involved in the oxidation of very long-chain fatty acids, serving

as precursors for bile acids, prostaglandins, leukotrienes and polyunsaturated fatty acids (Singh, 1997). Conversely, fatty acids ω-oxidation, which occurs in the endoplasmic reticulum, operates when mitochondria do not oxidize median-chain fatty acids. This process involves the activity of a crucial family of enzymes known as cytochrome P450 (CYP), which are essential to the liver by playing pivotal roles in both lipid metabolism and detoxification processes (Berthier et al., 2021). An operational and paired mitochondria-peroxisome activity is essential in hepatocytes for maintaining an appropriate lipid metabolism homeostasis in the liver (Islam et al., 2020).

According to Miura (2013) or Novelle et al. (2021), in a scenario in which the synthesis of fatty acid is significantly increased, such as in the livers of the F rats in this study (FASN; Fold change: 2.96), the abundance of carnitine palmitoyl transferase 1A (CPT1A) is inhibited. These authors found a correlation between decreased expression of hepatic CPT1A with decreased fatty acid oxidation associated with high fructose diets. Consistently, in the liver proteome from F rats, the abundance of CPT1A was significantly reduced as compared to the proteome from livers in C rats (CPT1A; Fold change: 0.82). Furthermore, the cited studies established a link between the activation of alternative pathways for fatty acid oxidation when hepatic fructose metabolism is presumably regulated. CPT1A is an essential protein for the translocation of fatty acids into the mitochondria (McGarry, 2002), and reduced levels of this enzyme may imply a diminished flux of fatty acids into the mitochondria for β-oxidation and might contribute to the development of steatosis (Herman & Birnbaum, 2021). This mechanism could plausibly explain the accumulation of lipid deposits in the liver from F rats as the chronic flux of fructose to the liver may compromise the oxidation of fatty acids in the mitochondria and hence, the onset of steatohepatitis.

In this study, the quantities of certain proteins associated with mitochondrial fatty acid β-oxidation, such as (family member 11) acyl-CoA dehydrogenase (ACAD11; Fold change: 0.80) and the mitochondrial hydroxyacyl-coenzyme A dehydrogenase (HADH; Fold change: 1.14) displayed an unclear trend. On one hand, ACAD11, that catalyses the first step of mitochondrial fatty acid oxidation, exhibited decreased amounts in the proteome of F livers, while HADH, implicated in the final step of the process, showed higher levels in the same samples. These results suggest an imbalance in the amounts and functions of mitochondrial proteins involved in the β-oxidation of fatty acids. In fact, an impairment in the concentration (and function) of a set of mitochondrial

proteins could play a key role in the pathogenesis associated with fructose consumption, as discussed below.

On the other hand, decreased quantities of proteins involved in peroxisomal fatty acid β-oxidation were found in the liver of F rats. Thus, the synthesis of acyl-CoA oxidases 1, 2 and 3 (ACOX1, ACOX2 and ACOX3: Fold change: 0.83, 0.80 and 0.74, respectively) and 3-ketoacyl-CoA thiolases A and B (ACAA1A and ACAA1B; Fold change: 0.87) was significantly downregulated in the livers from F rats compared to those from the C counterparts. ACOX1, ACOX2 and ACOX3 encode enzymes that play pivotal roles in the first step of the peroxisomal fatty acid β-oxidation, while ACAA1 and ACAA2 play important functions catalysing the last steps of peroxisomal fatty acid β-oxidation (Berthier et al., 2021; Singh, 1997). Both CPT1A and ACOX are involved in the rate-limiting steps of mitochondrial and peroxisomal β-oxidation, respectively. According to the scientific reported evidence (Islam et al., 2020), a peroxisomal dysfunction would be a molecular hallmark in the pathogenesis of non-alcoholic steatohepatitis. The results from the present study indicate that chronic fructose consumption may facilitate the onset of this condition by triggering this molecular mechanism. Yet, other authors such as Janevski et al. (2012), did not observe changes in the expression of these proteins in the livers from SD rats fed a diet containing 60% fructose for 4 weeks. In contrast, they did report an increased expression of genes of proteins closely involved in fat synthesis such as *FASN*, which partly concurs with our results. Besides the differences in the methodological approach (transcriptomics *vs.* proteomics), other divergences in terms of dose and time of exposure would explain that animals from both studies were at different stages of a potential NAFLD.

Furthermore, our results revealed a significant and highly meaningful decrease in the abundance of numerous proteins from the CYP family in the livers of F rats, including CYP2A1, CYP2C6, CYP2C23, CYP2C70, CYP2D26, CYP2E1, CYP3A2, CYP4F1 and CYP4F4. The literature in this field is controversial regarding the relationship between the expression of genes encoding CYP proteins and the development of NAFLD. Whereas some studies indicate that ω-fatty acid oxidation catalysed by enzymes such as CYP2E1, CYP4A10, or CYP4A14 contributes to lipid-induced cellular damage in NAFLD through the formation of ROS (Browning & Horton, 2004), other studies state that ω-oxidation of fatty acids represents a compensatory pathway for the management of fatty acid oxidation disorders, and particularly when mitochondrial/peroxisomal β-oxidation pathways

are impaired (Novelle et al., 2021). For instance, Li et al. (2013) observed an elevated expression of *CYP2A5* in hepatocytes from a C57BL/6J mice model suffering from a dietary-induced NAFLD. Conversely, Fisher et al. (2009) reported diverging results when analysed explanting hepatic tissue from adult humans. They found that microsomal *CYP1A2*, *CYP2D6*, and *CYP2E1* mRNA levels decreased with the progression of NAFLD, while *CYP2A6, CYP2B6*, and *CYP2C9* gene expression increased.

From a global perspective, the level of proteins associated with fatty acid oxidation was found to be diminished in the hepatic proteome of our fructose-consuming experimental subjects, in line with established knowledge. Nonetheless, to the best of our knowledge, this is the first time that the level of the CYP enzyme family has been associated with the consumption of high levels (30 % in drinking water) of fructose in the context of the development of NAFLD. This association may bear implications for the capacity of the cell to execute detoxification mechanisms in response to the increased oxidative stress generated by all the enhanced fructose-induced mechanisms.

Mitochondrial dysfunction and oxidative stress

In addition to the decrease in mitochondrial proteins involved in the β-oxidation of fatty acids in livers from F rats, the sustained consumption of fructose also led to disruptions in the quantities of other key hepatic mitochondrial enzymes participating in the electron transport chain and oxidative phosphorylation. The β-subunit of the electron transfer flavoprotein (ETFB; Fold change: 1.19) was found in significantly higher quantities in the liver proteome of F rats than that in C, which could mean that the electron transfer to the main mitochondrial respiratory chain in the liver may be increased due to the fructose supplementation. The elevated concentration of other enzymes implicated in mitochondrial energy supply such as the C2 subunit of NADH dehydrogenase [ubiquinone] 1 (NDUFC2; Fold change: 1.34), the subunit 8 of the cytochrome b-c1 complex (UQCRQ; Fold change: 1.59) and γ-subunit of the mitochondrial ATP synthase, (ATP5C1; Fold change; 1.15) would support this hypothesis. NDUFC2, UQCRQ, and ATP5C1 are subunits of the NADH dehydrogenase complex (Complex I), cytochrome b-c1 complex (Complex III), and ATP synthase complex (Complex V), respectively. These results are consistent with the observations made by other authors who previously noted that fructose increases the substrate for oxidative phosphorylation (reviewed by Hannou et al., 2018). Since fructolysis is an ATP-dependent process

(Herman & Birnbaum, 2021), enzymes involved in energy generation are expected to be increased in subjects exposed to sustained fructose consumption. Cioffi et al. (2017) associated hepatic damage in SD rats on a high fructose diet (20%/8 weeks) and mitochondrial DNA damage owing to the pro-oxidative environment caused by fructose occurrence and degradation and the concomitant depletion of antioxidant defences. In contrast, the results also suggested decreased amounts of proteins that are also part of the electron transport chain, *i.e.*, ubiquinone biosynthesis protein COQ9, mitochondrial (COQ9; Fold change: 0.69) and cytochrome c oxidase subunits 5A and 6A1, mitochondrial (COX5A and COX6A1; Fold changes: 0.73 and 0.72, respectively). COQ9 is a protein involved in the biosynthesis of coenzyme Q (ubiquinone), an essential lipid-soluble electro-transporter for aerobic cellular respiration. Likewise, COX5A and COX6A1 are indispensable subunits from the cytochrome c oxidase complex (Complex IV). An inefficient energetic metabolism in mitochondria is known to lead to oxidative stress and liver damage. Consistently, in a previous study, we found significant levels of glycoxidative stress in the livers of F rats than in the C counterparts (Figure 1).

It is plausible that under adverse physiological circumstances, an inherent cellular response to restore homeostasis, mitochondrial proteins, and functions may experience disruption. The abovementioned observations consequently suggest an elevation of ROS levels in the liver from F rats, which subsequently might have led to increased mitochondrial oxidative stress. Oxidative stress has been linked by far with the onset of several hepatic disorders, such as NAFLD (Ajaz et al., 2021), among others.

Depletion of antioxidant defences

Even though fructose stimulated an increase in the abundance of glutathione synthetase (GSS; Fold change: 1.45), an enzyme responsible for glutathione production and essential for protecting against oxidative damage, the proteome of livers from F rats displayed an overall decreased concentration of hepatic proteins involved in the antioxidant defences of hepatocytes. Consequently, proteins such as peroxiredoxin-1 (PRDX1; Fold change: 0.82), which plays a role in cell protection against oxidative stress; or glutathione peroxidase (GPX1; Fold change: 0.43), involved in glutathione metabolism, exhibited significantly reduced abundance in livers from F rats. Furthermore, different subunits of glutathione S-transferase (A6, alpha-3, kappa-1, Mu-2, theta-2, theta-3, and P) (GST;

Fold changes: 0.67, 0.77, 0.81, 0.77, 0.79, 0.48, 0.83, respectively), which are involved in detoxification process using glutathione as substrate, all showed decreased levels in the livers of F rats. Studies about the expression of both PRDX1 and GST in livers under certain pathological conditions are inconclusive. Our results are, in fact, inconsistent with those reported by Hsieh et al. (2016), who observed an increase in the expression of PRDX1 and α-3 glutathione S-transferase in SD rats fed a diet containing 60% fructose for 10 weeks. However, it is overall generally accepted that sustained fructose consumption leads to decreased antioxidant defences in hepatocytes. Cioffi et al. (2017) revealed that hepatic catalase content experienced a decrease when SD rats consumed a 20% fructose diet for 8 weeks.

Based on the available evidence, it is now well-established that oxidative stress plays a role in various pathological processes, including atherosclerosis, neurodegenerative diseases, and cancer (Matsuda & Shimomura, 2013). Moreover, the onset of MetS, closely associated with sustained consumption of fructose and other reducing sugars, is associated with oxidative and carbonyl stress, which in turn induces increased expression of antioxidant proteins. The depletion of proteins with antioxidant-related properties in a chronic condition featured by elevated concentration of pro-oxidative species (such as fructose and their degradation products) could potentially contribute to triggering the initial stages of liver damage, as indicated by our previous results.

Restorative impact of punicalagin-rich extract supplementation

In order to analyse how the addition of 0.2% P to 30% fructose solution affected the negative outcomes related to fructose consumption, we conducted a previous study examining the fat depots, hepatic fat contents, glycoxidative stress markers, and blood markers of liver injury and immunological response in rats supplemented with either drinking water (C), 30% fructose solution (F) or 0.2% P along with 30% fructose solution (F+P). Our preliminary findings indicated the ability of PU-rich pomegranate extract to counteract the fructose-induced physiological impairments, with a reduction of the weight of fat depots, hepatic fat content, and reduced levels of markers of liver injury (Sánchez-Terrón et al., *submitted*). To gain further knowledge on the molecular basis underlying the effects of the fruit-derived bioactive compounds, we compared the hepatic proteome of rats supplemented with P (F+P) with that of livers from F rats.

A total of 1479 proteins were identified by the LFQ algorithm in groups F or F+P. Quantitative (p<0.05) and qualitative (only detected in one experimental group) changes in protein abundance were identified (Table S2 of supporting information). Three hundred and seventy proteins were significantly affected by F+P treatment as compared to their F counterparts. Among those, 100 proteins were detected in higher quantity in the livers from F+P, while 51 were only found in the livers from F+P. Conversely, 48 were found in lower abundance in F+P livers, while 9 were only found in the livers of F rats. In the following sections, the most relevant biological functions and metabolic pathways influenced by the addition of P to the diet of *Wistar* rats exposed to sustained fructose intake, are discussed.

Reduced fructose metabolism in liver

The incorporation of P in the diet of F rats counteracted the effect of dietary fructose increasing the concentration of several proteins involved in both fructolysis and lipid metabolism, such as KHK, TKFC, FBP1, PKLR, FASN, or ACLY (Table 1). While the differences in these proteins were non-significant, the trend in reducing fructose-related metabolic enzymes seems to be biologically relevant as P might have changed the mechanisms with which hepatic cells would have dealt with elevated fructose concentration. A decreased hepatic first-pass fructose metabolism could have been induced by PU-rich pomegranate extract.

Fructose metabolism via KHK is an essential pathway for the onset of fructose-induced metabolic diseases (Herman & Birnbaum, 2021). Through our previous findings (Sánchez-Terrón et al., *submitted*) we learnt that P consumption decreased both intra- and extra-cavitary lipid depots in *Wistar* rats. The fat content of the livers as well as the hepatic histopathological images, revealed the hepatoprotective effects of PU-rich pomegranate in animals suffering from fructose-induced lipid deposition, which included, reduced lipid content in the liver, reduced inflammation and reduced glycoxidative stress (Sánchez-Terrón et al., *submitted*). Our current proteomic study suggests that lower amounts of fructose may be reaching the liver when P was consumed due to the diminished amounts of the main protein involved in its clearance. This would have potential consequences on the associated metabolic pathways since the proteins previously described as fructose-affected proteins showed a trend similar to that exhibited by C after the treatment with P. Thus, it could be considered that P consumption may decrease the hepatic processes of fructolysis,

gluconeogenesis, and *de novo* lipogenesis. This might account for the reduction in uric acid levels observed in the urine of F+P rats in our previous study (Sánchez-Terrón et al., *submitted*). The decreased amounts of proteins involved in the above-mentioned pathways could reduce the formation of intermediate products such as GA3P. GA3P is involved in purine metabolism, and decreased amounts could similarly lead to diminished levels of proteins associated with uric acid production when high amounts of fructose were supplemented with P. In fact, this may explain the anti-hyperuricemic effect linked to urolithins by other researchers (Adachi et al., 2020).

On the other hand, the pomegranate supplement might have a role in improving the oxidation of fatty acids. The most remarkable change in this regard was the increased amounts of CYP displayed by the hepatic proteome of F+P as compared to F rats. These results are in line with findings reported by Novelle et al. (2021), which suggested that the inhibition of a hepatic fructose transporter may be linked to improved fructose-induced damage as a consequence of the stimulation of ω-fatty acid oxidation by the CYP family of enzymes, serving as an alternative to the conventional hepatic fat oxidation.

Improved mitochondrial dynamics

Compared to the C rats, the mitochondrial proteins from hepatocytes were altered when high concentrations of fructose were consumed. Figure 2 shows the main changes promoted by P in the abundance of several fructose-altered mitochondrial proteins.

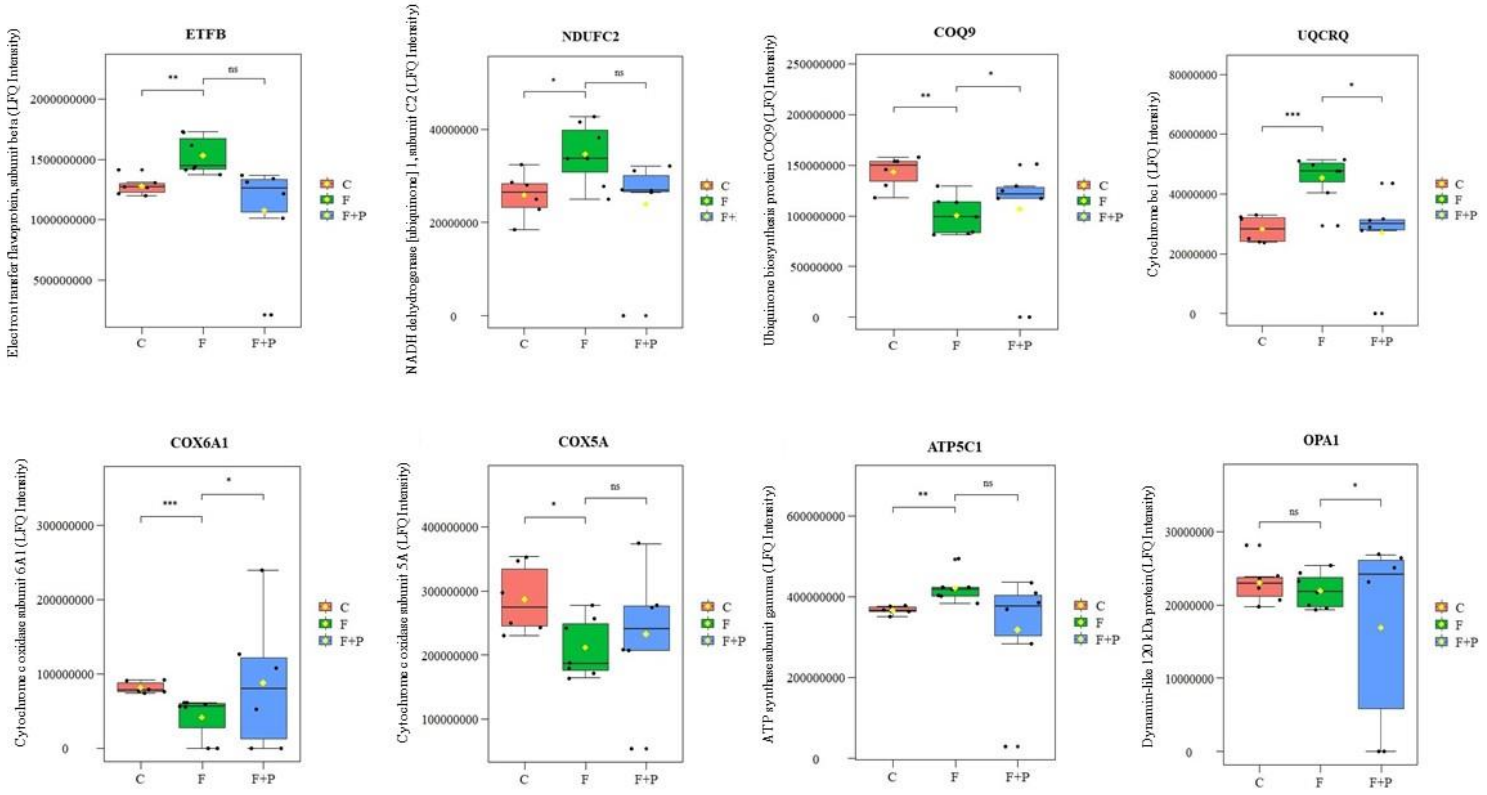

FIGURE 2. Differential abundance from the main mitochondrial proteins affected by the different supplementations: drinking water (C), 30% (w/v) fructose in drinking water (F) and 0.2% (w/v) P and 30% (w/v) fructose solution (F+P).

The simultaneous intake of P along with fructose decreased the abundance of UQCRQ in the liver of F+P rats with respect to F. Moreover, the levels of COX6A1 and COQ9 were increased in the livers of F+P rats. These effects were expected given the influence of P on the quantities of proteins involved in ATP-dependent metabolic pathways described above.

Surprisingly, fructose consumption appeared to alter the abundance of proteins implicated in normal mitochondrial physiology. The mitochondrial dynamin-like 120 kDa protein, [OPA1; Fold change: 1.16 (F+P *vs.* F)] is an essential protein involved in mitochondrial fusion located in the inner membrane of the mitochondria. The abundance of this protein showed a significant increase in the hepatic proteome of F+P rats compared to F counterparts, which is a clear counterbalance of the effect of F in the liver of experimental animals. The potential role of fructose inducing mitochondrial dysfunctions through the boost of oxidative stress was reported in some tissues by several authors, such as García-Berumen et al. (2019) or Jaiswal et al. (2015). Mamikutty et al. (2015) noted mitochondrial structural alteration coinciding with the onset of NAFLD in a similar assay, although we did not observe hepatic steatosis symptoms at higher fructose concentration used (30% *vs.* 20%) in a larger cohort and over an additional two-week duration. The mitochondrial structural impairments were studied through the changes accounted in mitochondrial parameters such as size, cristae, and matrix density. However, to the best of our knowledge, this constitutes the first occurrence in which a mitochondrial protein, involved in organelle dynamics, is related to high fructose consumption. Fission and fusion are both key mechanisms that regulate mitochondrial division during cell cycle and during the mitophagy when its functionally fails (Archer, 2013). When the fusion mediator is downregulated or dysfunctional, there is a reduction in the mitochondrial oxidative capacity. This could potentially account for the increase in the abundance of other proteins involved in the electron transport chain in the hepatic proteome of F. Moreover, changes in the abundance of OPA1 could be related to the negative effects attributed to fructose, since severe impairments of fusion promote diabetes (Archer, 2013).

Furthermore, there are assays that dilucidated the protective effects of PU against liver injury and diabetes-related outcomes in mice through their action regulating mitophagy. However, the study revealed decreased expression of several genes in a diabetic group of rats as regulators of the mitochondrial fusion processes without considering *OPA1 (Z*hang et al., 2022*)*. Furthermore, Cao et al. (2020) also showed in their *in vivo* assay that punicalagin promoted hepatic mitochondrial

fusion in C57BL/6J mice with induced acute hyperlipidaemia. The evidence that a PU-rich pomegranate-derived supplement increased the fructose-altered amounts of OPA1 could be a possible progress in comprehending the molecular mechanisms of action of the polyphenolic compound.

Protein turn-over and repair

Cellular protein levels and bioactivity depend on a regulatory cycle of protein synthesis, folding and degradation, and failure to accurately coordinate these processes could lead to physiological impairments and disease (Rousseau & Bertolotti, 2018). Proteins that are unnecessary or are damaged within the cell are tagged for degradation by the proteasome. The organelle, formed by several subunits, is responsible for the selective degradation of specific proteins, previously marked by ubiquitin, contributing to cellular homeostasis maintenance. Cells adapt proteasome-mediated degradation according to their requirements by controlling proteasome levels (Rousseau & Bertolotti, 2018). The proteasomal proteins include regulatory proteins involved in cell cycle control, DNA repair, immune response, and protein quality control, which prevent the accumulation of aberrant, oxidized, and glycosylated proteins and their potential toxicity to the cell (Goldberg, 2003).

The amounts of several hepatic proteins involved in proteasome functions were affected by fructose consumption as compared to the levels of the same proteins in the livers of C rats. In particular, the subunits 1 and 2 of the proteasome activator complex (PSME1 and PSME2; Fold changes: 0.77 and 0.76, respectively) and subunits from both 26S proteasome non-ATPase regulatory (PSMD1, PSMD11 and PSMD13; Fold changes: 0.77, 0.90 and 0.79, respectively) and proteasome (PSMA5, PSMB3, PSMB8 and PSMB10; Fold changes: 0.84, 0.90, 0.85 and 0.73, respectively) were decreased in quantity in the hepatic cells of F rats.

To the best of our knowledge, this is the first study to relate fructose consumption with altered proteasome-involved protein quantities. Decreased proteasome function has been related to chronic neurodegenerative diseases that occurred in brain tissues, plausibly due to the accretion of damaged proteins (Thibaudeau et al., 2018). The target of rapamycin complex I (TORC1) indirectly regulates the expression of proteasome subunits via nuclear factor erythroid 2-related factor 1 (NRF1). TORC1 has been related to hepatic lipid metabolism impairments associated with fructose in some

studies such as that conducted by Sapp et al. (2014). The authors observed activation of TORC1 signalling in the liver samples from patients with NAFLD, which may also imply suppression of protein degradation through proteasome as a result of the promotion of protein synthesis (Waite et al., 2022). Meanwhile, P had the ability to change the trend of the proteomic results, and increased amounts of PSME2 and PSMD were displayed when the supplement were consumed. This supports the beneficial effects of P in the restoration of the impairments associated with fructose consumption.

Furthermore, small nuclear ribonucleoprotein U1, U1-A, and polypeptide B2 (SNRP70, SNRPA, and SNRPB2, respectively), all of them involved in the regulation of mRNA splicing, via spliceosome, were detected only in the hepatic proteasome of F+P rats. The spliceosome is a molecular machinery composed of small nuclear ribonucleoproteins and additional proteins responsible for conducting the RNA splicing during RNA maturation in eukaryotes. The organelle plays a crucial role in the regulation of genic expression and has been related to cancer diseases. A study of RNA splicing dysregulation was conducted to explore its potential impact on the development and progression of fatty liver disease in the assay carried out by Wu et al. (2021). Moreover, Del Río-Moreno et al. (2019) analyzed the expression of several splicing-related proteins in liver biopsies from women undergoing bariatric surgery with different levels of hepatic steatosis and concluded that there was a putative association between changes in the expression of certain spliceosome components and splicing factors and NAFLD-associated comorbidities. In the present assay, the experimental animals suffering from fructose-induced liver damage (F rats) did not seem to have affected the splicing machinery, when their proteome was compared to C rats. However, there are authors that suggested methylglyoxal (MG)-induced damage to the spliceosome, promoting a depletion of the RNA splicing proteins in culture cells exposed to antitumour drugs (Alhujaily et al., 2021). MG is formed spontaneously by trace-level degradation of glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetonephosphate (DHAP). Both GA3P and DHAP are products resulting of fructose cleavage, and the hepatic enzymes responsible for their metabolism were increased in F rats, suggesting a regulated accretion of the intermediates. Under these conditions, larger amounts of MG may be forming, which could be promoting undetectable changes in the synthesis of spliceosomal proteins in the liver of F rats. The ability of P to reduce the hepatic glycoxidative stress promoted by fructose may imply diminished amounts of the above-mentioned

intermediates, as well as decreased protein and DNA damage, which would explain the expression of spliceosomal proteins in the liver of F+P rats. Irrespective of the effect of fructose intake on the mRNA splicing mechanism, the potential benefits of P supplementation on hepatocytes by regulating this enzymatic machinery, could have contributed to the clinical benefits observed in the liver of P-supplemented rats (Sánchez-Terrón et al., 2023, *submitted*; Figure 1).

Conclusions

As part of a broader study, this novel research provides, to the best of our knowledge, the first comprehensive insight into the changes induced in the livers of *Wistar* rats exposed to a precise concentration of dietary fructose (9 g fructose/kg live bodyweight/day) using an untargeted proteomic approach. Beyond the mechanisms underlying fructose-induced hepatic damage proposed in the literature, such as increased DNL or decreased fatty acid oxidation, the present assay shows that hepatic mitochondrial proteins implied in energy production, as well as in the organelle morphology, were affected when the animals were supplied with dietary fructose. Moreover, the amounts of antioxidant proteins and enzymes involved in the degradation of damaged proteins were diminished in the liver of animals challenged with dietary fructose.

The most remarkable outcomes allow us to elucidate how a punicalagin-rich extract supplementation (P) was able to restore the physiological concentration of hepatic proteins involved in fructose-induced metabolic impairments. The abundance of proteins related to damaged protein degradation and cellular DNA damage repair were increased by the consumption of P. Furthermore, it is worth noting that alternative lipid oxidation mechanisms were promoted due to the presence of the pomegranate supplement. The bioactive functions attributed to urolithin-derived compounds from PU against several oxidative stress-derived diseases may support these improvements, as in a concurrent study using the same animals, we were able to identify a urine urolithin-related metabolite. These results suggest that the consumption of this PU-rich supplement counteracted the noxious molecular mechanisms activated at the early stages of hepatic fructose metabolism, thus plausibly inhibiting the downstream enzymatic machinery and the associated mitochondrial failures (Figure 3). Pomegranate supplements and their bioactive compounds may be tested in clinical trials to alleviate the harmful effects of dietary reducing sugars in (pre-)diabetic patients.

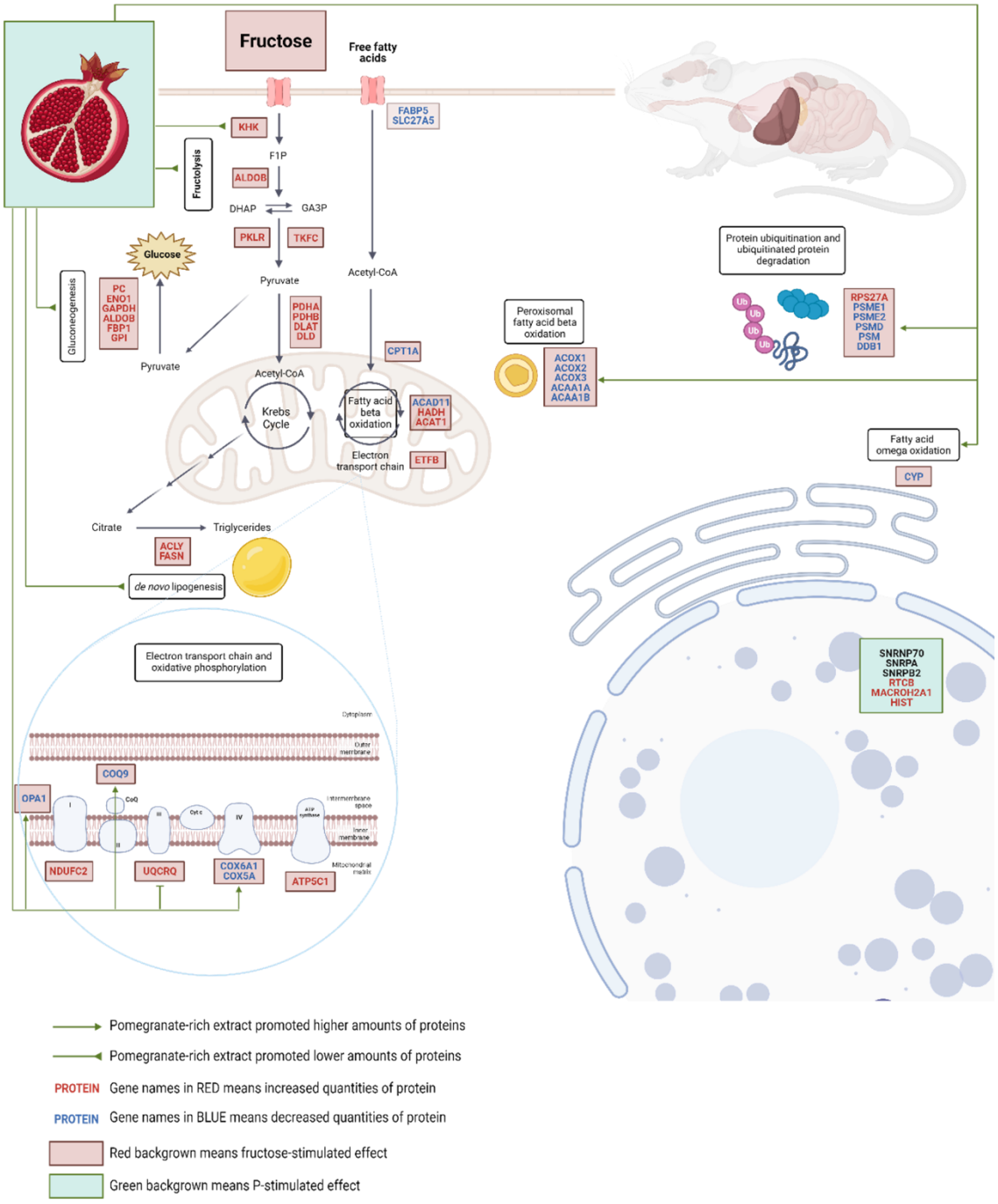

FIGURE 3. Proposed mechanism by which pomegranate could exert its beneficial effects on fructose-induced hepatic damage.

Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

E., M. Funding acquisition, project administration, conceptualization, investigation, methodology, supervision, data analysis, validation and writing─review and editing.

S.-T., G.: Investigation, methodology, data analysis, validation and writing─original draft.

M., R.: Investigation, methodology, supervision, data analysis, validation and writing─review and editing.

D., J.: Supervision, data analysis, validation and writing─review and editing.

M., J.: Supervision, data analysis, validation and writing─review and editing.

All authors made critical revisions to the manuscript for key intellectual content and read and approved the final manuscript.

Supporting information

Acknowledgments

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TABLE 1. Proteins from the livers of rats affected by the supplementation with either 30% fructose solution (F *vs*. C) or 0.2% P on a 30% fructose solution (F+P *vs*. F). Proteins are grouped by biological functions and/or molecular pathways (Continuación).

Protein name	*Gene name*	Biological function	FASTA accession number	Fold change	p value	Fold change	*p* value
Fructolysis				F *vs*. C		F+P *vs*. F	
Pyruvate kinase PKLR	*PKLR*	Catalyses the terminal step of glycolysis	P12928	1.94	<0.001	0.67	ns
Triokinase/FMN cyclase	*TKFC*	Catalyses the phosphorylation of both DHA and GA	Q4KLZ6	1.61	<0.001	0.69	ns
Glucokinase regulatory protein	*GCKR*	Regulates GCK activity	Q07071	1.34	<0.05	1.33	ns
Ketohexokinase	*KHK*	Catalyses the phosphorylation of fructose to fructose-1-phosphate	Q02974	1.38	<0.001	0.59	ns
Glycogen phosphorylase, liver form	*PYGL*	Inhibits glycogen synthesis	P09811	1.27	<0.001	0.68	ns
Fructose-bisphosphate aldolase	*ALDOB*	Catalyses F1P cleavage	Q66HT1	1.23	<0.001	0.64	ns
Gluconeogenesis				F vs. C		F+P vs. F	
Glucose-6-phosphate isomerase	*GPI*	Catalyses the second step of glycolysis	Q6P6V0	1.69	<0.001	1.07	ns
Pyruvate carboxylase, mitochondrial	*PC*	Involved in the first step of gluconeogenesis	P52873	1.34	<0.001	0.40	ns
Alpha-enolase	*ENO1*	Catalyses the generation of phosphoenolpyruvate at the lasts step of glycolysis	P04764	1.30	<0.001	0.97	ns
Glyceraldehyde-3-phosphate dehydrogenase	*GADPH*	Involving in the phosphorylation of GAP and DHAP	P04797	1.21	<0.01	-	-
Fructose-1,6-bisphosphatase 1	*FBP1*	Modulates gluconeogenesis in liver, as well as appetite and adiposity.	P19112	1.14	<0.01	0.58	ns

TABLE 1. Proteins from the livers of rats affected by the supplementation with either 30% fructose solution (F *vs.* C) or 0.2% P on a 30% fructose solution (F+P *vs.* F). Proteins are grouped by biological functions and/or molecular pathways.

Protein name	*Gene name*	Biological function	FASTA accession number	Fold change	p value	Fold change	*p* value
De novo lipogeneses				F vs. C		F+P vs. F	
Fatty acid synthase	*FASN*	Catalyses the *de novo* lipogenesis	P12785	2.96	<0.05	0.66	ns
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	*DLAT*	Catalyses the overall conversion of pyruvate to acetyl-CoA and CO_2	P08461	1.81	<0.001	0.76	ns
ATP-citrate synthase	*ACLY*	Catalyses the cleavage of citrate	A0A0G2K5E7	1.77	<0.01	0.85	ns
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	*PDHB*	Component beta of pyruvate dehydrogenase complex	A0A0G2KAM3	1.37	<0.001	0.84	ns
Pyruvate dehydrogenase E1 component subunit alpha	*PDHA1*	Component alpha of pyruvate dehydrogenase complex	D4A5G8	1.19	<0.05	0.89	ns
Dihydrolipoyl dehydrogenase, mitochondrial	*DLD*	Required for the complete reaction of pyruvate dehydrogenase complex	Q6P6R2	1.08	<0.05	0.77	ns
Fatty acid oxidation				F vs. C		F+P vs. F	
Acetyl-CoA acetyltransferase, mitochondrial	*ACAT1*	Catalyses the last step of the mitochondrial β-oxidation pathway	P17764	1.19	<0.01	0.71	ns
Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	*HADH*	Catalyses the third step of the mitochondrial β-oxidation. Plays a role in the control of insulin secretion by inhibiting the activation of GLUD1	Q9WVK7	1.14	<0.01	0.57	ns
3-ketoacyl-CoA thiolase A y B, peroxisomal	*ACAA1A; ACAA1B*	Role in fatty acid peroxisomal β-oxidation	P21775; P07871	0.87	<0.01	0.56	ns
Acyl-coenzyme A oxidase	*ACOX1*	Catalyses peroxisomal fatty acid β-oxidation	F1LQC1	0.83	<0.05	1.05	ns

TABLE 1. Proteins from the livers of rats affected by the supplementation with either 30% fructose solution (F *vs*. C) or 0.2% P on a 30% fructose solution (F+P *vs*. F). Proteins are grouped by biological functions and/or molecular pathways (Continuación).

Protein name	*Gene name*	Biological function	FASTA accession number	Fold change	p value	Fold change	*p* value
Carnitine O-palmitoyl transferase 1, liver isoform	*CPT1A*	Translocation of fatty acids into mitochondria	P32198	0.82	<0.01	1.03	ns
Peroxisomal acyl-coenzyme A oxidase 2	*ACOX2*	Catalyses fatty acid β-oxidation	F1LNW3	0.80	<0.01	1.05	ns
Acyl-CoA dehydrogenase family member 11	*ACAD11*	Probably participates in β-oxidation and energy production (involved in the metabolism of specific fatty acids)	B3DMA2	0.80	<0.01	1.14	<0.05
Long-chain fatty acid transport protein 5	*SLC27A5*	Plays an important role in hepatic long chain fatty acids uptake	Q9ES38	0.78	<0.05	1.43	<0.05
Peroxisomal acyl-coenzyme A oxidase 3	*ACOX3*	Catalyses peroxisomal fatty acid β-oxidation	Q63448	0.74	<0.01	0.54	ns
Cytochrome P450 (2A1, 2C6, 2C23, 2C70, 2D26, 2E1, 3A2, 4F1, 4F4)	*CYP*	Involved in ω-fatty acid oxidation	P11711; P05178; P24470; P19225; P10634; P05182; P05183; P33274; P51869	Down	*	Up	*
Mitochondrial proteins				F vs. C		F+P vs. F	
Cytochrome b-c1 complex subunit 8	*UQCRQ*	Component of the complex III of the mitochondrial electron transport chain	Q7TQ16	1.59	<0.001	0.72	<0.05
NADH dehydrogenase [ubiquinone] 1 subunit C2	*NDUFC2*	Accessory subunit of the mitochondrial membrane respiratory chain Complex I	A6I691	1.34	<0.05	0.84	ns
Electron transfer flavoprotein subunit beta	*ETFB*	Transfers the electrons to the main mitochondrial respiratory chain	Q68FU3	1.19	<0.01	0.6	ns

TABLE 1. Proteins from the livers of rats affected by the supplementation with either 30% fructose solution (F *vs*. C) or 0.2% P on a 30% fructose solution (F+P *vs*. F). Proteins are grouped by biological functions and/or molecular pathways.

Protein name	*Gene name*	Biological function	FASTA accession number	Fold change	p value	Fold change	*p* value
ATP synthase subunit gamma, mitochondrial	*ATP5F1C*	ATP-producer (Complex V)	P35435	1.15	<0.01	0.58	ns
Cytochrome c oxidase subunit 6A1, mitochondrial	*COX6A1*	Components of the cytochrome c oxidase, which drives oxidative phosphorylation (Complex IV of electron transport chain)	P10818	0.72	<0.001	1.96	<0.05
Cytochrome c oxidase subunits 5A, mitochondrial	*COX5A*		P11240	0.73	<0.05	0.97	ns
Ubiquinone biosynthesis protein COQ9, mitochondrial	*COQ9*	Involved in the biosynthesis of coenzyme Q	Q68FT1	0.69	<0.01	1.28	<0.05
Dynamin-like 120kDa protein, mitochondrial	*OPA1*	Essential for normal mitochondrial morphology	Q2TA68	0.95	ns	1.16	<0.05
Protection against oxidative damage				F vs. C		F+P vs. F	
Glutathione synthetase	*GSS*	Catalyses the production of glutathione, involved in process such as protection against oxidative damage o detoxification	P46413	1.45	<0.001	0.78	ns
Complement component 1 Q subcomponent-binding protein, mitochondrial	*C1QBP*	Multifunctional and multicompartmental protein involved in inflammation and infection processes	O35796	1.22	<0.01	0.89	ns
Peroxiredoxin-1	*PRDX1*	Plays a role in cell protection against oxidative stress	Q63716	0.82	<0.05	1.15	ns
Glutathione peroxidase	*GPX1*	Involved in glutathione metabolism	M0RAM5	0.43	<0.001	1.19	ns
Glutathione S-transferase (A6, alpha-3, kappa-1, Mu-2, theta-2, theta-3, P)	*GST*	Implied in detoxification processes through by glutathione as substrate	Q6AXY0; P04904; P24473; P30713; D3Z8I7; P04906	Down	*	Up	ns

TABLE 1. Proteins from the livers of rats affected by the supplementation with either 30% fructose solution (F *vs*. C) or 0.2% P on a 30% fructose solution (F+P *vs*. F). Proteins are grouped by biological functions and/or molecular pathways (Continuación).

Protein name	*Gene name*	Biological function	FASTA accession number	Fold change	p value	Fold change	*p* value
Proteasome and spliceosome				F vs. C		F+P vs. F	
Proteasome activator complex subunit 1	*PSME1*	Implicated in immunoproteasome, that degrades ubiquitin-labelled proteins found in the cytoplasm in cell exposed to oxidative stress and proinflammatory stimuli	Q63797	0.77	<0.001	1.28	ns
Proteasome activator complex subunit 2	*PSME2*		Q63798	0.76	<0.001	1.39	<0.05
26S proteasome non-ATPase regulatory (subunits 1, 11, 13)	*PSMD*	Involved in the degradation of ubiquitinated proteins	O88761; F1LMZ8; B0BN93	Down	*	Up	**
Proteasome (subunits: alpha type 5, beta type-3, beta type-8, beta type-10)	*PSM*	Subunits of proteasome complex, which degrades unneeded or damaged proteins by proteolysis	P34064; P40112; P28064; Q4KM35	Down	*	Up	ns
Small nuclear ribonucleoprotein U1	*SNRNP70*	Regulation of mRNA splicing, via spliceosome	D3Z8H0	-	-	F+P	-
U1 small nuclear ribonucleoprotein A	*SNRPA*		Q5U214	-	-	F+P	-
Small nuclear ribonucleoprotein polypeptide B2	*SNRPB2*		B5DEQ4	-	-	F+P	-
DNA-damage-binding protein	*DDB1*	Involved in DNA repair and protein ubiquitination	Q9ESW0	0.87	<0.01	1.10	ns
RNA-splicing ligase RtcB homolog	*RTCB*	Catalytic subunit of the tRNA-splicing ligase complex	Q6AYT3	0.81	<0.01	1.26	<0.05
Core histone macro-H2A.1	*MACROH2A1*	Variant histone H2A, plays a central role in DNA repair	A0A140TAB4	0.81	<0.001	1.50	<0.001

TABLE 1. Proteins from the livers of rats affected by the supplementation with either 30% fructose solution (F *vs*. C) or 0.2% P on a 30% fructose solution (F+P *vs*. F). Proteins are grouped by biological functions and/or molecular pathways.

Protein name	*Gene name*	Biological function	FASTA accession number	Fold change	p value	Fold change	*p* value
Histone (H2A, H2B type 1, H3, H4,)		Play a role in gene regulation and DNA replication, protecting DNA from DNA damage	D3ZXP3; G3V9C7; P84245; P62804	Down	*	Up	**

References

Adachi, S. I., Sasaki, K., Kondo, S., Komatsu, W., Yoshizawa, F., Isoda, H., & Yagasaki, K. (2020). Antihyperuricemic effect of urolithin a in cultured hepatocytes and model mice. Molecules, 25(21), 1–15. https://doi.org/10.3390/molecules25215136

Ajaz, S., McPhail, M. J., Gnudi, L., Trovato, F. M., Mujib, S., Napoli, S., Carey, I., & Agarwal, K. (2021). Mitochondrial dysfunction as a mechanistic biomarker in patients with non-alcoholic fatty liver disease (NAFLD). Mitochondrion, 57(December 2020), 119–130. https://doi.org/10.1016/j.mito.2020.12.010

Alhujaily, M., Abbas, H., Xue, M., de la Fuente, A., Rabbani, N., & Thornalley, P. J. (2021). Studies of Glyoxalase 1-Linked Multidrug Resistance Reveal Glycolysis-Derived Reactive Metabolite, Methylglyoxal, Is a Common Contributor in Cancer Chemotherapy Targeting the Spliceosome. Frontiers in Oncology, 11(November). https://doi.org/10.3389/fonc.2021.748698

Archer, S. L. (2013). Mitochondrial Dynamics — Mitochondrial Fission and Fusion in Human Diseases. New England Journal of Medicine, 369(23), 2236–2251. https://doi.org/10.1056/nejmra1215233

Bai, Q., Liu, Y., Wang, C.-M., Wang, J.-R., Feng, Y., Ma, X., Yang, X., Shi, Y.-N., & Zhang, W. J. (2023). Hepatic but not Intestinal FBP1 Is Required for Fructose Metabolism and Tolerance. Endocrinology, 164(6), bqad054. https://doi.org/10.1210/endocr/bqad054

Berthier, A., Johanns, M., Zummo, F. P., Lefebvre, P., & Staels, B. (2021). PPARs in liver physiology. Biochimica et Biophysica Acta - Molecular Basis of Disease, 1867(5), 166097. https://doi.org/10.1016/j.bbadis.2021.166097

Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. Journal of Clinical Investigation, 114(2), 147–152. https://doi.org/10.1172/JCI200422422

Caballero, V., Estévez, M., Tomás-Barberán, F. A., Morcuende, D., Martín, I., & Delgado, J. (2022). Biodegradation of Punicalagin into Ellagic Acid by Selected Probiotic Bacteria: A Study of the Underlying Mechanisms by MS-Based Proteomics. Journal of Agricultural and Food Chemistry, 70(51), 16273–16285. https://doi.org/10.1021/acs.jafc.2c06585

Cao, K., Wang, K., Yang, M., Liu, X., Lv, W., & Liu, J. (2020). Punicalagin improves hepatic lipid metabolism: Via modulation of oxidative stress and mitochondrial biogenesis in hyperlipidemic mice. Food and Function, 11(11), 9624–9633. https://doi.org/10.1039/d0fo01545h

Cioffi, F., Senese, R., Lasala, P., Ziello, A., Mazzoli, A., Crescenzo, R., Liverini, G., Lanni, A., Goglia, F., & Iossa, S. (2017). Fructose-rich diet affects mitochondrial DNA damage and repair in rats. Nutrients, 9(4), 1–14. https://doi.org/10.3390/nu9040323

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnology, 26(12), 1367–1372. https://doi.org/10.1038/nbt.1511

Del Río-Moreno, M., Alors-Pérez, E., González-Rubio, S., Ferrín, G., Reyes, O., Rodríguez-Perálvarez, M., Sánchez-Frías, M. E., Sánchez-Sánchez, R., Ventura, S., López-Miranda, J., Kineman, R. D., De La Mata, M., Castaño, J. P., Gahete, M. D., & Luque, R. M. (2019). Dysregulation of the Splicing Machinery Is Associated to the Development of Nonalcoholic Fatty Liver Disease. Journal of Clinical Endocrinology and Metabolism, 104(8), 3389–3402. https://doi.org/10.1210/jc.2019-00021

Delgado, J., Núñez, F., Asensio, M. A., & Owens, R. A. (2019). Quantitative proteomic profiling of ochratoxin A repression in Penicillium nordicum by protective cultures. International Journal of Food Microbiology, 305(June), 108243. https://doi.org/10.1016/j.ijfoodmicro.2019.108243

Dharmalingam, M., & Yamasandhi, P. G. (2018). Nonalcoholic fatty liver disease and Type 2 diabetes mellitus. Indian Journal of Endocrinology and Metabolism, 22(3), 421–428. https://doi.org/10.4103/ijem.IJEM-585-17

Díaz-Velasco, S., Delgado, J., Peña, F. J., & Estévez, M. (2022). Protein oxidation marker, α-amino adipic acid, impairs proteome of differentiated human enterocytes: Underlying toxicological mechanisms. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1870(7), 140797. https://doi.org/10.1016/j.bbapap.2022.140797

Fisher, C. D., Lickteig, A. J., Augustine, L. M., Ranger-Moore, J., Jackson, J. P., Ferguson, S. S., & Cherrington, N. J. (2009). Hepatic cytochrome P450 enzyme alterations in humans with

progressive stages of nonalcoholic fatty liver disease. Drug Metabolism and Disposition, 37(10), 2087–2094. https://doi.org/10.1124/dmd.109.027466

García-Berumen, C. I., Ortiz-Avila, O., Vargas-Vargas, M. A., Del Rosario-Tamayo, B. A., Guajardo-López, C., Saavedra-Molina, A., Rodríguez-Orozco, A. R., & Cortés-Rojo, C. (2019). The severity of rat liver injury by fructose and high fat depends on the degree of respiratory dysfunction and oxidative stress induced in mitochondria. Lipids in Health and Disease, 18(1), 1–11. https://doi.org/10.1186/s12944-019-1024-5

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. Nature, 426(6968), 895–899. https://doi.org/10.1038/nature02263

Hannou, S. A., Haslam, D. E., McKeown, N. M., & Herman, M. A. (2018). Fructose metabolism and metabolic disease. Journal of Clinical Investigation, 128(2), 545–555. https://doi.org/10.1172/JCI96702

Herman, M. A., & Birnbaum, M. J. (2021). Molecular aspects of fructose metabolism and metabolic disease. Cell Metabolism, 33(12), 2329–2354. https://doi.org/10.1016/j.cmet.2021.09.010

Herman, M. A., & Samuel, V. T. (2016). The Sweet Path to Metabolic Demise: Fructose and Lipid Synthesis. Trends in Endocrinology and Metabolism, 27(10), 719–730. https://doi.org/10.1016/j.tem.2016.06.005

Hsieh, C. C., Liao, C. C., Liao, Y. C., Hwang, L. S., Wu, L. Y., & Hsieh, S. C. (2016). Proteomic changes associated with metabolic syndrome in a fructose-fed rat model. Journal of Food and Drug Analysis, 24(4), 754–761. https://doi.org/10.1016/j.jfda.2016.03.005

Islam, S. M. T., Won, J., Khan, M., Chavin, K. D., & Singh, I. (2020). Peroxisomal footprint in the pathogenesis of nonalcoholic steatohepatitis. Annals of Hepatology, 19(5), 466–471. https://doi.org/10.1016/j.aohep.2019.11.007

Jaiswal, N., Maurya, C. K., Arha, D., Avisetti, D. R., Prathapan, A., Raj, P. S., Raghu, K. G., Kalivendi, S. V., & Tamrakar, A. K. (2015). Fructose induces mitochondrial dysfunction and triggers apoptosis in skeletal muscle cells by provoking oxidative stress. Apoptosis, 20(7), 930–947. https://doi.org/10.1007/s10495-015-1128-y

Janevski, M., Ratnayake, S., Siljanovski, S., McGlynn, M. A., Cameron-Smith, D., & Lewandowski, P. (2012). Fructose containing sugars modulate mRNA of lipogenic genes ACC and FAS and protein levels of transcription factors ChREBP and SREBP1c with no effect on body weight or liver fat. Food and Function, 3(2), 141–149. https://doi.org/10.1039/c1fo10111k

Jang, C., Hui, S., Lu, W., Cowan, A. J., Morscher, R. J., Lee, G., Liu, W., Tesz, G. J., Birnbaum, M. J., & Rabinowitz, J. D. (2018). The small intestine converts dietary fructose into glucose and organic acids. Cell Metabolism, 27(2), 351-361.e3. https://doi.org/10.1016/j.cmet.2017.12.016

Janssens, S., Ciapaite, J., Wolters, J. C., van Riel, N. A., Nicolay, K., & Prompers, J. J. (2017). An in vivo magnetic resonance spectroscopy study of the effects of caloric and non-caloric sweeteners on liver lipid metabolism in rats. Nutrients, 9(5), 1–19. https://doi.org/10.3390/nu9050476

Jensen, T., Abdelmalek, M. F., Sullivan, S., Nadeau, K. J., Green, M., Roncal, C., Nakagawa, T., Kuwabara, M., Sato, Y., Kang, D. H., Tolan, D. R., Sanchez-Lozada, L. G., Rosen, H. R., Lanaspa, M. A., Diehl, A. M., & Johnson, R. J. (2018). Fructose and sugar: A major mediator of non-alcoholic fatty liver disease. Journal of Hepatology, 68(5), 1063–1075. https://doi.org/10.1016/j.jhep.2018.01.019

Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. P. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with non-alcoholic fatty liver disease. Gastroenterology, 146(3), 726–735. https://doi.org/1doi:10.1053/j.gastro.2013.11.049.

Li, R., Guo, G., Cao, Y., Wang, Y., Liu, F., & Zhang, X. (2013). Expression of cytochrome P450 2A5 in a C57BL/6J mouse model of nonalcoholic fatty liver disease. Pharmacology, 92(1–2), 26–31. https://doi.org/10.1159/000348575

Luber, C. A., Cox, J., Lauterbach, H., Fancke, B., Selbach, M., Tschopp, J., Akira, S., Wiegand, M., Hochrein, H., O'Keeffe, M., & Mann, M. (2010). Quantitative Proteomics Reveals Subset-Specific Viral Recognition in Dendritic Cells. Immunity, 32(2), 279–289. https://doi.org/10.1016/j.immuni.2010.01.013

Mamikutty, N., Thent, Z. C., & Haji Suhaimi, F. (2015). Fructose-drinking water induced nonalcoholic fatty liver disease and ultrastructural alteration of hepatocyte mitochondria in Male wistar rat. BioMed Research International, 2015. https://doi.org/10.1155/2015/895961

Mandal, A., Bhatia, D., & Bishayee, A. (2017). Anti-inflammatory mechanism involved in pomegranate-mediated prevention of breast cancer: The role of NF-κB and Nrf2 signaling pathways. Nutrients, 9(5), 1–13. https://doi.org/10.3390/nu9050436

Mastrocola, R., Ferrocino, I., Liberto, E., Chiazza, F., Cento, A. S., Collotta, D., Querio, G., Nigro, D., Bitonto, V., Cutrin, J. C., Rantsiou, K., Durante, M., Masini, E., Aragno, M., Cordero, C., Cocolin, L., & Collino, M. (2018). Fructose liquid and solid formulations differently affect gut integrity, microbiota composition and related liver toxicity: a comparative in vivo study. Journal of Nutritional Biochemistry, 55, 185–199. https://doi.org/10.1016/j.jnutbio.2018.02.003

Matsuda, M., & Shimomura, I. (2013). Increased oxidative stress in obesity: Implications for metabolic syndrome, diabetes, hypertension, dyslipidemia, atherosclerosis, and cancer. Obesity Research and Clinical Practice, 7(5), e330–e341. https://doi.org/10.1016/j.orcp.2013.05.004

McGarry, J. D. (2002). Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes. In Diabetes (Vol. 51, Issue 1, pp. 7–18). https://doi.org/10.2337/diabetes.51.1.7

Merino, B., Fernández-Díaz, C. M., Cózar-Castellano, I., & Perdomo, G. (2020). Intestinal fructose and glucose metabolism in health and disease. Nutrients, 12(1), 1–35. https://doi.org/10.3390/nu12010094

Miura, Y. (2013). The biological significance of ω-oxidation of fatty acids. Proceedings of the Japan Academy Series B: Physical and Biological Sciences, 89(8), 370–382. https://doi.org/10.2183/pjab.89.370

Muriel, P., López-sánchez, P., & Ramos-tovar, E. (2021). Fructose and the liver. International Journal of Molecular Sciences, 22(13). https://doi.org/10.3390/ijms22136969

Novelle, M. G., Bravo, S. B., Deshons, M., Iglesias, C., García-Vence, M., Annells, R., da Silva Lima, N., Nogueiras, R., Fernández-Rojo, M. A., Diéguez, C., & Romero-Picó, A. (2021).

Impact of liver-specific GLUT8 silencing on fructose-induced inflammation and omega oxidation. IScience, 24(2). https://doi.org/10.1016/j.isci.2021.102071

Olvera-Sandoval, C., Fabela-Illescas, H. E., Fernández-Martínez, E., Ortiz-Rodríguez, M. A., Cariño-Cortés, R., Ariza-Ortega, J. A., Hernández-González, J. C., Olivo, D., Valadez-Vega, C., Belefant-Miller, H., & Betanzos-Cabrera, G. (2022). Potential Mechanisms of the Improvement of Glucose Homeostasis in Type 2 Diabetes by Pomegranate Juice. Antioxidants, 11(3), 1–13. https://doi.org/10.3390/antiox11030553

Rajasekar, P., & Anuradha, C. V. (2007). Fructose-induced hepatic gluconeogenesis: Effect of l-carnitine. Life Sciences, 80(13), 1176–1183. https://doi.org/10.1016/j.lfs.2006.12.010

Rousseau, A., & Bertolotti, A. (2018). Regulation of proteasome assembly and activity in health and disease. Nature Reviews Molecular Cell Biology, 19(11), 697–712. https://doi.org/10.1038/s41580-018-0040-z

Sánchez-Terrón, G., Martínez, R., Morcuende, D., Caballero, V., & Estévez, M. (n.d.). Pomegranate supplementation alleviates the dyslipidemia and the onset of non-alcoholic fatty liver disease caused by chronic dietary fructose in Wistar rats. Molecular Nutrition & Food Research.

Sánchez-Terrón, G., Martínez, R., Ruiz, J., Luna, C., & Estévez, M. (2023). Impact of sustained fructose consumption on gastrointestinal function and health in Wistar rats: glycoxidative stress, impaired protein digestion and shifted faecal microbiota. Journal of Agricultural and Food Chemistry. https://doi.org/10.1021/acs.jafc.3c04515

Sapp, V., Gaffney, L., Eauclaire, S. F., & Matthews, R. P. (2014). Fructose leads to hepatic steatosis in zebrafish that is reversed by mechanistic target of rapamycin (mTOR) inhibition. Hepatology, 60(5), 1581–1592. https://doi.org/10.1002/hep.27284

Singh, I. (1997). Biochemistry of peroxisomes in health and disease. Molecular and Cellular Biochemistry, 167(1–2), 1–29. https://doi.org/10.1023/A:1006883229684

Softic, S., Cohen, D. E., & Kahn, C. R. (2016). Role of Dietary Fructose and Hepatic De Novo Lipogenesis in Fatty Liver Disease. Digestive Diseases and Sciences, 61(5), 1282–1293. https://doi.org/10.1007/s10620-016-4054-0

Softic, S., Gupta, M. K., Wang, G. X., Fujisaka, S., O'Neill, B. T., Rao, T. N., Willoughby, J., Harbison, C., Fitzgerald, K., Ilkayeva, O., Newgard, C. B., Cohen, D. E., & Kahn, C. R. (2017). Divergent effects of glucose and fructose on hepatic lipogenesis and insulin signaling. Journal of Clinical Investigation, 127(11), 4059–4074. https://doi.org/10.1172/JCI94585

Ter Horst, K. W., & Serlie, M. J. (2017). Fructose consumption, lipogenesis, and non-alcoholic fatty liver disease. Nutrients, 9(9), 1–20. https://doi.org/10.3390/nu9090981

Thibaudeau, T. A., Anderson, R. T., & Smith, D. M. (2018). A common mechanism of proteasome impairment by neurodegenerative disease-associated oligomers. Nature Communications, 9(1). https://doi.org/10.1038/s41467-018-03509-0

Tomás-Barberán, F. A., González-Sarrías, A., García-Villalba, R., Núñez-Sánchez, M. A., Selma, M. V., García-Conesa, M. T., & Espín, J. C. (2017). Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. Molecular Nutrition and Food Research, 61(1). https://doi.org/10.1002/mnfr.201500901

Tow, W. K., Chee, P. Y., Sundralingam, U., & Palanisamy, U. D. (2022). The Therapeutic Relevance of Urolithins, Intestinal Metabolites of Ellagitannin-Rich Food: A Systematic Review of In Vivo Studies. In Nutrients (Vol. 14, Issue 17). https://doi.org/10.3390/nu14173494

Waite, K. A., Burris, A., Vontz, G., Lang, A., & Roelofs, J. (2022). Proteaphagy is specifically regulated and requires factors dispensable for general autophagy. Journal of Biological Chemistry, 298(1), 101494. https://doi.org/10.1016/j.jbc.2021.101494

Wickham, H. (2008). Elegant Graphics for Data Analysis: ggplot2. In Applied Spatial Data Analysis with R.

Wu, P., Zhang, M., & Webster, N. J. G. (2021). Alternative RNA Splicing in Fatty Liver Disease. Frontiers in Endocrinology, 12(February), 1–14. https://doi.org/10.3389/fendo.2021.613213

Yanpar, E., Yildirim, M., Akkapulu, M., Değirmenci, U., Könen Adigüzel, S., Yalin, S., & Yalin, A. E. (2021). Possible protective role of punicalagin on oxidative stress, inflammation and genotoxicity in ethanol-induced liver toxicity. Journal of Research in Pharmacy, 25(5), 600–608. https://doi.org/10.29228/jrp.51

Yin, Y., Martínez, R., Zang, W., & Estévez, M. (2023). Crosstalk between dietary pomegranate and gut microbiota: Evidence of health benefits. https://doi.org/https://doi.org/10.1080/10408398.2023.2219763

Zahin, M., Ahmad, I., Gupta, R. C., & Aqil, F. (2014). Punicalagin and ellagic acid demonstrate antimutagenic activity and inhibition of benzo[a]pyrene induced DNA adducts. BioMed Research International, 2014. https://doi.org/10.1155/2014/467465

Zhang, Y., Tan, X., Cao, Y., An, X., Chen, J., & Yang, L. (2022). Punicalagin Protects against Diabetic Liver Injury by Upregulating Mitophagy and Antioxidant Enzyme Activities. Nutrients, 14(14). https://doi.org/10.3390/nu14142782

Zhao, S., Jang, C., Liu, J., Uehara, K., Gilbert, M., Izzo, L., Zeng, X., Trefely, S., Fernandez, S., Carrer, A., Miller, K. D., Schug, Z. T., Snyder, N. W., Gade, T. P., Titchenell, P. M., Rabinowitz, J. D., & Wellen, K. E. (2020). Dietary fructose feeds hepatic lipogenesis via microbiota-derived acetate. Nature, 579(7800), 586–591. https://doi.org/10.1038/s41586-020-2101-7

DISCUSIÓN

5. Discusión conjunta

Como punto de partida de la presente tesis doctoral se llevó a cabo un experimento *in vitro* que simuló una digestión gástrica y que permitió conocer de forma preliminar los cambios que sufrieron las proteínas de un sistema modelo cárnico en presencia de diferentes compuestos (Capítulo I), entre ellos glucosa y glioxal. Ambos compuestos fueron elegidos para evaluar en qué medida la presencia tanto de azúcares reductores como de compuestos derivados de las reacciones en las que participan impulsan la glicoxidación de proteínas, mediante el incremento de ROS (del inglés, *reactive oxygen species*) como consecuencia de su degradación o mediante la presencia de RCS (del inglés, *reactive carbonyl species*) por su implicación en reacciones de Maillard. Las conclusiones obtenidas en este primer capítulo sentaron las bases para el diseño y desarrollo del resto de capítulos.

Así, en los capítulos posteriores, se planteó un experimento *in vivo* en el que ratas *Wistar* fueron suplementadas a través del agua de bebida con un 30% de fructosa y con un 30% de fructosa y un suplemento comercial rico en punicalagina derivado de granada (P) durante 10 semanas. Gracias a este ensayo, se pudieron describir los efectos perjudiciales que la fructosa ocasionó en el organismo de las ratas que la consumieron (Capítulos II y III), así como el efecto protector que P ejerció sobre estos daños (Capítulos III y IV).

5.1. Efecto de glucosa, glioxal y nitritos sobre la glicoxidación de proteínas y la digestibilidad proteica: digestión in vitro

Varios estudios han descrito un aumento de los procesos oxidativos de los componentes dietéticos en el estómago durante la digestión, debido al ambiente prooxidante que se genera en el compartimento como consecuencia del bajo pH y de las condiciones de oxigenación (Oueslati *et al*., 2016; Van Hecke *et al.*, 2021). A lo largo de los estudios de la presente tesis doctoral se ha podido corroborar este hecho, tanto por los resultados del experimento que engloba el Capítulo I como por los que recoge el Capítulo II, los cuales serán descritos con más detalle en el subapartado 5.2.

La presencia de glucosa en el sistema modelo utilizado para la digestión *in vitro* promovió un aumento tanto de los marcadores de oxidación proteica y de lípidos (carbonilos proteicos y TBARs, respectivamente) como de APOPs (del inglés, *advanced protein oxidation products*) en los lotes en los que el compuesto apareció combinado con la

mioglobina (Mb+GLU). Es sabido que la formación de radicales férricos proteicos como consecuencia de la cascada de reacciones en las que se ve envuelta la mioglobina tiene un impacto directo sobre la formación de ROS durante la digestión (Bechaux *et al*., 2018). Este proceso se vio aún más potenciado en presencia de glucosa, como queda reflejado en los mayores niveles de ROS observados en el lote Mb+GLU. Este incremento de ROS, potenciado por la presencia del azúcar reductor, tuvo un impacto directo sobre los procesos oxidativos que afectaron a las proteínas durante la digestión *in vitro*, enfatizando el papel que se le atribuye a la degradación de azúcares simples en la generación de ROS (Wang *et al*., 2016). Sin embargo, el lote con Mb+GLY pareció no mostrar un aumento tan significativo de los marcadores de oxidación de proteínas, si bien los niveles de APOPs como consecuencia de la presencia de GLY aumentaron. Esto concuerda con el papel que algunos autores atribuyen al GLY, implicándolo directamente en la formación de productos avanzados de glicación, también conocidos como AGEs (del inglés *advanced glycation end-products*) (Aragno & Mastrocola, 2017).

La presencia de nitritos redujo la oxidación de proteínas miofibrilares tras el proceso de digestión, si bien solo tuvo ningún efecto sobre las mismas en los lotes que contenían mioglobina. El efecto estabilizador de la mioglobina por parte de los nitritos se ha descrito con anterioridad (Skibsted, 2011), al igual que sus capacidades antioxidantes durante el procesado y el almacenamiento de la carne (Ruiz-Carrascal, 2015). Una vez combinados con la mioglobina, parece que los nitritos disminuyen la capacidad de ésta para formar radicales libres según la química de Fenton, como así lo sugiere también la disminución de ROS en los lotes que se trataron con nitritos y en los que se añadió mioglobina. Sin embargo, en presencia de nitritos y tras los procesos digestivos, resulta inevitable que se produzca la nitrosación de proteínas. Si bien es cierto, cuando los nitritos se combinan con la mioglobina, esta última podría disminuir la capacidad nitrosante de los nitritos mediante el bloqueo de la acción de las especies nitrogenadas con otros compuestos mientras estimula su propia nitrosación (Bourassa *et al*., 2001). La conexión establecida entre la formación de ROS y RNS por autores como Skibsted (2011) podría explicar, por otra parte, el alto grado de nitrosación presentado por el lote GLU [N+].

Por último, desde un punto de vista nutricional, se analizó la digestibilidad de las proteínas tras el proceso simulado de digestión en presencia de los diferentes compuestos. Los grupos Mb+GLU, GLY y Mb+GLY fueron los que presentaron los niveles más bajos de proteínas degradadas, de lo que infiere una disminución de la digestibilidad de las

mismas. Según Estévez *et al.* (2021), los cambios ocasionados en la estructura de las proteínas como consecuencia de procesos glicoxidativos podrían explicar la posible falta de habilidad por parte las enzimas gastrointestinales de reconocer los puntos de escisión proteicos durante la digestión. Puesto que los tres grupos presentaron los niveles más elevados de carbonilos y APOPs, esto podría explicar el origen de una digestión proteica alterada.

Por tanto, gracias a este estudio se pudieron describir las bases y las consecuencias de los procesos glicoxidativos que tuvieron lugar sobre proteínas miofibrilares durante una digestión gástrica simulada en presencia de compuestos como la mioglobina, inherentemente presente en las matrices cárnicas, y azúcares reductores, los cuales promovieron un aumento de ROS a consecuencia de los procesos de autodegradación en los que se ven envueltos.

Sin embargo, uno de los aspectos más complejos que limita el uso de las plataformas *in vitro*, ya sean estáticas o dinámicas, es la simulación de la permeabilidad de los tejidos gastrointestinales. Poniendo en común los resultados de carbonilos proteicos obtenidos en este primer ensayo después de la digestión gástrica simulada del lote con glucosa (GLU) con los que se obtuvieron a partir del contenido gástrico de las ratas suplementadas con fructosa (Capítulo II), se pudo observar una gran diferencia entre los valores. Así, en el ensayo *in vitro*, el bolo simulado después de la digestión gástrica presentó 8.56 nmol carbonilos/mg proteína, mientras que el contenido gástrico de las ratas que consumieron fructosa en el experimento *in vivo* mostró 1.29 nmol de carbonilos/mg proteína. Existen estudios en los que se ha señalado a la fructosa como más reactiva que la glucosa por la estabilidad de su cadena abierta y su grupo *keto* (Gugliucci, 2017; Semchyshyn, 2013). Por esto, podría considerarse que los resultados del experimento *in vitro* se alejan aún más de lo que fisiológicamente ocurre en el estómago sobre la glicoxidación de proteínas. Además, comparando los niveles de carbonilos proteicos del contenido gástrico de las ratas que consumieron fructosa con respecto a los de su tejido gástrico (Capítulo II), se pudo comprobar que fueron similares (1.29 *vs.* 1.15 nmol carbonilos/mg proteína, respectivamente). De existir plausiblemente una relación lineal entre los niveles de carbonilos que se observaron en el contenido gástrico simulado del estudio *in vitro* y una acreción/formación en tejido gástrico, se estaría haciendo referencia a una concentración de carbonilos en el tejido que sin duda estaría muy por encima del rango patológico descrito por algunos autores (Ma *et al*., 2013).

Este rango tan elevado de marcadores de glicoxidación proteica en el trabajo *in vitro* podría deberse a la ausencia de dinámica y absorción gastrointestinal inherente a los organismos vivos y que en el primer ensayo no se simularon. Incluso con un modelo para realizar digestiones *in vitro* que permitiese simular la absorción mediante membranas de diálisis permeables, poder imitar la medida en que se produce la acreción y/o la formación de carbonilos en los tejidos del tracto gastrointestinal resulta muy complejo, ya que se trata de un proceso que puede verse afectado por numerosos factores individuales y del entorno.

Como un acercamiento a lo que ocurre en el compartimento gástrico, el experimento *in vitro* resultó ser muy ilustrativo, pues en todo caso mostró cómo inequívocamente la glucosa potenció un ambiente prooxidante que afectó a la digestibilidad de las proteínas. Sin embargo, no son resultados útiles para cuantificar y fijar valores que sirvan como estándares de salud/enfermedad y que puedan utilizarse como referencia para otros estudios. Así, se hace necesario un acercamiento más preciso a la fisiología humana, como puede hacerse mediante el uso de animales de experimentación.

5.2. Efecto de la fructosa sobre el metabolismo: digestión y asimilación, metabolismo hepático

Dado el impacto de la glucosa sobre la digestibilidad de las proteínas miofibrilares que se observó en el ensayo *in vitro* (Capítulo I), se realizó una revisión bibliográfica para elaborar el planteamiento del experimento *in vivo*. Puesto que la fructosa está descrita por varios autores como más reactiva que la glucosa (Gugliucci, 2017; Semchyshyn, 2013), sumado a su relación con problemas metabólicos tan prevalentes actualmente como la obesidad, la T2DM (del inglés *type 2 diabetes mellitus*) o la NAFLD (del inglés *non-alcoholic fatty liver disease*), se concluyó que sería más relevante utilizar la fructosa como azúcar reductor en la investigación.

A pesar de que los resultados anteriores señalaron a la mioglobina como un prooxidante en las condiciones simuladas, en esta ocasión se optó por evaluar únicamente el efecto del consumo de fructosa sobre las funciones metabólicas de los animales. Partiendo de esta base, tanto o más relevante que hablar del efecto prooxidante del consumo de azúcares reductores junto con carne roja fresca rica en mioglobina, sería también hablar de sus efectos cuando se consume junto con productos ultraprocesados. Este tipo de productos resultan interesantes desde el punto de vista nutricional por ser alimentos que

tras los procedimientos a los que se ven sometidos durante su elaboración, son ingeridos con elevadas cantidades de proteínas estructuralmente alteradas. Por otro lado, con respecto al uso de nitritos como compuestos antioxidantes, debido a la potencial interacción con la mioglobina que sustenta su efecto y en ausencia de la misma, se optó por el estudio del efecto que tendría sobre la fisiopatología asociada al consumo de fructosa, la ingesta sostenida de un suplemento derivado de granada rico en punicalagina como agente antioxidante (P).

Como ya se adelantó en el subapartado anterior, la presencia de fructosa en el lumen del GIT promovió la formación tanto de carbonilos proteicos como de APOPs en el contenido estomacal de los animales (Capítulo II). Sin embargo, la presencia del azúcar no promovió ningún efecto significativo sobre la oxidación de lípidos. Esto coincide con otros estudios en los que también las proteínas fueron descritas como los objetivos principales de las reacciones de oxidación tanto durante digestiones *in vitro* como *in vivo* (Van Hecke *et al.*, 2018, 2021). La glicoxidación de proteínas en el estómago pareció afectar al patrón fisiológico de digestión, haciendo que mayores cantidades de proteínas sin digerir llegaran al yeyuno. Esto explicaría las mayores tasas de degradación proteica que parecieron tener lugar en el tramo intestinal de las ratas que consumieron fructosa con respecto a las ratas control, como se expone en el Capítulo II de la presente tesis doctoral. También podría estar detrás de los mayores niveles de APOPs registrados en el yeyuno, así como de la disminución de los niveles de carbonilos que se registraron en este tramo. Estos resultados refuerzan las conclusiones del Capítulo I de la presente tesis doctoral, en el que se pudo observar que los procesos de glicoxidación de proteínas durante una digestión simulada en presencia de un azúcar reductor afectaron a la digestibilidad de las mismas, al igual que mostraron otros autores en sus trabajos (Bains *et al*., 2017).

Además de su papel sobre la función intestinal y la salud, los carbonilos proteicos pueden verse envueltos en la formación de APOPs, lo cual también subyace tanto la disminución de los primeros como el aumento de los segundos en el contenido yeyunal de los animales que consumieron fructosa (Capítulo II). Bains *et al.* (2017) describieron, mediante una digestión gástrica e intestinal simulada, la formación intestinal de compuestos de glicación avanzada cuando la fructosa formaba parte de la matriz digerida. Basándonos en nuestro conocimiento actual, los resultados expuestos vinculan por primera vez *in vivo* la ingesta excesiva de fructosa con la formación de APOPs en el lumen intestinal. Esto resulta muy relevante desde el punto de vista nutricional, pues la presencia de productos

de glicación avanzada de proteínas podría tener una implicación directa en la activación de mecanismos que promueven la inflamación intestinal (Bains *et al.*, 2017).

La falta de degradación de proteínas en los compartimentos iniciales del tracto gastrointestinal pudo haber facilitado igualmente que proteínas glicoxidadas sin digerir llegaran al ciego y al colon de los animales que consumieron fructosa, como así lo sugieren los mayores valores de TDP (del inglés, *total dietary protein*) en el contenido colónico de las ratas F con respecto al de las ratas C (Capítulo II). En el colon, las proteínas dañadas no digeridas que llegan son fermentadas por la microbiota. Estas fermentaciones pueden tener un impacto negativo sobre la salud del hospedador como consecuencia de la formación de metabolitos perjudiciales. Estos metabolitos, en última instancia, podrían verse involucrados en el daño tanto a tejidos adyacentes como a órganos periféricos tras su paso al torrente sanguíneo (Rodríguez-Romero *et al*., 2022).

Las tasas de actividad microbiana que tienen lugar en el colon ocasionan un ambiente prooxidante intenso (Tian *et al.*, 2017). Según los resultados expuestos en el Capítulo II de la presente tesis doctoral, el ambiente prooxidante del colon de las ratas que consumieron fructosa durante 10 semanas pareció potenciarse como consecuencia de la llegada de proteínas dañadas sin digerir. Además, esto pudo favorecer el daño *in situ* de las proteínas que sortearon la digestión enzimática. Por su parte, cuando se consumen cantidades excesivas de fructosa, éstas pueden saturar la capacidad del transportador de fructosa intestinal encargado de la primera etapa de su metabolismo y hacer que mayores cantidades del compuesto lleguen a colon e hígado (Jang *et al.*, 2018), como se ilustra en la Figura 5.1. Así, junto con la hipotética presencia de proteínas glicoxidadas no digeridas en el colon, la presencia de fructosa pudo promover un intenso estrés oxidativo en el compartimento. Esto explicaría los elevados niveles de marcadores de glicoxidación proteica (PPC y APOPs) en el contenido colónico de los animales suplementados con fructosa revelados en el Capítulo II de la presente tesis doctoral.

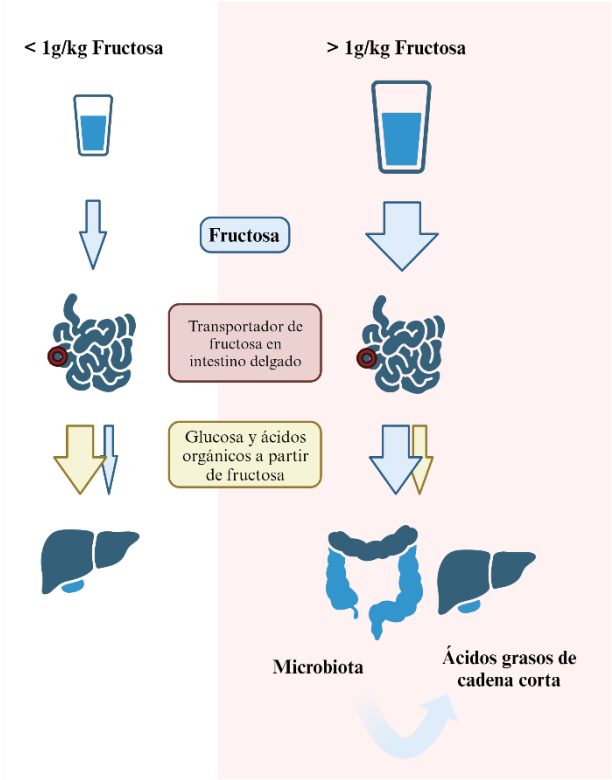

Figura 5.1. Mecanismo intestinal de metabolismo de fructosa, basado en el trabajo de Jang *et al.* (2018). Elaboración propia.

La activación de mecanismos proinflamatorios en el colon podría estar conectada con un estrés oxidativo crónico en este compartimento. Esto podría conducir a procesos de inflamación en los tejidos colónicos, los cuales son la base de muchas de las enfermedades intestinales que prevalecen en la actualidad, como enfermedad de Crohn o cáncer colorrectal (Tian *et al.*, 2017; Wang *et al.*, 2016). El incremento del estrés glicoxidativo en el lumen del tracto gastrointestinal contribuye al daño de los tejidos en contacto (Estévez & Luna, 2017; Soladoye *et al.*, 2015). Por tanto, resulta de gran interés tanto el estudio de los cambios que tienen lugar en el contenido intraluminal del GIT como en los tejidos, lo cual sin duda mejoraría la comprensión de los mecanismos que subyacen el comienzo y desarrollo de estas enfermedades.

En este aspecto, cuando se analizaron marcadores de glicoxidación de proteínas en los tejidos del GIT de los animales que consumieron fructosa, éstos mostraron un incremento en sus niveles en todos los casos (Capítulo II). Este aumento pudo ser debido tanto por una acreción de los carbonilos formados durante la digestión como consecuencia de los procesos de absorción, como por una carbonilación *in situ* de las proteínas que forman parte de los tejidos (Estévez & Luna, 2017). El incremento más pronunciado de los

niveles de carbonilos proteicos y APOPs se registró en el tejido del colon de los animales que consumieron fructosa (Capítulo II), el cual pudo haber sido promovido por el ambiente intraluminal pernicioso descrito con anterioridad en el compartimento. Estos niveles destacan por aproximarse a los rangos descritos por otros autores en tejidos colónicos humanos con diferentes grados de tumores primarios colorrectales *(*Mehrabi *et al.*, 2015).

Además del efecto sobre los tejidos gastrointestinales, el consumo de fructosa tuvo un efecto sobre el metaboloma del contenido intraluminal colónico y la microbiota intestinal, tal y como otros autores describieron previamente en sus estudios (Beisner *et al.*, 2020; Kawano *et al.*, 2022). Comparando con los niveles de las heces, los resultados sugieren que mayores cantidades de carbonilos y APOPs fueron metabolizados y/o absorbidos en el compartimento colónico de las ratas que consumieron fructosa con respecto a las ratas control (Capítulo II). La cantidad de metabolitos implicados en el metabolismo energético, como el ácido láctico o el ácido pirúvico, se vieron aumentadas en dicho contenido intraluminal. Esto sugiere que efectivamente mayores tasas de metabolismo tuvieron lugar en este tramo, bien como consecuencia de los mecanismos inherentes al hospedador implicados en la absorción de compuestos, bien a partir de mecanismos derivados de la actividad microbiana.

Algunos géneros de bacterias probióticas como *Lactobacillus* o *Bifidobacterium* presentaron una menor expresión en las heces de las ratas que consumieron fructosa durante las 10 semanas del experimento (Capítulo II). Tanto trabajos que evaluaron el impacto de un consumo excesivo de fructosa sobre la microbiota intestinal (Cho et al., 2021; Song *et al.*, 2023), como trabajos que consideraron el efecto del consumo de proteínas oxidadas sobre el microbioma (Van Hecke *et al.*, 2021; Yin *et al.*, 2022) vieron el mismo efecto depresivo sobre estos géneros de bacterias. También especies de bacterias pertenecientes al género *Ruminococcaceae* y a géneros no clasificados de la familia *Lachnospiraceae* aumentaron de forma significativa en el microbioma de las ratas que fueron suplementadas con fructosa de forma sostenida, al igual que en otros trabajos que evaluaron su expresión tanto en presencia de fructosa (Lambertz *et al.*, 2017; Song *et al.*, 2023; Wang *et al.*, 2020), como en presencia de proteínas oxidadas (Amaretti *et al*., 2019; Van Hecke *et al.*, 2021).

La disminución de los niveles de *Bifidobacterium animalis* como consecuencia del consumo sostenido de altas concentraciones de fructosa resulta muy interesante desde la

perspectiva de la suplementación probiótica. Curiosamente, se trata además de una especie cuya disminución otros autores han relacionado con la presencia de AGEs en el colon (Snelson & Coughlan, 2019). Este hecho, junto con todo lo anterior, sustenta la hipótesis planteada que relaciona el consumo de fructosa con la presencia de proteínas glicoxidadas sin digerir en el colon, las cuales fomentarían un incremento del estrés glicoxidativo en el compartimento y la formación de este tipo de compuestos.

Cambios en la abundancia de los géneros bacterianos mencionados como consecuencia del consumo de fructosa pudieron ser los responsables de la menor presencia de compuestos implicados en el normal desarrollo de la mucosa intestinal, como la espermidina. Además, la mayor frecuencia detectada de especies del género *Desulfovibrio*, descrita cuando proteínas oxidadas son fermentadas en el colon, podría afectar a la integridad de la mucosa intestinal (Gilbert *et al.*, 2018). Este hecho podría explicar además la asociación que algunos autores han hecho entre el consumo de fructosa y la producción alterada de mucus por parte de los enterocitos (Song *et al*., 2023).

También se analizaron las alteraciones en el metabolismo colónico de diferentes aminoácidos cuando consumieron cantidades elevadas de fructosa de forma sostenida (Capítulo II). Así, se registraron mayores niveles de triptófano y ácido glutámico en el contenido luminal del colon de las ratas F, lo que sugiere un menor metabolismo de estos aminoácidos. Esto podría ser debido de forma plausible a la disminución de algunos géneros de microorganismos implicados en el mismo. También resulta interesante destacar la menor abundancia de histamina revelada por los análisis metabolómicos en el contenido colónico. Una mayor concentración de histamina se encuentra relacionada con una reducción de la producción de citoquinas proinflamatorias (Oliphant & Allen-Vercoe, 2019), lo cual podría explicar la sobreexpresión de algunos de los marcadores de inflamación descritos en los hígados de las ratas que consumieron fructosa (Capítulo III, cuyos resultados se discutirán con más detalle a continuación).

Por otro lado, son varios los trabajos que relacionan cambios en la microbiota como consecuencia del consumo sostenido de cantidades elevadas de fructosa con el desarrollo de dislipidemia y con un aumento del contenido graso hepático, entre otras alteraciones, debido a una mayor producción de metabolitos bacterianos como el ácido acético (Beisner *et al.*, 2020; Herman & Birnbaum, 2021; Zhao *et al.*, 2020). Sin embargo, el consumo de un 30% de fructosa en el agua de bebida por parte de las ratas reveló una disminución de la cantidad de ácido acético en el contenido intraluminal del colon de los animales,

relacionada posiblemente con la menor abundancia de los géneros probióticos descritos. A pesar de esto, los resultados mostrados en el Capítulo III de la presente tesis doctoral revelaron mayores contenidos de grasa en los hígados de las ratas que consumieron fructosa. Por ello, en este caso no se pudo establecer una relación directa entre ambos factores.

Como se viene describiendo, cuando se consumen excesivas cantidades de fructosa, el compuesto también podría alcanzar el hígado al saturar la capacidad del transportador intestinal implicado en las primeras etapas de su degradación (Jang *et al.*, 2018). El hígado es el órgano por excelencia relacionado con los efectos metabólicos perjudiciales asociados al consumo excesivo de fructosa. A pesar de esto, cada vez hay más evidencia de que el metabolismo intestinal juega un papel importante en muchos de estos efectos metabólicos negativos. Entre ellos destacarían la activación de mecanismos defensivos y proinflamatorios en respuesta al estrés glicoxidativo desencadenado por el azúcar. También la endotoxemia, promovida por el daño que la fructosa podría originar en la permeabilidad de la membrana intestinal, puede ser otro de los efectos perjudiciales asociados al consumo intenso de fructosa (Cho *et al*., 2021). Tanto la fructolisis intestinal como la hepática podrían incrementar el pool de triosas-fosfato hepático, el cual es compartido tanto por mecanismos implicados en el metabolismo energético como en el lipídico (Herman & Birnbaum, 2021). Las alteraciones de estas rutas metabólicas son las que relacionan el consumo excesivo de fructosa con diversas patologías como obesidad, hiperlipidemia, resistencia a la insulina o NAFLD, junto con las alteraciones previamente descritas de la microbiota intestinal (Beisner *et al*., 2020; Herman & Birnbaum, 2021; Miller & Adeli, 2008; Softic *et al.*, 2020).

Los resultados tratados en el Capítulo III de la presente tesis doctoral, el cual actualmente se encuentra en proceso de revisión por la revista *Journal of Nutritional Biochemistry*, revelaron que el mayor consumo calórico que las ratas ingirieron a partir de la solución con fructosa no supuso en ningún caso un aumento del peso corporal de los animales al final del estudio. Esto coincide con otros estudios que observaron una falta de efecto del consumo de altas cantidades de fructosa sobre el peso corporal de los individuos (Hsieh *et al.*, 2016; Ramos *et al.*, 2017; Wang *et al.*, 2020), a pesar de que otros autores relacionaron un alto consumo de fructosa con un aumento del peso de los animales, como Bray (2010) o Sandeva *et al.* (2015).

Por otra parte, la hiperlipidemia asociada al consumo excesivo de fructosa descrita en la bibliografía pudo evidenciarse en varios de los parámetros que se analizaron a lo largo del Capítulo III de la presente tesis doctoral. Entre ellos, el peso de los depósitos grasos de las ratas que consumieron fructosa, los cuales aumentaron en todos los casos, o el contenido de grasa hepático, que también mostró mayores niveles de forma significativa en los hígados de los animales que fueron suplementados únicamente con el azúcar. Este último hecho pudo evidenciarse de una manera más precisa a través de los análisis microscópicos. Estudios como el de Jürgens *et al.* (2005) o Sandeva *et al.* (2015) también relacionaron un consumo excesivo de fructosa con un aumento de los depósitos grasos de sus animales, si bien las condiciones de los ensayos no fueron exactamente las mismas. Otros estudios, por su parte, refirieron un aumento de la grasa hepática a consecuencia de consumos sostenidos y excesivos de fructosa, en línea con nuestros resultados (Varghese & Thomas, 2019; Meneses *et al.*, 2022; Sellmann *et al.*, 2015). Es importante destacar que el hígado graso, como un estadio previo a la NAFLD, resulta más vulnerable a cualquier daño originado por diversas causas, incrementando la probabilidad de que el órgano progrese a estadios más avanzados de la enfermedad (Sellmann *et al.*, 2015).

Los mecanismos moleculares que subyacen estos fenómenos según la bibliografía podrían estar relacionados con mayores tasas de lipogénesis *de novo* (DNL, del inglés *de novo lipogenesis),* así como con una disminución de los mecanismos de oxidación de ácidos grasos. Estos últimos participarían de alguna manera en la detoxificación de compuestos sintetizados celularmente en exceso (Herman & Birnbaum, 2021; Muriel *et al.*, 2021). Mediante un análisis proteómico de las células hepáticas descrito en el Capítulo IV de la presente tesis doctoral, se pudo dilucidar que efectivamente algunas proteínas involucradas tanto en la fructolisis como en la síntesis de lípidos, vieron aumentado su contenido tras un consumo excesivo de fructosa. Esto sustenta en todo caso la teoría de que llegan mayores cantidades de fructosa al hígado tras un consumo sostenido de un 30% de fructosa. Así, proteínas implicadas en la DNL, como la ATP citrato sintasa (ACLY) y la ácido graso sintasa (FASN), mostraron mayor abundancia en el proteoma hepático de las ratas que consumieron fructosa, con cantidades casi triplicadas, por ejemplo, en el caso de FASN. Estos resultados concuerdan con los expuestos por varios autores como Hsieh *et al*. (2016), Mastrocola et al. (2018) o Softic *et al.* (2017). A pesar de que se trata de resultados basados en transcriptómica y técnicas

dirigidas, a diferencia de nuestros resultados, que se apoyan en una robusta cuantificación diferencial de proteínas ya expresadas en el hígado de forma no dirigida.

Por otra parte, proteínas tanto mitocondriales, como peroxisomales y micosomales, relacionadas con la *β*- y la *ω*-oxidación de ácidos grasos en el hígado, vieron disminuida su abundancia en el proteoma hepático de las ratas F (Capítulo IV), lo cual está en consonancia con la bibliografía científica (Herman & Birnbaum, 2021; Muriel *et al*., 2021). La disminución de algunas de estas proteínas, como la carnitina palmitoiltransferasa 1A (CPT1A), encargada de la transposición de ácidos grasos a la mitocondria, o las subunidades 1, 2 y 3 de la acil-CoA oxidasa (ACOX), las cuales catalizan las primeras etapas de la *β*-oxidación peroxisomal, ha sido relacionada también por otros autores con un consumo excesivo de fructosa (Herman & Birnbaum, 2021; Miura, 2013; Novelle *et al.*, 2021). Sin embargo, gracias al ensayo no dirigido (Capítulo IV), se ha podido relacionar, por primera vez que nosotros sepamos, la disminución de algunas proteínas involucradas en la *ω*-oxidación de ácidos grasos con el consumo de fructosa en células hepáticas. Esto resulta de gran interés en el contexto de la instauración de la NAFLD, con la que algunos autores ya habían relacionado este tipo de oxidación (Browning & Horton, 2004; Fisher *et al.*, 2009).

El consumo de cantidades elevadas de fructosa aumentó la acreción de marcadores de glicación temprana de proteínas (pentosidina) en los hígados de los animales experimentales (Capítulo III de la presente tesis doctoral), en línea con otros trabajos que relacionaron un aumento hepático de AGEs ante consumos excesivos de fructosa (Hsieh *et al.*, 2016; Mastrocola *et al.*, 2016). El metabolismo de fructosa en el hígado podría promover un aumento del estrés glicoxidativo (Hsieh *et al.*, 2016), el cual podría jugar un papel en el daño al ADN y en la activación de mecanismos proinflamatorios *(Muriel et al.*, 2021). Así efectivamente lo sugiere la mayor infiltración de células inmunológicas revelada por los análisis microscópicos realizados en los hígados de los animales F descrita en el Capítulo III de la presente tesis doctoral.

Profundizando en los mecanismos moleculares que podrían verse implicados en estos cambios, el proteoma hepático de las ratas que consumieron fructosa durante el ensayo reveló una mayor abundancia de proteínas mitocondriales implicadas en la cadena transportadora de electrones (Capítulo IV). Siendo la fructolisis un proceso ATP-dependiente (Muriel *et al.,* 2021), el aumento de proteínas implicadas en la producción de energía y por ende en la producción de ROS era esperado. Todo esto, junto con las

menores cantidades de proteínas como la glutatión peroxidasa (GPX1) o diferentes subunidades de la glutatión S-transferasa (GST), involucradas en la defensa contra el estrés oxidativo reveladas por el análisis proteómico de los hígados F, podría ilustrar la conexión entre el consumo de fructosa y el daño hepático mediado por un aumento del estrés glicoxidativo. Además, este último efecto podría asociarse con respuestas inflamatorias y daños en el ADN, en consonancia con los trabajos de otros autores como Cioffi *et al.* (2017).

La interpretación de estos resultados podría resultar controvertida al compararse con los de otros autores, que relacionaron un aumento de GST cuando animales de experimentación consumieron cantidades elevadas de fructosa (Hsieh *et al.*, 2016). Los autores suministraron a ratas *Sprague-Dawley* (SD) una alimentación base con un 60% de fructosa durante 16 semanas, tras lo cual observaron un aumento de la cantidad de proteínas envueltas en la defensa antioxidante. Sin embargo, estas diferencias experimentales podrían explicar las diferencias los resultados expuestos en el Capítulo IV de la presente tesis doctoral.

Por otro lado, la disminución de la cantidad de proteínas hepáticas involucradas en la degradación de proteínas ubiquitinadas (proteasoma) como consecuencia del consumo sostenido de un 30% de fructosa durante 10 semanas podría sugerir que el daño glicoxidativo al que se hace mención en el párrafo anterior representaría la antesala de un acúmulo de proteínas hepáticas dañadas. En última instancia, este acúmulo podría llegar a resultar tóxico para las células (Goldberg, 2003).

A modo de resumen, la Figura 5.2 muestra de manera genérica los efectos perjudiciales promovidos por el consumo de fructosa sobre los parámetros analizados a lo largo de los Capítulos II, III y IV.

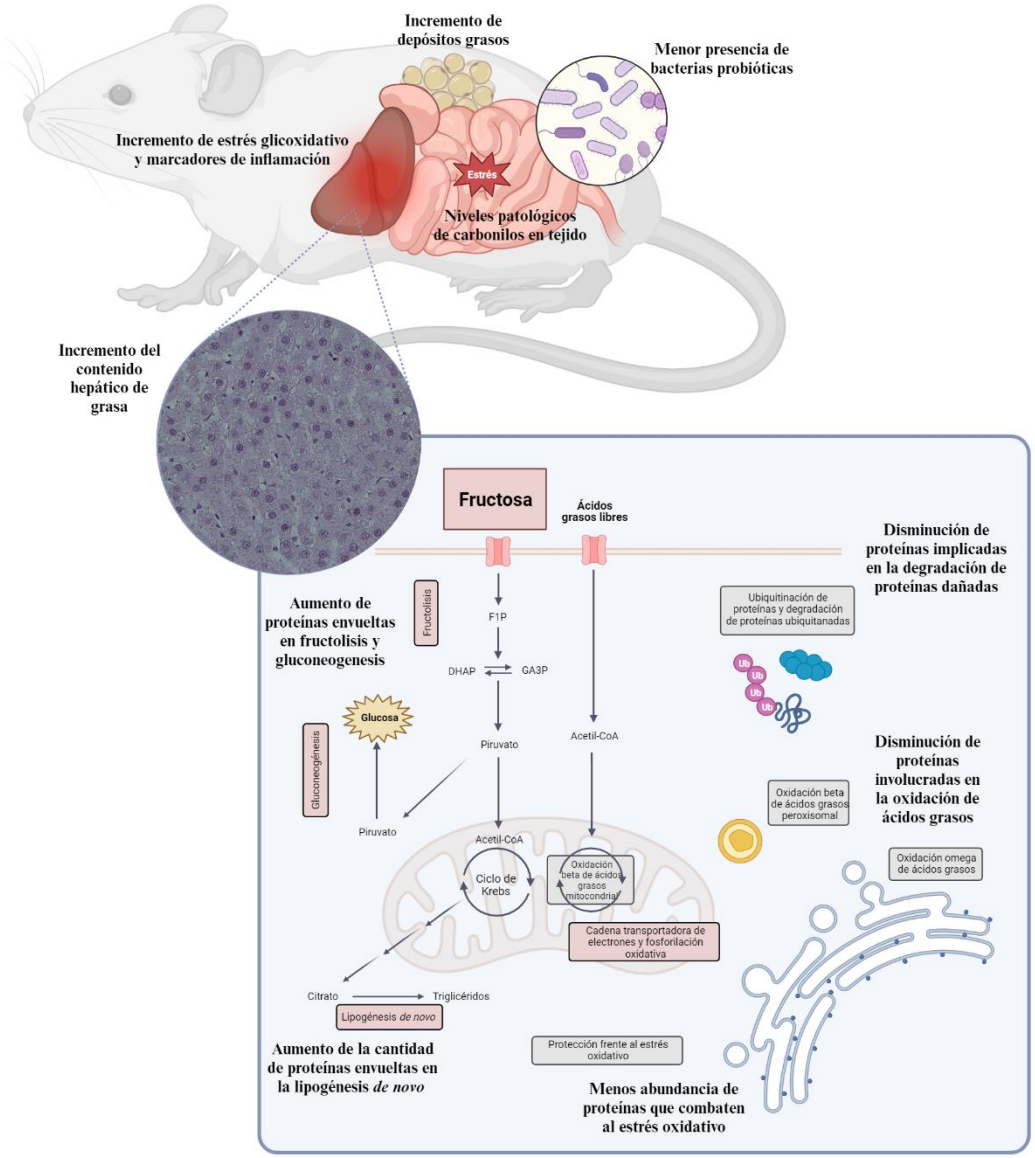

Figura 5.2. Resumen gráfico de los efectos de la fructosa sobre el metabolismo de las ratas (Capítulos II, III y IV). Elaboración propia.

5.3. Efecto del consumo de un suplemento rico en punicalagina sobre los efectos perjudiciales promovidos por la fructosa

La granada, considerada como un alimento funcional, es una de las frutas a la que más se ha atendido durante los últimos años en estudios que han tratado de analizar las propiedades bioactivas de algunos de sus polifenoles, como la punicalagina (PU) y el

ácido elágico (EA, del inglés *ellagic acid*) (Caballero *et al*., 2022; Jafarirad *et al.*, 2023; Noori *et al.*, 2017).

El Capítulo III de la presente tesis doctoral muestra los prometedores resultados que la suplementación con un extracto de granada rico en PU (P) en una solución de fructosa tuvo en el organismo de los animales que la consumieron, en comparación con los efectos indeseables que la solución de fructosa por sí sola ocasionó en el grupo correspondiente. Así, P promovió una disminución del peso de los depósitos grasos de los animales, al igual que una disminución del contenido hepático en grasa de estos.

Basándonos en los resultados proteómicos descritos en el Capítulo IV de la presente tesis doctoral, así como en trabajos previos que atribuyen a otros compuestos antioxidantes la capacidad de interrumpir la actividad de los transportadores intestinales de glucosa GLUT2 y GLUT5 (Varghese & Thomas, 2019; Merino *et al.*, 2020; Olvera-Sandoval *et al.*, 2022), los resultados sugieren que llegaron al hígado cantidades menores de fructosa tras el consumo de P. Tanto algunas proteínas envueltas en los procesos de fructolisis, como otras proteínas implicadas en la DNL y la oxidación de ácidos grasos, que tras el consumo de la solución de fructosa vieron afectada su abundancia, en presencia de P mostraron valores próximos a los del grupo control de animales.

Por otra parte, la suplementación con P también pareció estar detrás de la disminución de los valores hepáticos de pentosidina, uno de los marcadores de estrés glicoxidativo utilizado. Esto podría estar relacionado con un menor metabolismo de la fructosa en el hígado, reducido por las razones expuestas anteriormente, que en todo caso tendría un importante impacto disminuyendo la formación hepática de ROS y RCS. Igualmente, y posiblemente a consecuencia de la disminución de los niveles de estrés oxidativo, el consumo del suplemento rico en punicalagina pareció aliviar los recuentos hepáticos de células inmunológicas. Esto estaría en línea con diversos estudios que identifican en diferentes extractos de granadas diversas cantidades de compuestos bioactivos con propiedades antioxidantes y antiinflamatorias (Hou *et al.*, 2019; Mandal *et al.*, 2017). Si bien, a este respecto también podría atribuirse la acción de otros compuestos formados como consecuencia de la interacción de la microbiota con PU.

Sin embargo, que la captación de fructosa intestinal como primera etapa de la fructolisis se vea comprometida por una posible interacción entre los compuestos fenólicos del suplemento y los transportadores intestinales podría suponer que mayores cantidades fructosa lleguen a colon. De hecho, al analizar la microbiota de los animales que fueron

suplementados con P en la solución de fructosa, los resultados mostraron que efectivamente el impacto negativo de la fructosa sobre el microbioma estudiado en el Capítulo II se vio acentuado en este caso (Capítulo III). A pesar de la aún menor abundancia de especies de bacterias probióticas como *Lactobacillus* spp. o *Lactococcus* spp. en la microbiota de los animales F+P, la presencia de un género no clasificado perteneciente a la familia *Eggerthellaceae* spp. sugiere una potencial interacción entre PU y los microorganismos del tracto gastrointestinal. Varios autores, como Yin *et al*. (2023) o Zhang *et al*. (2023), describen en sus trabajos cómo la interacción entre compuestos fenólicos como PU o EA y microorganismos de los géneros *Gordonibacter* o *Eggerthellaceae* podría estar detrás de los efectos beneficiosos atribuidos a los compuestos fenólicos, principalmente mediante su conversión a urolitinas. Las urolitinas están descritas en la bibliografía como compuestos antioxidantes y con capacidad para mejorar la función de la barrera del tracto gastrointestinal (Singh *et al.*, 2019; Tomás-Barberán *et al.*, 2017).

Con el propósito de identificar algún metabolito potencialmente relacionado con las urolitinas, se llevó a cabo un análisis metabolómico de la orina de los animales de experimentación (Capítulo III). Mediante este análisis pudo detectarse un compuesto con fórmula y peso molecular idénticos a los de la Urolitina A: 1,3-dihidroxi-9H-xanten-9-ona. Este compuesto, identificado por primera vez en la orina de ratas suplementadas con un extracto rico en PU, reveló un patrón de fragmentación similar al descrito por García-Villalba *et al.* (2016) para la urolitina A, con algunas diferencias. De hecho, los espectros derivados de la cromatografía líquida mostraron un compuesto íntimamente relacionado con ella. La presencia por tanto de un derivado de urolitina A en la orina de los animales que consumieron P ayudaría a la comprensión de los efectos beneficiosos atribuidos al suplemento.

A este respecto, algunos de los resultados más relevantes de los análisis proteómicos en las células hepáticas de los animales que fueron suplementados con P y fructosa, además de los ya mencionados, fueron los siguientes: una mayor abundancia tanto de una proteína implicada en la fisiología normal de las mitocondrias, la proteína mitocondrial similar a la dinamina 120 kDa (OPA1), como de proteínas involucradas en la funcionalidad del espliceosoma, el cual es responsable de la regulación de la expresión génica y de la reparación del ADN. A pesar de que algunos autores han relacionado alteraciones mitocondriales con el comienzo de la NAFLD en condiciones similares a las expuestas

en la presente tesis doctoral (Mamikutty *et al.*, 2015), y otros con situaciones de diabetes e hiperlipidemia (Cao *et al.*, 2020; Zhang *et al.*, 2022), esta parece ser la primera ocasión en la que se relacionan alteraciones en la abundancia de proteínas mitocondriales con un efecto hepatoprotector por parte de un extracto rico en PU, frente a los efectos perjudiciales promovidos por un consumo excesivo de fructosa.

CONCLUSIONES

Conclusiones

- La presencia de glucosa en un sistema modelo a base de proteínas cárnicas promovió la glicoxidación de proteínas tras una digestión gástrica simulada, disminuyendo la digestibilidad de las mismas.

- El consumo de un 30% de fructosa en el agua de bebida durante 10 semanas (9 g/kg peso vivo/día) promovió la glicoxidación de proteínas en los compartimentos iniciales del tracto gastrointestinal de ratas *Wistar*, disminuyendo su digestibilidad y potenciando su paso a compartimentos posteriores en los cuales el ambiente prooxidante fue intensificado.

- El consumo de fructosa provocó cambios en la microbiota intestinal y el metaboloma del contenido colónico, disminuyendo géneros probióticos implicados en la síntesis de metabolitos neuroactivos y relacionados con la integridad de la mucosa intestinal.

- El metabolismo hepático de altas cantidades de fructosa pareció promover un aumento de los depósitos grasos de los animales de experimentación, así como del propio contenido graso de los hígados, lo cual podría desencadenar el desarrollo de la enfermedad del hígado graso no-alcohólico.

- Proteínas implicadas en el metabolismo hepático energético y lipídico vieron afectada su abundancia como consecuencia del consumo de fructosa. Esto promovió estrés oxidativo celular y alteraciones en los procesos de síntesis y oxidación de ácidos grasos, que en última instancia afectarían a la funcionalidad mitocondrial.

- Por su parte, la suplementación con un derivado de granada rico en punicalagina estaría implicada en la mejora de los parámetros afectados por la fructosa, siendo la plausible regulación de los niveles de fructosa que podrían estar llegando al hígado la responsable de ello.

- La punicalagina podría estar implicada en los efectos positivos atribuidos al suplemento por su potencial interacción con la microbiota. La detección de un metabolito relacionado con la urolitina A en la orina de los animales tratados con el suplemento de granada podría esclarecer la base molecular de los efectos beneficiosos

atribuidos al consumo del compuesto y la expresión de proteínas envueltas en la reparación celular.

REFERENCIAS

Referencias

Adachi, S. I., Sasaki, K., Kondo, S., Komatsu, W., Yoshizawa, F., Isoda, H., & Yagasaki, K. (2020). Antihyperuricemic effect of urolithin a in cultured hepatocytes and model mice. *Molecules*, *25*(21), 1–15. https://doi.org/10.3390/molecules25215136

Amaretti, A., Gozzoli, C., Simone, M., Raimondi, S., Righini, L., Pérez-Brocal, V., García-López, R., Moya, A., & Rossi, M. (2019). Profiling of protein degraders in cultures of human gut microbiota. *Frontiers in Microbiology*, *10*(November), 1–13. https://doi.org/10.3389/fmicb.2019.02614

Aragno, M., & Mastrocola, R. (2017). Dietary sugars and endogenous formation of advanced glycation endproducts: Emerging mechanisms of disease. *Nutrients*, *9*(4), 1–16. https://doi.org/10.3390/nu9040385

Bains, Y., Gugliucci, A., & Caccavello, R. (2017). Advanced glycation endproducts form during ovalbumin digestion in the presence of fructose : Inhibition by chlorogenic acid. *Fitoterapia*, *120*(May), 1–5. https://doi.org/10.1016/j.fitote.2017.05.003

Bechaux, J., de La Pomélie, D., Théron, L., Santé-Lhoutellier, V., & Gatellier, P. (2018). Iron-catalysed chemistry in the gastrointestinal tract: Mechanisms, kinetics and consequences. A review. *Food Chemistry*, *268*(June), 27–39. https://doi.org/10.1016/j.foodchem.2018.06.018

Beisner, J., Gonzalez-granda, A., Basrai, M., & Damms-machado, A. (2020). Fructose-induced intestinal microbiota shift. *Nutrients*, *12*, 1–21.

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., & Crowe, S. E. (2014). Oxidative stress: An essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews*, *94*(2), 329–354. https://doi.org/10.1152/physrev.00040.2012

Bourassa, J. L., Ives, E. P., Marqueling, A. L., Shimanovich, R., & Groves, J. T. (2001). Myoglobin catalyzes its own nitration. *Journal of the American Chemical Society*, *123*(21), 5142–5143. https://doi.org/10.1021/ja015621m

Bray, G. A. (2010). Soft drink consumption and obesity: It is all about fructose. *Current Opinion in Lipidology*, *21*(1), 51–57. https://doi.org/10.1097/MOL.0b013e3283346ca2

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. *Journal of Clinical Investigation*, *114*(2), 147–152. https://doi.org/10.1172/JCI200422422

Caballero, V., Estévez, M., Tomás-Barberán, F. A., Morcuende, D., Martín, I., & Delgado, J. (2022). Biodegradation of punicalagin into ellagic acid by selected probiotic bacteria: A study of the underlying mechanisms by MS-based proteomics. *Journal of Agricultural and Food Chemistry*, *70*(51), 16273–16285. https://doi.org/10.1021/acs.jafc.2c06585

Cao, K., Wang, K., Yang, M., Liu, X., Lv, W., & Liu, J. (2020). Punicalagin improves hepatic lipid metabolism: Via modulation of oxidative stress and mitochondrial biogenesis in hyperlipidemic mice. *Food and Function*, *11*(11), 9624–9633. https://doi.org/10.1039/d0fo01545h

Cerdá, B., Tomás-Barberán, F. A., & Espín, J. C. (2005). Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: Identification of biomarkers and individual variability. *Journal of Agricultural and Food Chemistry*, *53*(2), 227–235. https://doi.org/10.1021/jf049144d

Chen, P., Chen, F., Lei, J., & Zhou, B. (2023). Pomegranate polyphenol punicalagin improves learning memory deficits, redox homeostasis, and neuroinflammation in aging mice. *Phytotherapy Research*, *37*(9), 3655–3674. https://doi.org/10.1002/ptr.7848

Cho, Y. E., Kim, D. K., Seo, W., Gao, B., Yoo, S. H., & Song, B. J. (2021). Fructose promotes leaky gut, endotoxemia, and liver fibrosis through ethanol-inducible Cytochrome P450-2E1–mediated oxidative and nitrative stress. *Hepatology*, *73*(6), 2180–2195. https://doi.org/10.1002/hep.30652

Chusyd, D. E., Wang, D., Huffman, D. M., & Nagy, T. R. (2016). Relationships between

rodent white adipose fat pads and human white adipose fat depots. *Frontiers in Nutrition*, *3*(April). https://doi.org/10.3389/fnut.2016.00010

Cigliano, L., Spagnuolo, M. S., Crescenzo, R., Cancelliere, R., Iannotta, L., Mazzoli, A., Liverini, G., & Iossa, S. (2018). Short-term fructose feeding induces inflammation and oxidative stress in the hippocampus of young and adult rats. *Molecular Neurobiology*, *55*(4), 2869–2883. https://doi.org/10.1007/s12035-017-0518-2

Cioffi, F., Senese, R., Lasala, P., Ziello, A., Mazzoli, A., Crescenzo, R., Liverini, G., Lanni, A., Goglia, F., & Iossa, S. (2017). Fructose-rich diet affects mitochondrial DNA damage and repair in rats. *Nutrients*, *9*(4), 1–14. https://doi.org/10.3390/nu9040323

Davies, M. J. (2005). The oxidative environment and protein damage. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1703*(2), 93–109. https://doi.org/10.1016/j.bbapap.2004.08.007

de La Pomélie, D., Santé-Lhoutellier, V., & Gatellier, P. (2017). Mechanisms and kinetics of tryptophan N-nitrosation in a gastro-intestinal model. *Food Chemistry*, *218*, 487–495. https://doi.org/10.1016/j.foodchem.2016.08.131

de La Pomélie, D., Santé-Lhoutellier, V., & Gatellier, P. (2018). Mechanisms and kinetics of heme iron nitrosylation in an in vitro gastro-intestinal model. *Food Chemistry*, *239*, 86–93. https://doi.org/10.1016/j.foodchem.2017.06.092

de La Pomélie, D., Santé-Lhoutellier, V., Sayd, T., & Gatellier, P. (2018). Oxidation and nitrosation of meat proteins under gastro-intestinal conditions: Consequences in terms of nutritional and health values of meat. *Food Chemistry*, *243*(September 2017), 295–304. https://doi.org/10.1016/j.foodchem.2017.09.135

de La Pomélie, D., Santé-Lhoutellier, V., Sayd, T., Théron, L., & Gatellier, P. (2019). Using a dynamic artificial digestive system to investigate heme iron nitrosylation during gastro-intestinal transit. *Food Chemistry*, *281*(January), 231–235. https://doi.org/10.1016/j.foodchem.2018.12.094

Ding, R. xue, Goh, W. R., Wu, R. na, Yue, X. qing, Luo, X., Khine, W. W. T., Wu, J. rui, & Lee, Y. K. (2019). Revisit gut microbiota and its impact on human health and disease. *Journal of Food and Drug Analysis*, *27*(3), 623–631. https://doi.org/10.1016/j.jfda.2018.12.012

Dupont, D., Alric, M., Blanquet-Diot, S., Bornhorst, G., Cueva, C., Deglaire, A., Denis, S., Ferrua, M., Havenaar, R., Lelieveld, J., Mackie, A. R., Marzorati, M., Menard, O., Minekus, M., Miralles, B., Recio, I., & Van den Abbeele, P. (2019). Can dynamic in vitro digestion systems mimic the physiological reality? *Critical Reviews in Food Science and Nutrition*, *59*(10), 1546–1562. https://doi.org/10.1080/10408398.2017.1421900

Estévez, M. (2011). Protein carbonyls in meat systems: A review. *Meat Science*, *89*(3), 259–279. https://doi.org/10.1016/j.meatsci.2011.04.025

Estévez, M., Díaz-Velasco, S., & Martínez, R. (2021). Protein carbonylation in food and nutrition: a concise update. *Amino Acids*, *0123456789*. https://doi.org/10.1007/s00726-021-03085-6

Estévez, M., Kylli, P., Puolanne, E., Kivikari, R., & Heinonen, M. (2008). Fluorescence spectroscopy as a novel approach for the assessment of myofibrillar protein oxidation in oil-in-water emulsions. *Meat Science*, *80*(4), 1290–1296. https://doi.org/10.1016/j.meatsci.2008.06.004

Estévez, M., & Luna, C. (2017). Dietary protein oxidation: A silent threat to human health? *Critical Reviews in Food Science and Nutrition*, *57*(17), 3781–3793. https://doi.org/10.1080/10408398.2016.1165182

Estévez, M., & Xiong, Y. (2019). Intake of oxidized proteins and amino acids and causative oxidative stress and disease: Recent scientific evidences and hypotheses. *Journal of Food Science*, *84*(3), 387–396. https://doi.org/10.1111/1750-3841.14460

Fan, P., Li, L., Rezaei, A., Eslamfam, S., Che, D., & Ma, X. (2015). Metabolites of dietary protein and peptides by intestinal microbes and their impacts on gut. *Current Protein and Peptide Science*, *16*(7), 646–654.

Feng, X., Li, C., Jia, X., Guo, Y., Lei, N., Hackman, R. M., Chen, L., & Zhou, G. (2016). Influence of sodium nitrite on protein oxidation and nitrosation of sausages subjected to processing and storage. *Meat Science*, *116*, 260–267. https://doi.org/10.1016/j.meatsci.2016.01.017

Fisher, C. D., Lickteig, A. J., Augustine, L. M., Ranger-Moore, J., Jackson, J. P., Ferguson, S. S., & Cherrington, N. J. (2009). Hepatic cytochrome P450 enzyme alterations in humans with progressive stages of nonalcoholic fatty liver disease.

Drug Metabolism and Disposition, *37*(10), 2087–2094. https://doi.org/10.1124/dmd.109.027466

García-Berumen, C. I., Ortiz-Avila, O., Vargas-Vargas, M. A., Del Rosario-Tamayo, B. A., Guajardo-López, C., Saavedra-Molina, A., Rodríguez-Orozco, A. R., & Cortés-Rojo, C. (2019). The severity of rat liver injury by fructose and high fat depends on the degree of respiratory dysfunction and oxidative stress induced in mitochondria. *Lipids in Health and Disease*, *18*(1), 1–11. https://doi.org/10.1186/s12944-019-1024-5

García-Villalba, R., Espín, J. C., & Tomás-Barberán, F. A. (2016). Chromatographic and spectroscopic characterization of urolithins for their determination in biological samples after the intake of foods containing ellagitannins and ellagic acid. *Journal of Chromatography A*, *1428*, 162–175. https://doi.org/10.1016/j.chroma.2015.08.044

García-Villalba, R., Giménez-Bastida, J. A., Cortés-Martín, A., Ávila-Gálvez, M. Á., Tomás-Barberán, F. A., Selma, M. V., Espín, J. C., & González-Sarrías, A. (2022). Urolithins: a comprehensive update on their metabolism, bioactivity, and associated gut microbiota. *Molecular Nutrition and Food Research*, *66*(21). https://doi.org/10.1002/mnfr.202101019

Gilbert, M. S., Ijssennagger, N., Kies, A. K., & van Mil, S. W. C. (2018). Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs, and poultry. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *315*(2), G159–G170. https://doi.org/10.1152/ajpgi.00319.2017

Giromini, C., Cheli, F., Rebucci, R., & Baldi, A. (2019). Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. *Journal of Dairy Science*, *102*(2), 929–942. https://doi.org/10.3168/jds.2018-15163

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature*, *426*(6968), 895–899. https://doi.org/10.1038/nature02263

González-Sarrías, A., Espín, J. C., & Tomás-Barberán, F. A. (2017). Non-extractable polyphenols produce gut microbiota metabolites that persist in circulation and show anti-inflammatory and free radical-scavenging effects. *Trends in Food Science and Technology*, *69*, 281–288. https://doi.org/10.1016/j.tifs.2017.07.010

Gugliucci, A. (2017). Formation of fructose-mediated advanced glycation end products and their roles in metabolic and inflammatory diseases. *Advances in Nutrition*, *8*(1), 54–62. https://doi.org/10.3945/an.116.013912

Guo, P., Wang, H., Ji, L., Song, P., & Ma, X. (2021). Impacts of fructose on intestinal barrier function, inflammation and microbiota in a piglet model. *Nutrients*, *13*(10), 1–12. https://doi.org/10.3390/nu13103515

Handa, O., Naito, Y., & Yoshikawa, T. (2010). Helicobacter pylori: A ROS-inducing bacterial species in the stomach. *Inflammation Research*, *59*(12), 997–1003. https://doi.org/10.1007/s00011-010-0245-x

Hannou, S. A., Haslam, D. E., McKeown, N. M., & Herman, M. A. (2018). Fructose metabolism and metabolic disease. *Journal of Clinical Investigation*, *128*(2), 545–555. https://doi.org/10.1172/JCI96702

He, J., Zhou, G., Bai, Y., Wang, C., Zhu, S., Xu, X., & Li, C. (2018). The effect of meat processing methods on changes in disulfide bonding and alteration of protein structures: Impact on protein digestion products. *RSC Advances*, *8*(31), 17595–17605. https://doi.org/10.1039/c8ra02310g

Hecker, M., & Wagner, A. H. (2018). Role of protein carbonylation in diabetes. *Journal of Inherited Metabolic Disease*, *41*(1), 29–38. https://doi.org/10.1007/s10545-017-0104-9

Herman, M. A., & Birnbaum, M. J. (2021). Molecular aspects of fructose metabolism and metabolic disease. *Cell Metabolism*, *33*(12), 2329–2354. https://doi.org/10.1016/j.cmet.2021.09.010

Honikel, K. O. (2008). The use and control of nitrate and nitrite for the processing of meat products. *Meat Science*, *78*(1–2), 68–76. https://doi.org/10.1016/j.meatsci.2007.05.030

Hou, C., Zhang, W., Li, J., Du, L., Lv, O., Zhao, S., & Li, J. (2019). Beneficial Effects of Pomegranate on Lipid Metabolism in Metabolic Disorders. *Molecular Nutrition and Food Research*, *63*(16), 1–12. https://doi.org/10.1002/mnfr.201800773

Hsieh, C. C., Liao, C. C., Liao, Y. C., Hwang, L. S., Wu, L. Y., & Hsieh, S. C. (2016). Proteomic changes associated with metabolic syndrome in a fructose-fed rat model. *Journal of Food and Drug Analysis*, *24*(4), 754–761.

https://doi.org/10.1016/j.jfda.2016.03.005

Hua, Q., Han, Y., Zhao, H., Zhang, H., Yan, B., Pei, S., He, X., Li, Y., Meng, X., Chen, L., Zhong, F., & Li, D. (2022). Punicalagin alleviates renal injury: Via the gut-kidney axis in high-fat diet-induced diabetic mice. *Food and Function*, *13*(2), 867–879. https://doi.org/10.1039/d1fo03343c

Jafarirad, S., Goodarzi, R., Mohammadtaghvaei, N., Dastoorpoor, M., & Alavinejad, P. (2023). Effectiveness of the pomegranate extract in improving hepatokines and serum biomarkers of non-alcoholic fatty liver disease: A randomized double blind clinical trial. *Diabetes and Metabolic Syndrome: Clinical Research and Reviews*, *17*(1), 102693. https://doi.org/10.1016/j.dsx.2022.102693

Jaiswal, N., Maurya, C. K., Arha, D., Avisetti, D. R., Prathapan, A., Raj, P. S., Raghu, K. G., Kalivendi, S. V., & Tamrakar, A. K. (2015). Fructose induces mitochondrial dysfunction and triggers apoptosis in skeletal muscle cells by provoking oxidative stress. *Apoptosis*, *20*(7), 930–947. https://doi.org/10.1007/s10495-015-1128-y

Jang, C., Hui, S., Lu, W., Cowan, A. J., Morscher, R. J., Lee, G., Liu, W., Tesz, G. J., Birnbaum, M. J., & Rabinowitz, J. D. (2018). The small intestine converts dietary fructose into glucose and organic acids. *Cell Metabolism*, *27*(2), 351-361.e3. https://doi.org/10.1016/j.cmet.2017.12.016

Janssens, S., Ciapaite, J., Wolters, J. C., van Riel, N. A., Nicolay, K., & Prompers, J. J. (2017). An in vivo magnetic resonance spectroscopy study of the effects of caloric and non-caloric sweeteners on liver lipid metabolism in rats. *Nutrients*, *9*(5), 1–19. https://doi.org/10.3390/nu9050476

Jiang, H., Lin, Q., Ma, L., Luo, S., Jiang, X., Fang, J., & Lu, Z. (2021). Fructose and fructose kinase in cancer and other pathologies. *Journal of Genetics and Genomics*, *48*(7), 531–539. https://doi.org/10.1016/j.jgg.2021.06.006

Jürgens, H., Haass, W., Castañeda, T. R., Schürmann, A., Koebnick, C., Dombrowski, F., Otto, B., Nawrocki, A. R., Scherer, P. E., Spranger, J., Ristow, M., Joost, H. G., Havel, P. J., & Tschöp, M. H. (2005). Consuming fructose-sweetened beverages increases body adiposity in mice. *Obesity Research*, *13*(7), 1145–1156. https://doi.org/10.1038/oby.2005.136

Kandylis, P., & Kokkinomagoulos, E. (2020). Food applications and potential health

benefits of pomegranate and its ferivates. *Foods*, *9*(122). https://doi.org/doi:10.3390/foods9020122

Kanner, J., & Lapidot, T. (2001). The stomach as a bioreactor: Dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biology and Medicine*, *31*(11), 1388–1395. https://doi.org/10.1016/S0891-5849(01)00718-3

Kanner, J., Selhub, J., Shpaizer, A., Rabkin, B., Shacham, I., & Tirosh, O. (2017). Redox homeostasis in stomach medium by foods: The postprandial oxidative stress index (POSI) for balancing nutrition and human health. *Redox Biology*, *12*(April), 929–936. https://doi.org/10.1016/j.redox.2017.04.029

Karwowska, M., & Kononiuk, A. (2020). Nitrates/nitrites in food—risk for nitrosative stress and benefits. *Antioxidants*, *9*(3), 1–17. https://doi.org/10.3390/antiox9030241

Kawano, Y., Edwards, M., Huang, Y., Bilate, A. M., Araujo, L. P., Tanoue, T., Atarashi, K., Ladinsky, M. S., Reiner, S. L., Wang, H. H., Mucida, D., Honda, K., & Ivanov, I. I. (2022). Microbiota imbalance induced by dietary sugar disrupts immune-mediated protection from metabolic syndrome. *Cell*, *185*(19), 3501-3519.e20. https://doi.org/10.1016/j.cell.2022.08.005

Kim, B. R., Shin, J., Guevarra, R. B., Lee, J. H., Kim, D. W., Seol, K. H., Lee, J. H., Kim, H. B., & Isaacson, R. E. (2017). Deciphering diversity indices for a better understanding of microbial communities. *Journal of Microbiology and Biotechnology*, *27*(12), 2089–2093. https://doi.org/10.4014/jmb.1709.09027

Lambertz, J., Weiskirchen, S., Landert, S., & Weiskirchen, R. (2017). Fructose: A dietary sugar in crosstalk with microbiota contributing to the development and progression of non-alcoholic liver disease. *Frontiers in Immunology*, *8*(SEP). https://doi.org/10.3389/fimmu.2017.01159

Li, C. Q., Pignatelli, B., & Ohshima, H. (2001). Increased oxidative and nitrative stress in human stomach associated with cagA+ Helicobacter pylori infection and inflammation. *Digestive Diseases and Sciences*, *46*(4), 836–844. https://doi.org/10.1023/A:1010764720524

Li, L., Liu, Y., Zou, X., He, J., Xu, X., Zhou, G., & Li, C. (2017). In vitro protein digestibility of pork products is affected by the method of processing. *Food*

Research International, *92*, 88–94. https://doi.org/10.1016/j.foodres.2016.12.024

Liu, F., Smith, A. D., Wang, T. T. Y., Pham, Q., Yang, H., & Li, R. W. (2023). Multi-omics analysis detected multiple pathways by which pomegranate punicalagin exerts its biological effects in modulating host-microbiota interactions in murine colitis models. *Food and Function*, *14*(8), 3824–3837. https://doi.org/10.1039/d3fo00286a

Luna, C., & Estévez, M. (2019). Formation of allysine in β-lactoglobulin and myofibrillar proteins by glyoxal and methylglyoxal: Impact on water-holding capacity and in vitro digestibility. *Food Chemistry*, *271*(April 2018), 87–93. https://doi.org/10.1016/j.foodchem.2018.07.167

Lund, M. N., Heinonen, M., Baron, C. P., & Estévez, M. (2011). Protein oxidation in muscle foods: A review. *Molecular Nutrition and Food Research*, *55*(1), 83–95. https://doi.org/10.1002/mnfr.201000453

Ma, Y., Zhang, L., Rong, S., Qu, H., Zhang, Y., Chang, D., Pan, H., & Wang, W. (2013). Relation between gastric cancer and protein oxidation, DNA damage, and lipid peroxidation. *Oxidative Medicine and Cellular Longevity*, *2013*. https://doi.org/10.1155/2013/543760

Maillard, L. C. (1912). Action des acides amines sur les sucres; formation des melanoidies par voie methodique. *CR. Acad. Sci.*, *154*, 66–68.

Mamikutty, N., Thent, Z. C., & Haji Suhaimi, F. (2015). Fructose-drinking water induced nonalcoholic fatty liver disease and ultrastructural alteration of hepatocyte mitochondria in Male wistar rat. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/895961

Mandal, A., Bhatia, D., & Bishayee, A. (2017). Anti-inflammatory mechanism involved in pomegranate-mediated prevention of breast cancer: The role of NF-κB and Nrf2 signaling pathways. *Nutrients*, *9*(5), 1–13. https://doi.org/10.3390/nu9050436

Martinez-Saez, N., Fernandez-Gomez, B., Cai, W., Uribarri, J., & del Castillo, M. D. (2019). In vitro formation of Maillard reaction products during simulated digestion of meal-resembling systems. *Food Research International*, *118*(September 2017), 72–80. https://doi.org/10.1016/j.foodres.2017.09.056

Mary Varghese, S., & Thomas, J. (2019). The effect of *M. latifolia* leaf extract on high-fructose corn syrup (HFCS)-induced non-alcoholic fatty liver disease in rat models

. *Nonalcoholic Fatty Liver Disease - An Update*. https://doi.org/10.5772/intechopen.82200

Mastrocola, R., Collino, M., Rogazzo, M., Medana, C., Nigro, D., Boccuzzi, G., & Aragno, M. (2013). Advanced glycation end products promote hepatosteatosis by interfering with SCAP-SREBP pathway in fructose-drinking mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *305*(6), 398–407. https://doi.org/10.1152/ajpgi.00450.2012

Mastrocola, R., Ferrocino, I., Liberto, E., Chiazza, F., Cento, A. S., Collotta, D., Querio, G., Nigro, D., Bitonto, V., Cutrin, J. C., Rantsiou, K., Durante, M., Masini, E., Aragno, M., Cordero, C., Cocolin, L., & Collino, M. (2018). Fructose liquid and solid formulations differently affect gut integrity, microbiota composition and related liver toxicity: a comparative in vivo study. *Journal of Nutritional Biochemistry*, *55*, 185–199. https://doi.org/10.1016/j.jnutbio.2018.02.003

Mastrocola, R., Nigro, D., Chiazza, F., Medana, C., Dal Bello, F., Boccuzzi, G., Collino, M., & Aragno, M. (2016). Fructose-derived advanced glycation end-products drive lipogenesis and skeletal muscle reprogramming via SREBP-1c dysregulation in mice. *Free Radical Biology and Medicine*, *91*, 224–235. https://doi.org/10.1016/j.freeradbiomed.2015.12.022

Mehrabi, S., Wallace, L., Cohen, S., Yao, X., & Aikhionbare, F. O. (2015). Differential measurements of oxidatively modified proteins in colorectal adenopolyps. *International Journal of Clinical Medicine*, *6*(4), 288–299. https://doi.org/10.4236/ijcm.2015.64037.

Mehta, R., Sonovane, M., Migaud, M. E., & Gassman, N. R. (2017). Exogenous exposure to dihydroxyacetone mimics high fructose induced oxidative stress and mitochondrial dysfunction. *Environ Mol Mutagen*, *62*(3), 185–202. https://doi.org/10.1002/em.22425.Exogenous

Meneses, M. J., Sousa-Lima, I., Jarak, I., Raposo, J. F., Alves, M. G., & Macedo, M. P. (2022). Distinct impacts of fat and fructose on the liver, muscle, and adipose tissue metabolome: An integrated view. *Frontiers in Endocrinology*, *13*(August), 1–16. https://doi.org/10.3389/fendo.2022.898471

Merino, B., Fernández-Díaz, C. M., Cózar-Castellano, I., & Perdomo, G. (2020). Intestinal fructose and glucose metabolism in health and disease. *Nutrients*, *12*(1),

1–35. https://doi.org/10.3390/nu12010094

Miller, A., & Adeli, K. (2008). Dietary fructose and the metabolic syndrome. *Current Opinion in Gastroenterology*, *24*, 204–209. https://doi.org/10.3390/nu11091987

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., MacIerzanka, A., MacKie, A., … Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food-an international consensus. *Food and Function*, *5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j

Mitra, B., Lametsch, R., Akcan, T., & Ruiz-Carrascal, J. (2018). Pork proteins oxidative modifications under the influence of varied time-temperature thermal treatments: A chemical and redox proteomics assessment. *Meat Science*, *140*(March), 134–144. https://doi.org/10.1016/j.meatsci.2018.03.011

Miura, Y. (2013). The biological significance of ω-oxidation of fatty acids. *Proceedings of the Japan Academy Series B: Physical and Biological Sciences*, *89*(8), 370–382. https://doi.org/10.2183/pjab.89.370

Moreno, I., Ladero, L., & Cava, R. (2020). Effect of the Iberian pig rearing system on blood plasma antioxidant status and oxidative stress biomarkers. *Livestock Science*, *235*(March), 104006. https://doi.org/10.1016/j.livsci.2020.104006

Muriel, P., López-sánchez, P., & Ramos-tovar, E. (2021). Fructose and the liver. *International Journal of Molecular Sciences*, *22*(13). https://doi.org/10.3390/ijms22136969

Namdar, A. B., Ghafouridehgolan, F., Rakhshandeh, H., Sanaei, H., Jarrahi, L., Mohammadmousaei, F., & Rajabian, A. (2023). Effect of Punica granatum Extract Supplementation in Patients With Liver Cirrhosis: A Randomised, Double-Blind, Placebo-Controlled Trial. *Journal of Herbal Medicine*, *42*(October), 100795. https://doi.org/10.1016/j.hermed.2023.100795

Noori, M., Jafari, B., & Hekmatdoost, A. (2017). Pomegranate juice prevents development of non-alcoholic fatty liver disease in rats by attenuating oxidative stress and inflammation. *Journal of the Science of Food and Agriculture*, *97*(8), 2327–2332. https://doi.org/10.1002/jsfa.8042

Novelle, M. G., Bravo, S. B., Deshons, M., Iglesias, C., García-Vence, M., Annells, R., da Silva Lima, N., Nogueiras, R., Fernández-Rojo, M. A., Diéguez, C., & Romero-Picó, A. (2021). Impact of liver-specific GLUT8 silencing on fructose-induced inflammation and omega oxidation. *IScience*, *24*(2). https://doi.org/10.1016/j.isci.2021.102071

Oliphant, K., & Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. *Microbiome*, *7*(1), 1–15. https://doi.org/10.1186/s40168-019-0704-8

Olvera-Sandoval, C., Fabela-Illescas, H. E., Fernández-Martínez, E., Ortiz-Rodríguez, M. A., Cariño-Cortés, R., Ariza-Ortega, J. A., Hernández-González, J. C., Olivo, D., Valadez-Vega, C., Belefant-Miller, H., & Betanzos-Cabrera, G. (2022). Potential mechanisms of the improvement of glucose homeostasis in type 2 diabetes by pomegranate juice. *Antioxidants*, *11*(3), 1–13. https://doi.org/10.3390/antiox11030553

Oueslati, K., De La Pomélie, D., Santé-Lhoutellier, V., & Gatellier, P. (2016). Impact of the Fenton process in meat digestion as assessed using an in vitro gastro-intestinal model. *Food Chemistry*, *209*, 43–49. https://doi.org/10.1016/j.foodchem.2016.04.041

Papadopoulos, G., Legaki, A. I., Georgila, K., Vorkas, P., Giannousi, E., Stamatakis, G., Moustakas, I. I., Petrocheilou, M., Pyrina, I., Gercken, B., Kassi, E., Chavakis, T., Pateras, I. S., Panayotou, G., Gika, H., Samiotaki, M., Eliopoulos, A. G., & Chatzigeorgiou, A. (2023). Integrated omics analysis for characterization of the contribution of high fructose corn syrup to non-alcoholic fatty liver disease in obesity. *Metabolism: Clinical and Experimental*, *144*(July 2022), 155552. https://doi.org/10.1016/j.metabol.2023.155552

Portune, K. J., Beaumont, M., Davila, A. M., Tomé, D., Blachier, F., & Sanz, Y. (2016). Gut microbiota role in dietary protein metabolism and health-related outcomes: The two sides of the coin. *Trends in Food Science and Technology*, *57*, 213–232. https://doi.org/10.1016/j.tifs.2016.08.011

Rahbar, S., Blumenfeld, O., & Ranney, H. M. (1969). Studies of an unusual hemoglobin in patients with diabetes mellitus. *Biochemical Journal*, *36*(5).

Rajasekar, P., & Anuradha, C. V. (2007). Fructose-induced hepatic gluconeogenesis:

Effect of l-carnitine. *Life Sciences*, *80*(13), 1176–1183. https://doi.org/10.1016/j.lfs.2006.12.010

Rajesh, R., & Sreekala, V. (2020). High fructose diet-induced metabolic syndrome and the functional abnormalities in the liver and kidney of Wistar albino rats. *National Journal of Physiology, Pharmacy and Pharmacology*, *11*(0), 1. https://doi.org/10.5455/njppp.2021.11.10266202006102020

Ramos, V. W., Batista, L. O., & Albuquerque, K. T. (2017). Effects of fructose consumption on food intake and biochemical and body parameters in Wistar rats. *Revista Portuguesa de Cardiologia (English Edition)*, *36*(12), 937–941. https://doi.org/10.1016/j.repce.2017.04.009

Richards, J. L., Yap, Y. A., McLeod, K. H., MacKay, C. R., & Marinõ, E. (2016). Dietary metabolites and the gut microbiota: An alternative approach to control inflammatory and autoimmune diseases. *Clinical and Translational Immunology*, *5*(February). https://doi.org/10.1038/cti.2016.29

Rodríguez-Romero, J. de J., Durán-Castañeda, A. C., Cárdenas-Castro, A. P., Sánchez-Burgos, J. A., Zamora-Gasga, V. M., & Sáyago-Ayerdi, G. S. (2022). What we know about protein gut metabolites: Implications and insights for human health and diseases. *Food Chemistry: X*, *13*(November 2021). https://doi.org/10.1016/j.fochx.2021.100195

Ruiz-Carrascal, J. (2015). Cured foods: Health effects. *Encyclopedia of Food and Health*, 338–342. https://doi.org/10.1016/B978-0-12-384947-2.00207-5

Rysman, T., Van Hecke, T., Van Poucke, C., De Smet, S., & Van Royen, G. (2016). Protein oxidation and proteolysis during storage and in vitro digestion of pork and beef patties. *Food Chemistry*, *209*, 177–184. https://doi.org/10.1016/j.foodchem.2016.04.027

Sandeva, R. V., Mihaylova, S. M., Sandeva, G. N., Trifonova, K. Y., & Popova-Katsarova, R. D. (2015). Effect of high-fructose solution on body weight, body fat, blood glucose and triglyceride levels in rats. *Journal of Biomedical and Clinical Research*, *8*(1), 5–8. https://doi.org/10.1515/jbcr-2015-0143

Santé-Lhoutellier, V., Astruc, T., Marinova, P., Greve, E., & Gatellier, P. (2008). Effect of meat cooking on physicochemical state and in vitro digestibility of myofibrillar

proteins. *Journal of Agricultural and Food Chemistry*, *56*(4), 1488–1494. https://doi.org/10.1021/jf072999g

Sante-Lhoutellier, V., Aubry, L., & Gatellier, P. (2007). Effect of oxidation on in vitro digestibility of skeletal muscle myofibrillar proteins. *Journal of Agricultural and Food Chemistry*, *55*(13), 5343–5348. https://doi.org/10.1021/jf070252k

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. *Pharmacological Research*, *69*(1), 52–60. https://doi.org/10.1016/j.phrs.2012.10.020

Sellmann, C., Priebs, J., Landmann, M., Degen, C., Engstler, A. J., Jin, C. J., Gärttner, S., Spruss, A., Huber, O., & Bergheim, I. (2015). Diets rich in fructose, fat or fructose and fat alter intestinal barrier function and lead to the development of nonalcoholic fatty liver disease over time. *Journal of Nutritional Biochemistry*, *26*(11), 1183–1192. https://doi.org/10.1016/j.jnutbio.2015.05.011

Selma, M. V., Beltrán, D., García-Villalba, R., Espín, J. C., & Tomás-Barberán, F. A. (2014). Description of urolithin production capacity from ellagic acid of two human intestinal Gordonibacter species. *Food and Function*, *5*(8), 1779–1784. https://doi.org/10.1039/c4fo00092g

Semchyshyn, H. M. (2013). Fructation in vivo: Detrimental and protective effects of fructose. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/343914

Shi, J., Zhao, D., Song, S., Zhang, M., Zamaratskaia, G., Xu, X., Zhou, G., & Li, C. (2020). High-meat-protein high-fat diet induced dysbiosis of gut microbiota and tryptophan metabolism in Wistar rats. *Journal of Agricultural and Food Chemistry*, *68*(23), 6333–6346. https://doi.org/10.1021/acs.jafc.0c00245

Shi, Y. N., Liu, Y. J., Xie, Z., & Zhang, W. J. (2021). Fructose and metabolic diseases: Too much to be good. *Chinese Medical Journal*, *134*(11), 1276–1285. https://doi.org/10.1097/CM9.0000000000001545

Singh, R., Chandrashekharappa, S., Bodduluri, S. R., Baby, B. V., Hegde, B., Kotla, N. G., Hiwale, A. A., Saiyed, T., Patel, P., Vijay-Kumar, M., Langille, M. G. I., Douglas, G. M., Cheng, X., Rouchka, E. C., Waigel, S. J., Dryden, G. W., Alatassi, H., Zhang, H. G., Haribabu, B., … Jala, V. R. (2019). Enhancement of the gut barrier

integrity by a microbial metabolite through the Nrf2 pathway. *Nature Communications*, *10*(1). https://doi.org/10.1038/s41467-018-07859-7

Skibsted, L. H. (2011). Nitric oxide and quality and safety of muscle based foods. *Nitric Oxide - Biology and Chemistry*, *24*(4), 176–183. https://doi.org/10.1016/j.niox.2011.03.307

Snelson, M., & Coughlan, M. T. (2019). Dietary advanced glycation end products: Digestion, metabolism and modulation of gut microbial ecology. *Nutrients*, *11*(2). https://doi.org/10.3390/nu11020215

Softic, S., Cohen, D. E., & Kahn, C. R. (2016). Role of dietary fructose and hepatic de novo Lipogenesis in Fatty Liver Disease. *Digestive Diseases and Sciences*, *61*(5), 1282–1293. https://doi.org/10.1007/s10620-016-4054-0

Softic, S., Gupta, M. K., Wang, G. X., Fujisaka, S., O'Neill, B. T., Rao, T. N., Willoughby, J., Harbison, C., Fitzgerald, K., Ilkayeva, O., Newgard, C. B., Cohen, D. E., & Kahn, C. R. (2017). Divergent effects of glucose and fructose on hepatic lipogenesis and insulin signaling. *Journal of Clinical Investigation*, *127*(11), 4059–4074. https://doi.org/10.1172/JCI94585

Softic, S., Stanhope, K. L., Boucher, J., Divanovic, S., Lanaspa, M. A., Johnson, R. J., & Kahn, C. R. (2020). Fructose and hepatic insulin resistance. *Critical Reviews in Clinical Laboratory Sciences*, *57*(5), 308–322. https://doi.org/10.1080/10408363.2019.1711360

Soladoye, O. P., Juárez, M. L., Aalhus, J. L., Shand, P., & Estévez, M. (2015). Protein oxidation in processed meat: Mechanisms and potential implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, *14*(2), 106–122. https://doi.org/10.1111/1541-4337.12127

Song, G., Gan, Q., Qi, W., Wang, Y., & Xu, M. (2023). Fructose stimulated colonic arginine and proline metabolism dysbiosis, altered microbiota and aggravated intestinal barrier dysfunction in DSS-induced colitis Rats. *Nutrients*, *15*, 782. https://doi.org/10.3390/nu15030782

Song, P., Wu, L., & Guan, W. (2015). Dietary nitrates, nitrites, and nitrosamines intake and the risk of gastric cancer: A meta-analysis. *Nutrients*, *7*(12), 9872–9895. https://doi.org/10.3390/nu7125505

Spagnuolo, M. S., Iossa, S., & Cigliano, L. (2021). Sweet but bitter: Focus on fructose impact on brain function in rodent models. *Nutrients*, *13*(1), 1–18. https://doi.org/10.3390/nu13010001

Tian, T., Wang, Z., & Zhang, J. (2017). Pathomechanisms of oxidative stress in inflammatory bowel disease and potential antioxidant therapies. *Oxidative Medicine and Cellular Longevity*, *2017*. https://doi.org/10.1155/2017/4535194

Tomás-Barberán, F. A., González-Sarrías, A., García-Villalba, R., Núñez-Sánchez, M. A., Selma, M. V., García-Conesa, M. T., & Espín, J. C. (2017). Urolithins, the rescue of "old" metabolites to understand a "new" concept: Metabotypes as a nexus among phenolic metabolism, microbiota dysbiosis, and host health status. *Molecular Nutrition and Food Research*, *61*(1). https://doi.org/10.1002/mnfr.201500901

Twarda-clapa, A., Olczak, A., Białkowska, A. M., & Koziołkiewicz, M. (2022). Advanced Glycation End-Products (AGEs): Formation, chemistry, classification, receptors, and diseases related to AGEs. *Cells*, *11*(1312). https://doi.org/https://doi.org/ 10.3390/cells11081312

Van Hecke, T., Basso, V., & De Smet, S. (2018). Lipid and protein oxidation during in vitro gastrointestinal digestion of pork under Helicobacter pylori gastritis conditions. *Journal of Agricultural and Food Chemistry*, *66*(49), 13000–13010. https://doi.org/10.1021/acs.jafc.8b04335

Van Hecke, T., Van Camp, J., & De Smet, S. (2017). Oxidation during digestion of meat: Interactions with the diet and Helicobacter pylori gastritis, and implications on human health. *Comprehensive Reviews in Food Science and Food Safety*, *16*(2), 214–233. https://doi.org/10.1111/1541-4337.12248

Van Hecke, T., Vossen, E., Goethals, S., Boon, N., De Vrieze, J., & De Smet, S. (2021). In vitro and in vivo digestion of red cured cooked meat: oxidation, intestinal microbiota and fecal metabolites. *Food Research International*, *142*(September 2020), 110203. https://doi.org/10.1016/j.foodres.2021.110203

Ventanas, S., Estevez, M., Tejeda, J. F., & Ruiz, J. (2006). Protein and lipid oxidation in Longissimus dorsi and dry cured loin from Iberian pigs as affected by crossbreeding and diet. *Meat Science*, *72*(4), 647–655. https://doi.org/10.1016/j.meatsci.2005.09.011

Verzijl, N., Bank, R. A., TeKoppele, J. M., & DeGroot, J. (2003). AGEing and osteoarthritis: a different perspective. *Current Opinion in Rheumatology*, *15*(5), 616–622.

Villaverde, A., Parra, V., & Estévez, M. (2014). Oxidative and nitrosative stress induced in myofibrillar proteins by a hydroxyl-radical-generating system: Impact of nitrite and ascorbate. *Journal of Agricultural and Food Chemistry*, *62*(10), 2158–2164. https://doi.org/10.1021/jf405705t

Vona, R., Pallotta, L., Cappelletti, M., Severi, C., & Matarrese, P. (2021). The impact of oxidative stress in human pathology: Focus on gastrointestinal disorders. *Antioxidants*, *10*(2), 1–26. https://doi.org/10.3390/antiox10020201

Wang, Q., Durand, E., Elias, R. J., & Tikekar, R. V. (2016). Generation of reactive oxidative species from thermal treatment of sugar solutions. *Food Chemistry*, *196*, 301–308. https://doi.org/10.1016/j.foodchem.2015.09.044

Wang, X., Zhu, L., Li, X., Wang, X., Hao, R., & Li, J. (2022). Effects of high fructose corn syrup on intestinal microbiota structure and obesity in mice. *Npj Science of Food*, *6*(1), 2–5. https://doi.org/10.1038/s41538-022-00133-7

Wang, Y., Qi, W., Song, G., Pang, S., Peng, Z., Li, Y., & Wang, P. (2020). High-fructose diet increases inflammatory cytokines and alters gut microbiota composition in rats. *Mediators of Inflammation*, *2020*. https://doi.org/10.1155/2020/6672636

Wang, Z., Li, S., Cao, Y., Tian, X., Zeng, R., Liao, D. F., & Cao, D. (2016). Oxidative stress and carbonyl lesions in ulcerative colitis and associated colorectal cancer. *Oxidative Medicine and Cellular Longevity*, *2016*. https://doi.org/10.1155/2016/9875298

Whittaker, A. R. H., & Whittaker, R. H. (1972). Evolution and Measurement of Species Diversity Published by. *Taxon*, *21*(2/3), 213–251.

WHO. (2018). Guideline: Sugars intake for adults and children. *World Health Organization*, *57*(6), 1716–1722.

Xiong, Q., Zhang, M., Wang, T., Wang, D., Sun, C., Bian, H., Li, P., Zou, Y., & Xu, W. (2020). Lipid oxidation induced by heating in chicken meat and the relationship with oxidants and antioxidant enzymes activities. *Poultry Science*, *99*(3), 1761–1767. https://doi.org/10.1016/j.psj.2019.11.013

Yanpar, E., Yildirim, M., Akkapulu, M., Değirmenci, U., Könen Adigüzel, S., Yalin, S., & Yalin, A. E. (2021). Possible protective role of punicalagin on oxidative stress, inflammation and genotoxicity in ethanol-induced liver toxicity. *Journal of Research in Pharmacy*, *25*(5), 600–608. https://doi.org/10.29228/jrp.51

Yao, C. K., Muir, J. G., & Gibson, P. R. (2016). Review article: Insights into colonic protein fermentation, its modulation and potential health implications. *Alimentary Pharmacology and Therapeutics*, *43*(2), 181–196. https://doi.org/10.1111/apt.13456

Yarmush, M. L., & Jayaraman, A. (2002). Advances in proteomic technologies. *Annual Review of Biomedical Engineering*, *4*, 349–373. https://doi.org/10.1146/annurev.bioeng.4.020702.153443

Yin, Y., Cai, J., Zhou, L., Xing, L., & Zhang, W. (2022). Dietary oxidized beef protein alters gut microbiota and induces colonic inflammatory damage in C57BL/6 mice. *Frontiers in Nutrition*, *9*(3). https://doi.org/10.3389/fnut.2022.980204

Yin, Y., Martínez, R., Zang, W., & Estévez, M. (2023). *Crosstalk between dietary pomegranate and gut microbiota: Evidence of health benefits*. https://doi.org/https://doi.org/10.1080/10408398.2023.2219763

Yin, Y., Zhou, L., Pereira, J., Zhang, J., & Zhang, W. (2020). Insights into digestibility and peptide profiling of beef muscle proteins with different cooking Methods. *Journal of Agricultural and Food Chemistry*, *68*(48), 14243–14251. https://doi.org/10.1021/acs.jafc.0c04054

Zhang, D.-M., Jiao, R.-Q., & Kong, L.-D. (2017). High dietary fructose: Direct or indirect dangerous factors disturbing tissue and organ functions. *Nutrients*, *9*(4).

Zhang, L., Perdomo, G., Kim, D. H., Qu, S., Ringquist, S., Trucco, M., & Dong, H. H. (2008). Proteomic analysis of fructose-induced fatty liver in hamsters. *Metabolism: Clinical and Experimental*, *57*(8), 1115–1124. https://doi.org/10.1016/j.metabol.2008.03.017

Zhang, M. W., Cui, S. M., Mao, B. Y., Zhang, Q. X., Zhao, J. X., Zhang, H., Tang, X., & Chen, W. (2023). Ellagic acid and intestinal microflora metabolite urolithin A: A review on its sources, metabolic distribution, health benefits, and biotransformation. *Critical Reviews in Food Science and Nutrition*, *63*(24), 6900–6922. https://doi.org/10.1080/10408398.2022.2036693

Zhang, Y. B., Meng, Y. H., Chang, S., Zhang, R. Y., & Shi, C. (2016). High fructose causes cardiac hypertrophy via mitochondrial signaling pathway. *American Journal of Translational Research*, *8*(11), 4869–4880.

Zhang, Y., Tan, X., Cao, Y., An, X., Chen, J., & Yang, L. (2022). Punicalagin protects against diabetic liver injury by upregulating mitophagy and antioxidant enzyme activities. *Nutrients*, *14*(14). https://doi.org/10.3390/nu14142782

Zhao, S., Jang, C., Liu, J., Uehara, K., Gilbert, M., Izzo, L., Zeng, X., Trefely, S., Fernandez, S., Carrer, A., Miller, K. D., Schug, Z. T., Snyder, N. W., Gade, T. P., Titchenell, P. M., Rabinowitz, J. D., & Wellen, K. E. (2020). Dietary fructose feeds hepatic lipogenesis via microbiota-derived acetate. *Nature*, *579*(7800), 586–591. https://doi.org/10.1038/s41586-020-2101-7

ANEXOS

ANEXO I. Documentos de autorización del proyecto con animales de experimentación.

1. Visto bueno del Comité de Bioética de la Universidad de Extremadura.
2. Resolución de autorización de proyecto por la Junta de Extremadura.

1. *Visto bueno del Comité de Bioética de la Universidad de Extremadura.*

UNIVERSIDAD DE EXTREMADURA

VICERRECTORADO DE INVESTIGACIÓN, TRANSFERENCIA E INNOVACIÓN

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NºRegistro: 68/2017

Dª MARÍA REYES PANADERO, SECRETARIA DEL COMITÉ DE ÉTICA DE EXPERIMENTACIÓN ANIMAL DE LA UNIVERSIDAD DE EXTREMADURA.

INFORMA: Que una vez evaluada por este Comité la solicitud de Proyecto de Investigación titulado "**Food protein oxidation: from chemistry fundamentals to the impact on human nutrition and health** (*Oxidación de las proteínas alimentarias: de los fundamentos de la química al impacto en la nutrición y la salud humanas*)" cuyo investigador responsable es D. Mario Estévez García, y tras considerar su valor científico, su conformidad con los requisitos de reemplazo, refinamiento y reducción, teniendo en cuenta las consideraciones éticas que se ajustan a la normativa vigente al efecto, y de tal manera considerar clasificado el procedimiento como "*moderado*", y sin que sea necesario llevar a cabo una evaluación retrospectiva del mismo, este Comité ha decidido evaluar positivamente el precitado proyecto de investigación.

Y para que conste y surta los efectos oportunos firmo el presente informe en Badajoz a 7 de julio de 2017.

VºBº

Fdo.: Fernando Henao
Presidente por delegación del
Comité de Ética de
Experimentación animal

2. Resolución de autorización de proyecto por la Junta de Extremadura.

JUNTA DE EXTREMADURA

Consejería de Agricultura, Desarrollo Rural, Población y Territorio
Dirección General de Agricultura y Ganadería
Servicio de Sanidad Animal

Avda. Luis Ramallo s/n
06800 Mérida
Teléfono: 924002344

RESOLUCION DE AUTORIZACIÓN DE PROYECTO CON FINES CIENTÍFICOS Y/O DE DOCENCIA EN EL QUE SE REALIZAN PROCEDIMIENTOS SOBRE ANIMALES DE EXPERIMENTACIÓN

Vista la solicitud presentada por D. Mario Estévez García sobre la autorización del procedimiento de memoria técnica titulada: **"Food Protein Oxidation: From chemistry fundamentals to the impact on human nutrition and health"** que se llevará a cabo en el establecimiento usuario "Servicio de animalario de la Universidad de Extremadura" Unidad de Cáceres con código REGA: ES 100370001803, y siendo el responsable del proyecto D. Mario Estévez García con DNI 28948068-S.

Vistos los informes del Comité de ética de experimentación animal del establecimiento usuario, donde se determina que no existe necesidad de evaluación retrospectiva del mencionado proyecto, y del Servicio de Sanidad Animal;

Considerando que el citado proyecto se ajusta a lo establecido en el Real Decreto 53/2013 de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia;

Esta Dirección General de Agricultura y Ganadería ha resuelto: **autorizar** la realización del proyecto referenciado siempre que se mantengan las condiciones que dieron lugar a la autorización y que el personal que intervenga posea la preparación y formación adecuada que se especifica en el citado Real Decreto.

El código de autorización otorgado al presente proyecto de investigación es EXP-20200904.

Contra esta Resolución, que no agota la vía administrativa, podrán los interesados interponer Recurso de Alzada ante la Consejera de Agricultura, Desarrollo Rural, Población y Territorio en el plazo de UN MES, contados a partir del día siguiente a la recepción de la notificación de esta Resolución, tal y como disponen los artículos 121 y siguientes de la Ley 39/2015, de 01 de octubre del Procedimiento Administrativo Común de las Administraciones Públicas. Todo ello sin perjuicio de que el interesado pueda ejercitar cualquier otro recurso que estime procedente.

El Director General de Agricultura y Ganadería

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ANEXO II. Trabajos presentados a Congresos Internacionales.

1. 67th International Congress of Meat Science and Technology (2021). Cracovia, Polonia.
2. 4th International Symposium of Lipid Oxidation and Antioxidants. Vigo, España.
3. 4th International Symposium of Lipid Oxidation and Antioxidants. Vigo, España.
4. 69th International Congress of Meat Science and Technology (2023). Padua, Italia.
5. 69th International Congress of Meat Science and Technology (2023). Padua, Italia.

1. *67th International Congress of Meat Science and Technology (2021). Cracovia, Polonia.*

Co-oxidation of meat proteins and glucose during a simulated gastric digestion: consequences of a severe pro-oxidative environment

ID: 51794008124

Co-oxidation of meat proteins and glucose during a simulated gastric digestion: consequences of a severe pro-oxidative environment

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Introduction

- ✓ The occurrence of oxidative reactions during food digestion has become a topic of increasing interest .
- ✓ Protein oxidation is normally attributed to Fenton-like radical-mediated mechanisms

Objective

"Investigate the effect of GLUCOSE and GLYOXAL on the nature and intensity of oxidative reactions occurred during simulated gastric digestion of emulsions prepared with meat lipids and myofibrillar proteins (MP)."

Material & methods

- Emulsions were made (triplicate) with MP from porcine muscle (100 mg/mL) and a mixture of triolein and glyceryl trinoleate (1/0.5; w/w).
- Depending on the addition of myoglobin (Mb; 10mg/mL); glucose (GU; 10 mg/mL); Mb+GU (10 mg/mL of each species); glyoxal (GO; 10mg/mL) and GO+Mb (10 mg/mL of each species), 6 experimental groups were considered, along with a CONTROL group (only emulsion).
- A simulated gastric digestion was made (1). Samples were taken before the simulated gastric digestion (BD), and immediately after digestion (AD). They were analysed for TBARS, protein carbonyls, reactive oxygen species, advanced glycation end-products (AGEs) and protein digestibility according to Luna & Estévez (1). Data was analysed by repeated measures ANOVA and Tukey's tests by SPSS 15.0.

Results & discussion

- The gastric digestion INCREASED the concentration of protein carbonyls up to 30-fold times in the experimental emulsions.
- GU-induced lipid and protein oxidation depended on the reaction with Mb and the generation of ROS.
- GO-induced severe carbonylation and AGEs formation was independent of Mb and ROS generation.
- The oxidative damage caused by GLU and GO impaired digestibility of meat proteins

Conclusions

Maillard-mediated pathways play a major role in the oxidative damage to meat proteins during gastric digestion.

Table 1. Concentration of lipid and protein oxidation products, AGEs, ROS and protein digestibility (means o3 replicates) in emulsions as affected by reactants

	TBARS[1]	PC[2]	AGES[3]	ROS[4]	DIGESTIBILITY[5]
CONTROL	0.23d	1.75e	210e	69.5e	109a
Mb	0.45c	2.98d	337d	210c	99.8ab
GU	0.65b	8.56c	446c	298b	84.2bc
GU+Mb	1.29a	20.21a	822b	523a	73.2c
GO	0.51bc	15.99b	1147a	164d	76.9c
GO+MB	0.59bc	16.05b	1220a	256bc	81.0bc

1: mg MDA/L sample; 2: Protein Carbonyls (nmol protein hydrazones/mg protein); 3: Fluorescence units; 4: microM; 5: Non-protein nitrogen (mM). Means with different letters within the same column were significantly different (p<0.05).

REFERENCE: (1) Luna, C., Estévez, M. (2019). Formation of allysine in β-lactoglobulin and myofibrillar proteins by glyoxal and methylglyoxal: Impact on water-holding capacity and in vitro digestibility. Food Chemistry, 271, 87-93.

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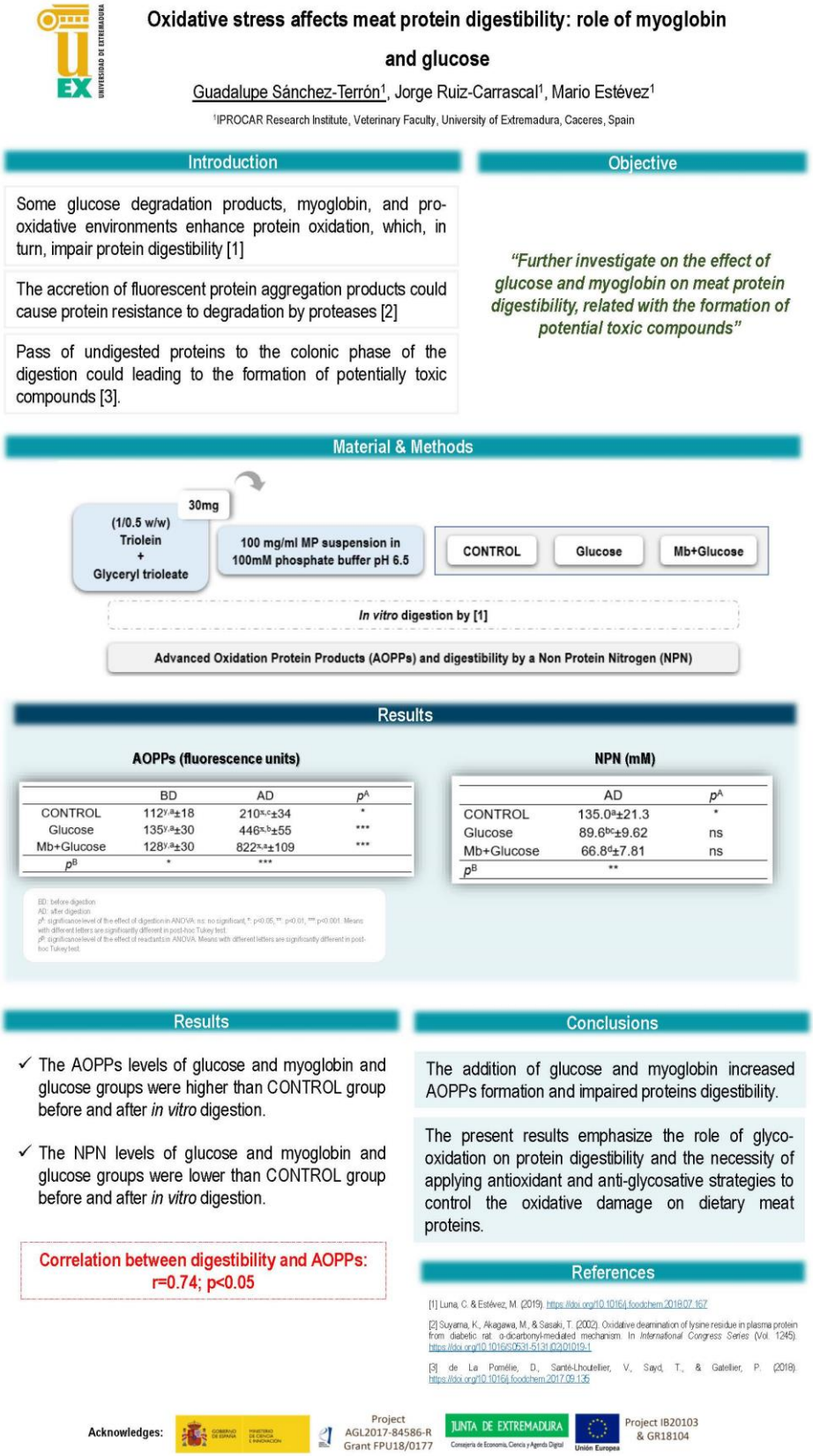
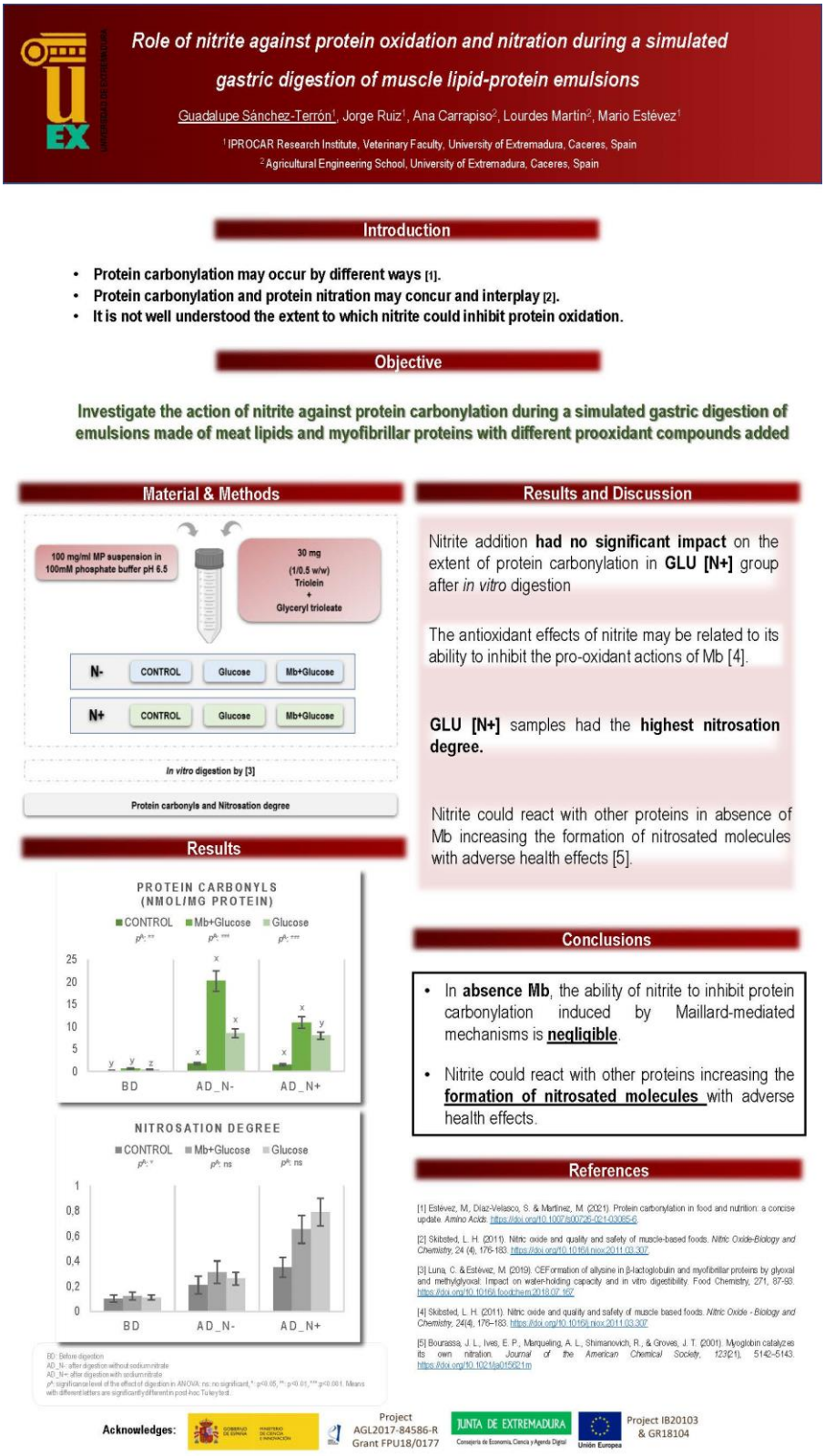
2. *4th International Symposium of Lipid Oxidation and Antioxidants. Vigo, España.*

Oxidative stress affects meat protein digestibility: role of Myoglobin and glucose



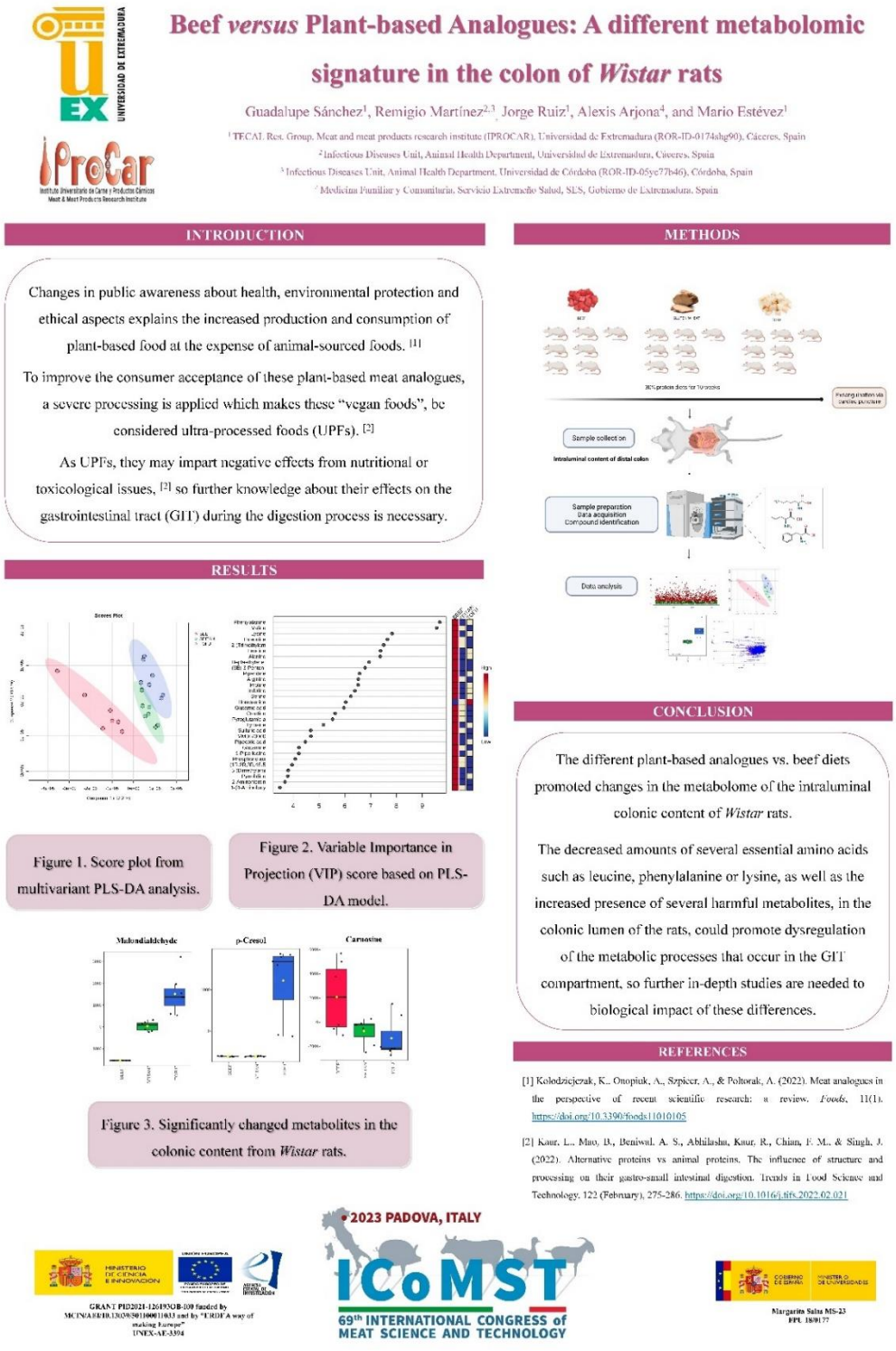
3. *4th International Symposium of Lipid Oxidation and Antioxidants. Vigo, España.*

Role of nitrite against protein oxidation and nitration during a simulated gastric digestion of muscle lipid-protein emulsions



4. *69th International Congress of Meat Science and Technology (2023). Padua, Italia.*

Beef versus plant-based analogues: a different metabolomic signature in the colon of Wistar rats



5. *69th International Congress of Meat Science and Technology (2023). Padua, Italia.*

Impact of ultraprocessed plant-based meat analogues on microbiota diversity and colonic amino acid metabolism in Wistar rats