Desarrollo de formulaciones alimentarias basadas en *Chenopodium quinoa* y *Salvia hispanica* L. con función preventivo/terapéutica de trastornos en el metabolismo de la glucosa

Programa de doctorado: Ciencias de la alimentación

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INFORMAN QUE:

La presente Tesis doctoral se enmarca en los proyectos Qui*Salhis*-Food (AGL2016-75687-C2-1-R) y Food4ImNut (ID2019-107650RB-C21 y C22), financiados por el Ministerio de Ciencia, Innovación y Universidades (MICIU) y CYTED, IA ValSe-Food (119RT0S67). Además, la doctoranda Raquel Selma Gracia ha podido disfrutar de un contrato de personal investigador en formación de carácter predoctoral dentro del Programa Prometeo (LINCE 2017/189) de la Conselleria d'Educación de la Generalitat Valenciana, en el periodo entre julio de 2018 y mayo de 2021 (Resolución de 28 de mayo de 2018).

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En Paterna, 18 de Octubre de 2021

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CERTIFICAN QUE:

La Graduada en Farmacia, Dra. Raquel Selma Gracia ha realizado el trabajo que tiene por título "Desarrollo de formulaciones alimentarias basadas en *Chenopodium quinoa* y *Salvia hispanica* L. con función preventivo/terapéutica de trastornos en el metabolismo de la glucosa", que reúne los requisitos necesarios para optar al Grado de Doctor por la Universidad de Valencia y que ha dado lugar a un compendio de publicaciones que se detallan a continuación:

- Selma-Gracia, R., Laparra, J. M., & Haros, C. M. (2020). Potential beneficial effect of hydrothermal treatment of starches from various sources on *in vitro* digestion. Food Hydrocolloids, 103, 6. Índice de impacto (2020): 9,147 Q1. Food Science & Technology
- Selma-Gracia R., Laparra J. M, & Haros C. M. (2020). Kinetic approach to the influence of chia flour on glucose bioaccessibility from hydrothermally treated maize and quinoa starch. Plant Foods for Human Nutrition, 75, 592–598. Índice de impacto (2020): 3,921 Q2. Food Science & Technology

- Selma-Gracia, R., Haros, C. M., & Laparra, J. M. (2020). Inclusion of *Salvia hispanica* L. and *Chenopodium quinoa* into bread formulations improve metabolic imbalances derived from a high fat intake in hyperglycae-mic mice. Food & function, 11, 7994–8002. Índice de impacto (2020): 5,396 Q1. Food Science & Technology
- Selma-Gracia, R., Megušar, P., Haros, C. M. & Llopis, J. M. L. (2021). Immunonutritional bioactives from *Chenopodium quinoa* and *Salvia hispanica* L. flour positively modulate insulin resistance and preserve alterations in peripheral myeloid population. Nutrients, 13, 1537. Índice de impacto (2020): 5,717 Q1. Nutrition & Dietetics

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RESUMEN

El incremento de enfermedades relacionadas con alteraciones en la homeostasis de la glucosa (i.e., obesidad, diabetes, glucogenosis), ha generado un continuo interés en el consumo de productos saludables, entre los que se encuentran los elaborados con pseudocereales como la quinoa (*Chenopodium quinoa*) y oleaginosas como la chía (*Salvia hispanica* L.) debido a su valor nutricional, propiedades saludables y tecno-funcionales. Es por ello que, en la presente tesis, se ha estudiado en un primer objetivo el almidón de quinoa comparado con otros almidones comerciales (i.e., patata, arroz, trigo, maíz), donde se ha determinado sus propiedades térmicas y de pasta y el efecto del tratamiento hidrotérmico en sus cinéticas de liberación de glucosa. Además, se ha evaluado el efecto de añadir harina de chía desgrasada en los flujos de glucosa, así como en la respuesta metabólica en células hepáticas de origen humano (HepG2). Como segundo objetivo de la tesis, se ha estudiado el efecto de remplazar harina de trigo por harina de quinoa roja (25 %), quinoa blanca (25 %), chía (20 %) y chía semidesgrasada (20 %) en formulaciones de pan. Se han administrado los diferentes productos a ratones para observar el impacto fisiológico de estos ingredientes en combinación con una dieta alta en grasa. Se han establecido tres modelos murinos (hiperglucemia, predisposición transgeneracional al acúmulo

lipídico hepático y deficiencia en hierro), centrándonos en la contribución inmunonutricional del eje enterohepático sobre el control de la homeostasis de la glucosa.

El almidón de quinoa mostró parámetros tecnológicos (como valores bajos de temperatura de inicio de gelatinización y de pasta, así como un alto pico de viscosidad) obteniendo una mayor susceptibilidad a la gelatinización y correlacionándose con su mayor digestibilidad comparada con otros almidones. La incorporación de harina de chía desgrasada no afectó significativamente al cálculo del coeficiente de difusión de glucosa, si bien, disminuyó la respuesta metabólica mitocondrial, en presencia de insulina, en los cultivos de células HepG2. En relación con los ensayos preclínicos *in vivo* con ratones, la administración de los panes con quinoa y chía a ratones hiperglucémicos redujo la resistencia a la insulina (HOMAir) en comparación con el pan de trigo. Sin embargo, en aquellos animales que se administró el pan con harina de chía semidesgrasada se determinaron niveles similares de peroxidación lipídica hepática a aquellos que recibieron el pan de trigo. Este efecto se asocia con el aporte de componentes bioactivos como los inhibidores de proteasa (STPIs), moduladores de la inmunidad innata, más que con el perfil nutricional (cantidad de almidón/omega-3). En animales con tendencia al acúmulo lipídico hepático, la administración de panes con quinoa blanca y chía (sin desgrasar) también mejoró el índice HOMAir respecto al

trigo, acompañado de una modulación de la expresión (ARNm) de biomarcadores lipogénicos hepáticos. Estos cambios se asociaron con una variación positiva de la población mieloide en el torrente circulatorio periférico. Esta misma tendencia en las células mieloides también se observó en un modelo de deficiencia en hierro entre los grupos administrados con las formulaciones a base de quinoa y chía (sin desgrasar). Estos grupos de tratamiento mantuvieron normalizados los niveles de glucosa, similares al grupo deficiente en hierro, previniendo el aumento de la glucemia después de la ingesta de dieta alta en grasa, a contraposición de lo que ocurrió con el pan de trigo.

Atendiendo a los resultados obtenidos, se concluye que la quinoa y la chía poseen propiedades beneficiosas a través del control inmunonutricional favoreciendo la prevención o la atenuación en el desarrollo de enfermedades metabólicas a través del control de la homeostasis de la glucosa en modelos metabólicos muridos.

ABSTRACT

The increase of diseases related to alterations in glucose homeostasis (i.e., obesity, diabetes, glycogen storage disease), has generated a continuous interest in the consumption of products made with pseudo-cereals such as quinoa (*Chenopodium quinoa*) and oilseeds such as chia (*Salvia hispanica* L.), due to its nutritional value, healthy and techno-functional properties. That is why, in the present thesis, quinoa starch has been studied as a first objective to compare to other commercial starches (i.e., potato, rice, wheat, maize), where theirs thermal and pasta properties and the effect of hydrothermal treatment in their glucose release kinetics have been determined. In addition, the effect of adding defatted chia flour on glucose fluxes has been evaluated, as well as on the metabolic response in liver cells of human origin (HepG2). As a second objective of the thesis, the effect of replacing wheat flour with red quinoa flour (25 %), white quinoa (25 %), chia (20 %) and semi-defatted chia (20 %) in bread formulations has been studied. The different products have been administered to mice to observe the physiological impact of these ingredients in combination with a high fat diet. Three murine models have been established (hyperglycaemia, transgenerational predisposition to hepatic lipid accumulation and iron deficiency), focusing on the

immunonutritional contribution of the enterohepatic axis on the control of glucose homeostasis.

Quinoa starch showed technological parameters (such as low gelatinisation and paste onset temperature values, as well as a high viscosity peak) obtaining a greater susceptibility to gelatinisation and is correlated to its greater digestibility compared to other starches. The incorporation of defatted chia flour did not significantly affect to calculate the glucose diffusion coefficient, although it decreased the mitochondrial metabolic response in presence of insulin in HepG2 cell cultures. In preclinical *in vivo* tests in mice, the administration of quinoa and chia breads to hyperglycaemic mice reduced insulin resistance (HOMAir) compared to wheat bread. However, similar levels of hepatic lipid peroxidation were found in animals fed bread with semi-defatted chia flour as in animals fed wheat bread. This effect is related to bioactive components such as protease inhibitors (STPIs), modulators of innate immunity, more than to the nutritional profile (amount of starch/omega-3). In animals with a tendency to hepatic lipid accumulation, the administration of bread with white quinoa and chia (undefatted) also improved the HOMAir index compared to wheat, accompanied by a modulation of the expression (mRNA) of hepatic lipogenic biomarkers. These changes are associated with a positive variation of the myeloid population in the peripheral bloodstream. This same trend in myeloid cells was also

observed in an iron deficiency model among the groups administered with the formulations based on quinoa and chia (undefatted). These treatment groups maintained normalized glucose levels, like the iron deficient group, preventing the increase of glycaemia after eating of high fat diet, in contrast to what happened with wheat bread.

Based on the results obtained, it is concluded that quinoa and chia have beneficial properties through immunonutritional control favouring the prevention or attenuation in the development of metabolic diseases through the control of glucose homeostasis in murine metabolic models.

RESUM

L'increment de malalties relacionades amb alteracions en l'homeòstasi de la glucosa (i.e., obesitat, diabetis, glucogenosis), ha generat un continu interés en el consum de productes elaborats amb pseudocereals com la quinoa (*Chenopodium quinoa*) i oleaginoses com la chia (*Salvia hispanica* L.), a causa del seu valor nutricional, propietats saludables i tecno-funcionals. És per això que, en la present tesi, s'ha estudiat en un primer objectiu el midó de quinoa comparat amb altres midons comercials (i.e., creïlla, arròs, blat, dacsa), on s'ha determinat les seues propietats tèrmiques i de pasta i l'efecte del tractament hidrotèrmic en les seues cinètiques d'alliberament de glucosa. A més, s'ha avaluat l'efecte d'afegir farina de chia desgreixada en els fluxos de glucosa, així com en la resposta metabòlica en cèl·lules hepàtiques d'origen humà (HepG2). Com a segon objectiu de la tesi, s'ha estudiat l'efecte de reemplaçar farina de blat per farina de quinoa roja (25 %), quinoa blanca (25 %), chia (20 %) i chia semidesgreixada (20 %) en formulacions de pa. S'han administrat els diferents productes a ratolins per a observar l'impacte fisiològic d'aquests ingredients en combinació amb una dieta alta en greix. S'han establit tres models murins (hiperglucèmia, predisposició transgeneracional al cúmul lipídic hepàtic i deficiència en ferro),

centrant-nos en la contribució inmunonutricional de l'eix enterohepàtic sobre el control de l'homeòstasi de la glucosa.

El midó de quinoa va mostrar paràmetres tecnològics (com valors baixos de temperatura d'inici de gelatinització i de pasta, així com un alt pic de viscositat) obtenint una major susceptibilitat a la gelatinització i correlacionant-se amb la seua major digestibilitat comparada amb altres midons. La incorporació de farina de chia desgreixada no va afectar significativament al càlcul del coeficient de difusió de glucosa, si bé, va disminuir la resposta metabòlica mitocondrial en presència d'insulina en els cultius de cèl·lules HepG2. En relació amb els assajos preclínics *in vivo* amb ratolins, l'administració dels pans amb quinoa i chia a ratolins hiperglucèmics va reduir la resistència a la insulina (HOMAir) en comparació amb el pa de blat. No obstant això, en aquells animals que es va administrar pa amb farina de chia semidesgreixada es van determinar nivells similars de peroxidació lipídica hepàtica a aquells que van rebre el pa de blat. Aquest efecte es relaciona amb components bioactius com els inhibidors de proteasa (STPIs), moduladors de la inmunitat innata, més que amb el perfil nutricional (quantitat de midó/omega-3). Els animals amb tendència al cúmul lipídic hepàtica, l'administració de pans amb quinoa blanca i chia (sense desgreixar) també va millorar l'índex HOMAir respecte al blat, acompanyat d'una modulació de l'expressió (ARNm) de biomarcadors lipogènics hepàtics. Aquests

canvis es van associar amb una variació positiva de la població mieloide en el torrent circulatori perifèric. Aquesta mateixa tendència en les cèl·lules mieloides també es va observar en un model de deficiència en ferro entre els grups administrats amb les formulacions a base de quinoa i chia (sense desgreixar). Aquests grups de tractament van mantenir normalitzats els nivells de glucosa, similars al grup deficient en ferro, prevenint l'augment de la glucèmia després de la ingesta de dieta alta en greix, a contraposició del que va ocórrer amb el pa de blat.

Atesos els resultats obtinguts, es conclou que la quinoa i la chia posseeixen propietats beneficioses a través del control inmunonutricional afavorint la prevenció o l'atenuació en el desenvolupament de malalties metabòliques a través del control de l'homeòstasi de la glucosa en models metabòlics murins.

ABREVIATURAS

ΔH_G	*Enthalpy of gelatinisation*
ΔH_R	*Enthalpy of retrogradation*
AGCC	*Short-chain fatty acids*
ALOX15	*Arachidonate 15-lipoxygenase*
AM	*Amylase*
AP	*Amylopectin*
AUC	*Area under the curve*
BA	*Bioaccessibility*
BW	*Body weight*
CF	*Chia flour*
Ch	*Chia bread*
Ch_D	*Semi-defatted chia bread*
CPV	*Cool paste viscosity*
Dapp	*Apparent diffusion coefficient*
DP	*Degree of polymerisation*
DSC	*Differential scanning calorimeter*
EFSA	*European Food Safety Authority*
FASN	*Fatty acid synthase*
GH	*Glucose homeostasis*
GI	*Glycaemic index*
GM-CSF	*Granulocyte macrophage colony-stimulating factor*
GR	*Granulocyte population*

GS	*Gelatinised starch*
GSD	*Glycogen storage disease*
Hb	*Haemoglobin*
HFD	*High fat diet*
HI	*Hydrolysis index*
HOMAir	*Homeostatic model assessment of insulin resistance*
HPV	*Hot paste viscosity*
HT	*Hydrothermal treatment*
ID	*Iron-deficient*
IL	*Interleukin*
IR	*Insulin resistance*
MDA	*Malondialdehyde*
MS	*Maize starch*
MS_70	*Treated maize starch at 70 °C*
MS_100	*Treated maize starch at 100 °C*
MS_CF	*Maize starch with chia flour*
MS_70_CF	*Treated maize starch with chia flour at 70 °C*
MS_100_CF	*Treated maize starch with chia flour at 100 °C*
MTT	*(3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide*
MY	*Myeloid cell population*
NAFLD	*Non-alcoholic fatty liver disease*
LDL	*Low-density lipoprotein*
LY	*Lymphocyte cell population*

PC	*Principal component*
PCA	*Principal component analysis*
PPAR	*Peroxisome proliferator-activated receptor*
P_{temp}	*Pasting temperature*
PTGS2	*Prostaglandin-endoperoxide synthase 2*
P_{time}	*Peak time*
PUFAs	*Polyunsaturated fatty acids*
PV	*Peak viscosity*
QS	*Quinoa starch*
QS_60	*Treated quinoa starch at 60 °C*
QS_100	*Treated quinoa starch at 100 °C*
QS_CF	*Quinoa starch with chia flour*
QS_60_CF	*Treated quinoa starch with chia flour at 60 °C*
QS_100_CF	*Treated quinoa starch with chia flour at 100 °C*
RBC	*Red blood cells*
RQ	*Red quinoa bread*
RVA	*Rapid Visco Analyser*
SH	*Starch hydrolysed*
SFM	*Phagocyte mononuclear system*
SREBP1	*Sterol regulatory element-binding protein 1*
STPIs	*Serine-type protease inhibitor*
STZ	*Streptozotocin*
T2D/DT2	*Type 2 diabetes*
TBT	*Tributyltin*

T_c	*Conclusion temperature*
TG	*Triglycerides*
TLR	*Toll-like receptor*
TNF	*Tumor necrosis factor*
T_o	*Onset temperature*
T_p	*Peak temperature*
TSH	*Total starch hydrolysed*
WB	*Wheat bread*
WBC	*Leukocytes counts*
WQ	*White quinoa bread*

ÍNDICE

I

INTRODUCCIÓN

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1. Semillas de chía y quinoa

1.1. Expansión de las semillas ancestrales

Existe una creciente preocupación de la sociedad por los hábitos alimenticios, lo cual, favorece un cambio en las tendencias de preferencia y consumo alimentario hacia un patrón cada vez más saludable. En este contexto, se han introducido en la dieta los cultivos 'ancestrales', por su amplia aceptación popular y por las características saludables y valor nutricional de sus semillas.

Las semillas 'ancestrales' son nombradas así porque su historia se remonta a miles de años atrás, siendo consumidas y, algunas de ellas, consideradas sagradas por antiguas civilizaciones como los Incas, Mayas o Aztecas. Los cultivos 'ancestrales' incluyen variedades de trigo: espelta, trigo khorasan, farro, einkorn, emmer; granos de mijo, cebada, teff, avena y sorgo; pseudocereales como la quinoa, amaranto y trigo sarraceno y semillas oleaginosas como el lino y la chía (Cooper, 2015). Dentro de este grupo de semillas 'ancestrales' se engloban a la quinoa (*Chenopodium quinoa*) y la chía (*Salvia hispanica* L.), que se diferencian de muchos otros cultivos porque no han sufrido selección agronómica y, por tanto,

apenas han sido expuestos a procesos de mejora en la producción y el producto.

En el marco legal en cuanto a uso autorizado en Europa, el uso de la chía está limitado. En 2009 la Autoridad Europea de Seguridad Alimentaria (EFSA), mediante la Decisión 2009/827/CE, declaró a la chía como "novel food" (nuevo alimento). Según la normativa de la Unión Europea (UE), un "novel food" es cualquier alimento que no ha sido consumido de manera "significativa" antes de mayo de 1997. Tras su incorporación como "novel food" en el 2009, se permitió la comercialización de semillas de chía (*Salvia hispanica* L.) en la UE como nuevo alimento para ser utilizadas en productos de panadería en contenido máximo de hasta un 5 % (p/p). Las especificaciones en cuanto al contenido en proteínas, carbohidratos, fibra cruda, lípidos y cenizas también aparecen especificadas en el decreto emitido por la Autoridad Europea en Seguridad Alimentaria (EFSA, 2009). En el 2013 se incrementó el porcentaje de un 5 % a 10 % en productos de panadería. Además, se extendió su uso hasta el 10 % a otras categorías de alimentos como son los productos de horno (normalmente tratados a temperaturas mayores a 120 °C), cereales de desayuno, frutos secos o mezclas de frutas (Decisión de Ejecución de la Comisión Europea, 2013/50/UE). En el 2017, se incorporó como "novel food" el aceite de chía. Por el momento, los usos aceptados del

consumo de aceite son 2 g/día en complementos alimenticios y un máximo del 10 % en grasas y aceites vegetales (Official Journal of the European Union, 2017). Además, se añadieron nuevas categorías de alimentos para el uso de las semillas de chía con ciertas restricciones en su uso (i.e., yogur, zumos de frutas, comidas preparadas esterilizadas a base de granos/legumbres) (Official Journal of the European Union, 2017). No obstante, en el decreto del 2020, se eliminan las restricciones máximas de estas últimas categorías, incorporando nuevas como productos de confitería o púdines que no requieran temperaturas mayores a 120 ºC, sin límite de uso máximo de las semillas (Official Journal of the European Union, 2020a). Finalmente, la última incorporación ha sido la autorización para la comercialización de 'polvo de semillas de chía' (*Salvia hispanica*) parcialmente desgrasados, obtenidos mediante el prensado y la molienda, como alimento nuevo en una categoría de alimentos específicos (i.e., lácteos, frutas o zumos) y en un contenido máximo entre 0,7–10 % en polvo, con una alta fracción en proteínas, y entre 2,5–4 % en polvo con alto contenido en fibra (Official Journal of the European Union, 2020b).

En cuanto a la seguridad alimentaria, ante el aumento del número de nuevas solicitudes de autorización para ampliar los usos de las semillas de chía, la EFSA concluyó en el 2019 que el uso de semillas de chía en alimentos que no requieren en su

fabricación, transformación o preparación un tratamiento térmico a una temperatura igual o superior a 120 °C, es seguro sin restricciones ni precauciones específicas en relación con los niveles de uso (Turck *et al*., 2019). Además, en el 2020 la EFSA realizó una declaración sobre la seguridad alimentaria de la chía donde aclaró que los datos obtenidos hasta la fecha no disponen de evidencia suficiente para concluir si la adición de semillas de chía a los alimentos sometidos a tratamientos térmicos (a temperaturas superiores a 120 °C), da como resultado una mayor formación de acrilamida en comparación con estos alimentos sin chía (Turck *et al*., 2020).

Por otro lado, la quinoa no presenta ninguna limitación sobre su comercialización en Europa, no es considerado un "novel food" debido a que su uso es ampliamente utilizado desde hace años. Este pseudocereal debido a la alta concentración de proteínas, la facilidad de uso y la versatilidad en su preparación, lleva siendo estudiado por la NASA desde finales del siglo pasado como ingrediente para ser incorporado en la dieta de los astronautas, incluyéndose comidas a base de quinoa para los viajes espaciales desde hace una década (Schlick, & Bubenheim, 1996; Jones, 2010) En el 2013, la FAO (Organización de las Naciones Unidas para la Alimentación y la Agricultura) declaró el "Año Internacional de la Quinoa" en reconocimiento a los pueblos andinos que han mantenido, protegido y preservado la quinoa

como alimento para las generaciones presentes (Bazile, & Baudon, 2015).

En los últimos años, la creciente popularidad de la quinoa y la chía se ha visto reflejada en el consumo en Europa a través del aumento notable de la importación de estas semillas, observándose un incremento del 69 % de chía y 72 % de quinoa entre los años 2015–2018 (Koekoek, 2019). Sin embargo, una gran diferencia entre estas dos semillas radica en su domesticación en Europa. Gracias a la alta adaptabilidad de la quinoa a diferentes condiciones agroecológicas, Europa se ha convertido en una importante productora de este pseudocereal, siendo Francia el primer país pionero en cultivarla (CBI, 2020). En el 2018, España se convirtió en un exportador neto de quinoa siendo mayor el valor de la mercancía exportada que la importada y, en el 2019, superó a los Países Bajos en el volumen total de exportación, convirtiéndose en el mayor exportador de quinoa de Europa (CBI, 2020). La superficie total de cultivo en España en 2020 fue de alrededor de 1.763 hectáreas, con la mayor producción en Andalucía (CBI, 2020). Por lo que concierne a la chía, aunque existen numerosos intentos de domesticación en Europa, a día de hoy, no se ha podido cultivar en condiciones adecuadas para producir semillas que cumplan con los requerimientos en cuanto a composición que exige la EFSA para su comercialización y consumo en la UE (CBI, 2021).

Estas dos semillas han ganado una atención creciente en todo el mundo por sus propiedades nutricionales como la ausencia de proteínas que conforman el gluten, así como su contenido alto en fibra, el aporte de proteínas de alto valor biológico y los ácidos grasos poliinsaturados (PUFAs). Todos estos factores han suscitado el interés de la población observándose un crecimiento de su búsqueda en internet relacionada con su implicación en la salud tras su consumo. Este incremento más marcado se observó a partir del año 2010 y fue en aumento hasta el día de hoy (Figura 1) (Angeli *et al*., 2020; Jamboonsri, Phillips, Geneve, Cahill, & Hildebrand, 2012).

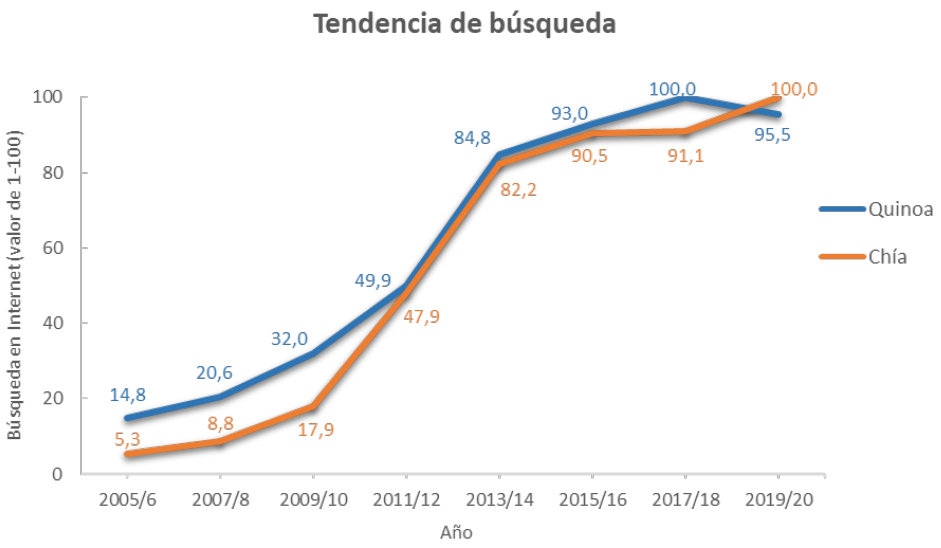

Figura 1: Tendencia de búsqueda global en Google (números de 0–100 según el interés de búsqueda en relación con el valor máximo) sobre la chía y quinoa en temas relacionados con la salud (Google Trends, 2021).

En la actualidad, se ha descrito que la ingesta de chía y quinoa produce impacto positivo sobre la salud en humanos, promoviendo la integración de estas semillas ancestrales en el desarrollo de alimentos más nutritivos y saludables. Algunos ejemplos se reflejan en la tabla 1.

Tabla 1: Impacto de marcadores del metabolismo sobre la ingesta de chía y quinoa en humanos.

Alimento	Diseño	Efecto	Referencias
Pan con harina de chía (7/15/24 g)	7/15/24 g, una dosis 11 personas sanas	↓ Índice glucémico	Vuksan *et al*., 2010
Pan con harina de chía y polvo de chía	30 g/1000 kcal, 6 meses 27 personas con diabetes en sobrepeso/obesas	↓ Circunferencia de cintura, proteína C reactiva, no diferencias en glucosa respecto al salvado de avena	Vuksan *et al*., 2017
Semilla molida de chía	25 g/día, 8 semanas 25 personas con NAFLD y resistencia a la insulina	↓ Peso corporal, colesterol, ácidos grasos libres	Medina-Urrutia *et al*., 2020
Semilla de chía	25 g/día, 12 semanas 76 adultos con sobrepeso/obesos	No diferencias en inflamación, peso corporal, marcadores de riesgo cardiovascular	Nieman *et al*., 2009
Semilla de chía entera o molida	25 g/día, 10 semanas 30 postmenopáusicas con sobrepeso	No diferencias en inflamación, peso, lipoproteínas respecto a las semillas de amapola	Nieman *et al*., 2012

NAFLD: Esteatosis hepática no alcohólica.

Alimento	Diseño	Efecto	Referencias
Copo de quinoa	25 g/día, 4 semanas 35 mujeres menopausia con exceso de peso	↓ Colesterol total, LDL respecto a los copos de maíz	De Carvalho *et al*., 2014
Barra de quinoa (39 % copos de quinoa)	19,5 g/día, 30 días 22 personas sanas	↓ Colesterol total, LDL, triglicéridos	Farinazzi-Machado *et al*., 2012
Envase con 20 g de harina de quinoa y 80 g de agua	40 g/día, 28 días 29 prediabéticos	↓ IMC, HbA1c, no diferencias en glucosa respecto al placebo (maltodextrina)	Ruiz *et al*., 2017
Pan de quinoa (12 % de harina de quinoa)	20 g de quinoa 4 semanas 19 personas con sobrepeso	↓ Glucosa, no diferencias en colesterol, LDL respecto al pan de trigo	Li *et al*., 2018
Galleta de quinoa (60 % de harina de quinoa)	15 g, 4 semanas 20 personas sanas	↓ Colesterol total, LDL, IMC	Pourshahidi *et al*., 2020
Semilla de quinoa cocida	20/50 g, 12 semanas 20 personas sobrepeso/obesas	25/50 g no cambios en LDL, glucosa, peso corporal 50g ↓ Triglicéridos plasmáticos	Navarro-Perez *et al*., 2017

LDL: Lipoproteína de baja densidad; IMC: Índice de masa corporal; HbA1c: Hemoglobina glicosilada.

A pesar de los estudios reflejados en la tabla 1, en el año 2009, la EFSA no atribuyó ningún efecto positivo sobre el control de la glucemia en diabéticos tras la ingesta de semillas de chía (EFSA, 2009). Más tarde, en el año 2019, la EFSA explicó las limitaciones de los estudios en humanos para afirmar y evaluar un verdadero beneficio en este sentido (Turck *et al*., 2019). Por otro lado, los escasos estudios en humanos observados en la quinoa y, sus efectos contrapuestos, dan lugar a la necesidad de una mayor investigación sobre sus propiedades.

1.2. Chía

La semilla de chía se obtiene de la planta *Salvia hispanica* L., la cual, se considera una oleaginosa debido a su alto contenido en lípidos (30–40 %) (Rubilar, Gutiérrez, Verdugo, Shene, & Sineiro, 2010). Además de su fracción lipídica, el otro componente mayoritario en la chía son los carbohidratos (27–42 %), donde la mayor parte corresponde a fibra dietética, careciendo prácticamente de almidón (Fernández-Espinar, Gil, Segura-Campos, & Haros, 2016; Kulczyński, Kobus-Cisowska, Taczanowski, Kmiecik, & Gramza-Michalowska, 2019).

Las semillas de chía también proporcionan un alto contenido en proteínas de alto valor biológico al tiempo que presentan una composición equilibrada de aminoácidos esenciales como leucina

y arginina. Es destacable, como se ha mencionado anteriormente, que carece de las proteínas que conforman el gluten y, por tanto, son aptas para celiacos (Kulczyński *et al*., 2019). Además, es rica en antioxidantes, principalmente en polifenoles como el ácido cafeico, gálico o clorogénico. El más abundante (27–31 μg/g de semilla), el ácido cafeico, es conocido por actuar en la regulación del metabolismo de la glucosa con actividad hipoglucémica, reduciendo las actividades de la glucosa-6-fosfatasa y la fosfoenolpiruvato carboxiquinasa hepáticas en ratones, ambas enzimas involucradas en la gluconeogénesis (Jung, Lee, Park, Jeon, & Choi, 2006). Se caracteriza también por su alto contenido en minerales como calcio, magnesio, fósforo y hierro. En concreto, el contenido en hierro es (~ 7,3 mg/100 g), similar a las legumbres, consideradas una de las mayores fuentes de hierro (1,8–8,2 mg/100 g) (Miranda-Ramos, Millán-Linares, & Haros, 2020; Cabrera, Lloris, Giménez, Olalla, & López, 2003). Sin embargo, la chía contiene componentes como los fitatos o los taninos que pueden interferir en la biodisponibilidad de los minerales (Miranda-Ramos *et al*., 2020; Melo, Machado, & Oliveira, 2019). Adicionalmente, contiene vitaminas tipo C, E y B como la vitamina B9 (folatos) y la vitamina B3 (niacina), esta última conocida por su influencia positiva reduciendo biomarcadores plasmáticos como los triglicéridos, el colesterol total o las lipoproteínas de baja densidad (LDL), íntimamente

asociados con el síndrome metabólico (Kulczyński *et al*., 2019; Adiels *et al*., 2018).

1.2.1. Fibra

Las semillas de chía contienen aproximadamente 35–40 g de fibra dietética total por cada 100 gramos, de las cuales alrededor de un 85–94 % pertenece a la fracción insoluble y el resto fibra soluble (Reyes-Caudillo, Tecante, & Valdivia-López, 2008; Marineli *et al*., 2014). Estos contenidos en fibra total son considerablemente más altos que los cuantificados en la quinoa (9–21 %) o siendo incluso el doble comparado con cereales como el arroz (3–10 %) (Prasadi, & Joye, 2020).

En general, un aspecto importante a destacar de la fibra dietética es su efecto prebiótico, principalmente asociada a la fibra soluble, fermentada por la microbiota intestinal con la producción de ácidos grasos de cadena corta (AGCC). Los efectos del consumo de fibra soluble se han asociado a la mejora de la sensibilidad a la insulina, regulación del peso corporal y reducción de la inflamación tisular, atribuidos al aumento de AGCC, reduciendo así el riesgo de desarrollar enfermedades metabólicas o enfermedades coronarias (Myhrstad, Tunsjø, Charnock, & Telle-Hansen, 2020; Jamshidi, Amato, Ahmadi, Bochicchio, & Rossi, 2019). En un estudio reciente, se investigó el efecto de la

inyección intraamniótica de diferentes porcentajes de los extractos solubles de la semilla de chía (0,5, 1, 2,5 y 5 %) en gallos, donde una gran parte corresponde a la fibra soluble, como potencial efecto prebiótico *in vivo* (da Silva, Kolba, Martino, Hart, & Tako, 2019). Se observaron cambios en la microbiota intestinal donde el grupo que recibió 0,5 % del extracto soluble incrementó la población de *Lactobacillus* spp. y *Bifidobacterium* spp. Sin embargo, a mayores concentraciones del extracto (1 y 2,5 %) no se obtuvieron diferencias con respecto al control, descendiendo significativamente el porcentaje de las dos poblaciones en el caso del grupo al 5 %.

Más concretamente, cabe destacar el contenido de mucílago en la fracción soluble de las semillas de chía (Reyes-Caudillo *et al*., 2008). Se ha demostrado que el mucílago de la chía tiene una alta capacidad de retención de agua, aproximadamente absorbe hasta 35 veces su peso en agua y resiste condiciones ácidas, lo que podría suponer que aumenta la viscosidad en el tracto digestivo e inhibir la accesibilidad de las enzimas digestivas (Lazaro, Puente, Zúñiga, & Muñoz, 2018; Hardacre, Yap, Lentle, & Monro, 2015). Tamargo *et al*., (2018) observaron *in vitro*, el impacto del mucílago de la chía sobre la composición de la microbiota, así como la producción de AGCC, principalmente acetato. Sin embargo, factores como la falta de correlación entre las distintas

cantidades de mucílago evaluado y los efectos observados, promueve la continua e incesante investigación en esta línea.

1.2.2. Lípidos

La chía es considerada una de las fuentes vegetales de mayor aporte de ácidos grasos omega-3. Se caracteriza por un alto contenido en PUFAs donde cabe destacar el alto contenido de ácido α-linolénico (englobado en el grupo de los omega-3), correspondiendo aproximadamente a un 60 % basándose en su aceite, mientras que el restante es prácticamente ácido linoleico (englobado en el grupo de los omega-6). En una menor medida, contiene ácidos grasos monoinsaturados como el oleico o saturados como el palmítico (Villanueva-Bermejo, Calvo, Castro-Gómez, Fornari, & Fontecha, 2019). Esta distribución de omega 3/6 difiere considerablemente de otras semillas como la quinoa, el trigo, el arroz o la avena donde principalmente predomina los ácidos grasos omega-6 (Figura 2).

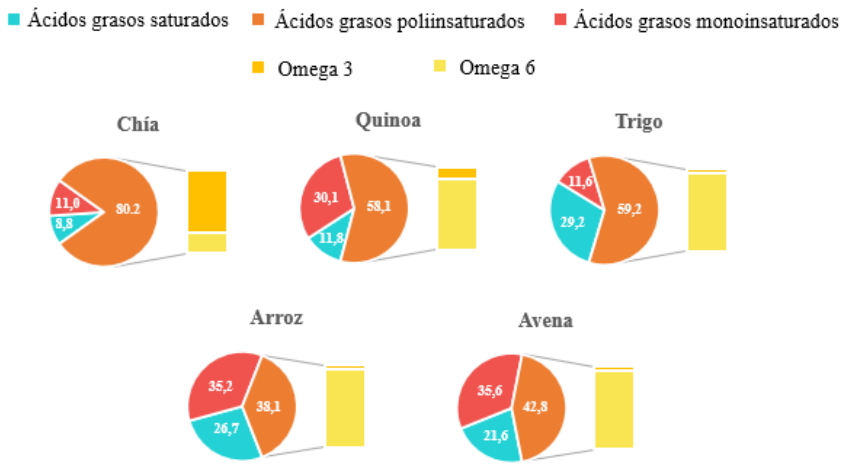

Figura 2: Representación de los porcentajes de ácidos grasos en el aceite de la chía, la quinoa, el trigo, el arroz y la avena (Ciftci, Przybylski, & Rudzińska, 2012; Ballester-Sánchez *et al*., 2019a; Liu, 2011).

El consumo de PUFA n-3 en suplementos de aceite de pescado o extractos de PUFA n-3, se ha correlacionado inversamente con la incidencia de enfermedades coronarias, asociándolas por sus efectos reductores en valores lipídicos como los triglicéridos, LDL o colesterol (Zuliani *et al*., 2009). Además, estudios con un modelo ratas Wistar han señalado que la ingesta de ácidos omega-3 (aceite de pescado) previenen el desarrollo de la resistencia a la

insulina en respuesta a una dieta alta en grasa regulando la expresión y secreción de adipocitocinas (Chacińska *et al*., 2019).

No obstante, a pesar del alto contenido de omega-3 en la chía, el efecto beneficioso sobre la resistencia a la insulina de los omega-3 podría no observarse cuando se ingiere la harina de chía (Enes *et al*., 2020). En un estudio donde se alimentaron a ratas con dieta alta en grasa y fructosa, se evaluó y se comparó el efecto de recibir harina de chía o aceite de chía. Solo el grupo de ratones alimentados con aceite de chía mejoró la tolerancia a la glucosa. A diferencia del grupo alimentado con harina de chía, el aceite de chía activó la vía de insulina a través del aumento de la expresión de ARNm del receptor de insulina y la proteina quinasa B (también conocida como AKT) seguido de un aumento de la glicólisis (incremento de la expresión de ARNm de enzimas como la glucoquinasa o fosfoquinasa), lo que podría indicar un aumento de la captación y oxidación de glucosa (Enes *et al*., 2020).

Aunque en el estudio de Enes *et al*., (2020) el contenido total de aceite en la dieta con harina de chía y aceite solo era similar, la disponibilidad de los compuestos del aceite podría depender de la matriz alimentaria. Componentes presentes en la chía como la fibra dietética o el calcio, podrían unir compuestos, como los ácidos grasos, disminuyendo su disponibilidad y absorción (Ayala-Bribiesca, Turgeon, & Britten, 2017; Kulczyński *et al*., 2019). Esta diferencia entre la harina y el aceite podría explicarse en

relación con los efectos del ácido α-linolénico que están mediados, en parte, por sus derivados (i.e., ácido eicosapentaenoico o docosahexaenoico) o sus metabolitos oxidados (i.e., eicosanoides u otras oxilipinas). Las oxilipinas son un grupo de metabolitos de ácidos grasos generados mediante la oxigenación de ácidos grasos poliinsaturados, involucrados en procesos como inflamación, inmunidad, dolor, tono vascular y coagulación (Nayeem, 2018). Por ejemplo, la ingesta dietética del ácido docosahexaenoico se asocia con varios beneficios para la salud, que incluyen una mejora en la sensibilidad a la insulina hepática favoreciendo la oxidación de los ácidos grasos y reduciendo la lipogénesis en pacientes con esteatosis hepática no alcohólica (NAFLD de sus siglas en inglés) (Hodson *et al*., 2017).

Por todo ello, a pesar de que existen un gran número de estudios que apuntan a un efecto beneficioso de los PUFAs incrementando la sensibilidad a la insulina, continúa existiendo una controversia en relación con estos efectos, alimentando un debate importante sobre el impacto potencial de los PUFAs en la función metabólica (Lalia, & Lanza, 2016).

1.3. Quinoa

La quinoa, *Chenopodium quinoa*, es considerada un pseudocereal, ya que, a diferencia de los cereales, no pertenece a la familia de las gramíneas (monocotiledóneas) sino a las dicotiledóneas. Existen diversos tipos según su color como pueden ser la quinoa blanca, la roja o la negra.

En cuanto a la fracción de carbohidratos, la quinoa contiene valores de almidón similares a los cereales como el trigo (62–68 %) y, por tanto, adquiere la capacidad de poder ser utilizados de la misma forma para la elaboración de productos de panadería (Ballester-Sánchez, Gil, Fernández-Espinar, & Haros, 2019a; Contreras-Jiménez, Torres-Vargas, & Rodríguez-García, 2019). Además, esta semilla contiene un valor alto de fibra dietética (entre 7–20 g/100 g) comparado con otros cereales como el trigo, el arroz o el maíz (0,5–5 g/100 g) (Ballester-Sánchez *et al*., 2019a; Hager, Wolter, Jacob, Zannini, & Arendt, 2012). Se ha observado que la suplementación con extractos de polisacáridos de quinoa mejora la hiperlipidemia y tienen un efecto sobre la microbiota en ratas alimentadas con dieta alta en grasa. Su ingesta redujo los niveles en plasma de triglicéridos, LDL, así como la distribución *Firmicutes/Bacteroides* (Cao *et al*., 2020).

En relación con el contenido de proteínas, la quinoa presenta una mayor cantidad respecto a otros cereales (maíz, arroz), siendo

similar o ligeramente superior, al obtenido en el trigo (Ballester-Sánchez *et al*., 2019a; Hager *et al*., 2012). No obstante, se diferencia de este último cereal por la presencia de aminoácidos esenciales como la lisina, encontrándose en mayor proporción en la quinoa mientras que en la mayoría de los cereales es un aminoácido limitante (Angeli *et al*., 2020; Hager *et al*., 2012; Ballester-Sánchez *et al*., 2019a). Una de las propiedades de mayor relevancia y, que cabe destacar de la quinoa, es que no contiene las proteínas que conforman el gluten y, *a priori*, son alimentos aptos para personas con intolerancias al gluten o celiacos. A pesar de que la fracción lipídica en estas semillas es alta (4–7 %), se caracteriza por su contenido en PUFAs como los ácidos α-linolénico y linoleico. Aproximadamente el 50 % de la fracción lipídica corresponde al ácido linoleico y entre el 4–12 % al ácido α-linolénico, entre el 25–30 % es ácido oleico y el restante correspondería a ácidos grasos saturados (Ballester-Sánchez *et al*., 2019a; Angeli *et al*., 2020; Haros, & Schoenlechner, 2017)

Respecto a sus micronutrientes, son buenas fuentes en minerales como hierro, potasio, magnesio y zinc (Angeli *et al*., 2020). Cabe destacar el aporte de hierro, siendo cuatro veces mayor al contenido que se puede encontrar en cereales como el trigo (Angeli *et al*., 2020; Ballester-Sánchez *et al*., 2019a). Sin embargo, el alto contenido que presenta la quinoa de sustancias tales como fitatos, taninos y oxalatos, puede influir en la

biodisponibilidad de los minerales (Filho *et al*., 2017). Además, contiene carotenoides, vitamina A, es particularmente una fuente de vitamina E (α-tocoferol, γ-tocoferol y β-tocotrienol) y betalaínas con un alto poder antioxidante (Haros, & Schoenlechner, *et al*., 2017; Ballester-Sánchez Gil, Haros, & Fernández-Espinar, 2019b). En estos últimos compuestos, las betalaínas, a parte de este efecto antioxidante, también se ha observado un efecto hipoglucemiante tanto en modelos murinos como humanos (Madadi *et al*., 2020).

Por otro lado, contienen concentraciones significativas de vitaminas del grupo B: B1 (tiamina), B2 (riboflavina), B6 (piridoxina), y B9 (folato) (Ruales, & Nair, 1993; Taylor, & Parker, 2002; Schoenlechner, Wendner, Siebenhandl-Ehn, & Berghofer, 2010). Los valores de piridoxina y folatos son más altos que los encontrados en la mayoría de los cereales tales como trigo, avena, cebada, arroz y maíz (Ruales, & Nair, 1993; Schoenlechner *et al*., 2010). El folato y la piridoxina son componentes nutricionales esenciales para la capacidad de metilación en el hígado. Por tanto, la disponibilidad de estas vitaminas puede modificar la metilación de fosfatidiletanolamina a fosfatidilcolina, interfiriendo en el transporte de PUFAs, en la secreción de lipoproteínas de muy baja densidad (VLDL) para evitar la acumulación de triglicéridos y la progresión hacia una

esteatosis, directamente relacionada con el síndrome metabólico (van Wijk *et al*., 2012 Zhao *et al*., 2009; Li *et al*., 2006).

1.4. Almidón

El almidón es el carbohidrato más común en la dieta humana y se encuentra en grandes cantidades en alimentos básicos como los cereales/pseudocereales, los tubérculos y las legumbres (Sajilata, Singhal, & Kulkarni, 2006). Como se ha comentado anteriormente, la chía carece prácticamente de almidón, mientras que en la quinoa es el compuesto mayoritario (Fernández-Espinar *et al*., 2016; Ballester-Sánchez *et al*., 2019a).

El almidón es un polisacárido que consta de dos glucanos: la amilosa (AM) y la amilopectina (AP). La AM es una molécula lineal (unidades de α-D-glucopiranosas unidas por enlaces α-(1 → 4), mientras que la AP, a diferencia de AM, es una molécula ramificada (unidades de α-D-glucopiranosas unidas por enlaces α-(1 → 4) con ramificaciones α-(1 → 6)) (Rooney, & Pflugfelder, 1986). En general, los almidones contienen entre un 20–30 % de AM y un 70–80 % de AP. Además de la AM y la AP, por lo general contienen pequeñas cantidades de proteínas y lípidos, que pueden influenciar en sus propiedades tecnológicas (Belitz, Grosch, & Schieberle, 2009; Srichuwong, & Jane, 2009). Por ejemplo, los fosfolípidos pueden formar complejos helicoidales

con la AM y las cadenas largas de AP pudiendo ser una restricción en el hinchamiento de los gránulos en tratamientos hidrotérmicos (Belitz, Grosch, & Schieberle, 2009; Srichuwong, & Jane, 2009).

Los gránulos de almidón son de tamaño (2–150 μm) y forma variable (Belitz *et al*., 2009). Se les considera que tienen un carácter semicristalino, aproximadamente el 70 % del gránulo de almidón se presenta como parte amorfa y el otro 30 % cristalina. Las regiones amorfas contienen la mayor parte de AM, pero también una parte considerable de AP. Las regiones cristalinas consisten principalmente en AP, presentando una mayor organización (Rooney, & Pflugfelder, 1986; Belitz *et al*., 2009).

Los almidones se pueden clasificar, dependiendo del patrón de difracción de rayos X, en tres tipos. Los patrones de tipo A (mayoritariamente en los cereales/pseudocereales) tienen una celda unitaria monoclínica, el canal central está ocupado por una doble hélice. El patrón de tipo B (en tubérculos) es una estructura similar al tipo A, excepto que está exento de la doble hélice en el canal central. Finalmente, el patrón de tipo C (en legumbres) es una mezcla del tipo A y B (Sajilata *et al*., 2006; Belitz *et al*., 2009; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005a).

Los almidones de tipo A se han relacionado con estructuras de AP mayoritariamente formadas por cadenas más cortas (grado de polimerización 6–12) y menor cristalinidad que los almidones de

tipo B (grado de polimerización 6–24) (Jane, Wong, & McPherson, 1997). Además, esta distribución de unidades de la AP parece ejercer un efecto importante en la capacidad de hinchamiento e índice de solubilidad de los almidones, obteniendo una menor capacidad cuando el grado de polimerización de la AP es menor y, repercutiendo así, en sus propiedades de gelatinización (Srichuwong *et al*., 2005a).

En los estudios de propiedades tecno-funcionales del almidón, estos son aislados por molienda seca. Sin embargo, esta molienda suele ir acompañada de alteraciones en la conformación del almidón dando lugar a una fracción de almidón dañado y más contaminada con el resto de componentes (Leewatchararongjaroen, & Anuntagool, 2016). Por otro lado, en procesos de molienda húmeda se ha observado una mejora de la preservación de la estructura del almidón a través de parámetros de gelatinización y pureza. Así, una menor entalpía de gelatinización (ΔH_G) en los almidones obtenidos por molienda seca se puede interpretar como una mayor destrucción de la cristalinidad que va a favorecer una mejor interacción con los enzimas digestivos (Leewatchararongjaroen, & Anuntagool, 2016; Shi, Gao, & Liu, 2018).

En estudios previos, se han optimizado las condiciones de maceración previo a la molienda húmeda de quinoa (6,5 h/30 °C), para obtener una mayor recuperación de almidón y de mayor

pureza en cuanto al contenido de lípidos (Ballester-Sánchez, Gil, Fernández-Espinar, & Haros, 2019c).

1.4.1. Propiedades que afectan a la digestibilidad del almidón

El almidón presenta diferentes características estructurales que, dependiendo del origen botánico y/o tratamiento, darán lugar a diferentes comportamientos en su digestibilidad. Los principales factores estructurales del gránulo que influyen en su digestibilidad, se muestran en la tabla 2.

Tabla 2: Propiedades estructurales del almidón que afectan a la digestibilidad.

Factores	Quinoa	Otros almidones	Efecto	Referencias
Tamaño del gránulo (µm)	0,4–2,0	Trigo/Maíz (10–21) Patata (35–60)	↓ Diámetro ↑ Superficie específica ↓ K_M → ↑ Hidrólisis	Li *et al*., 2018; Warren *et al*., 2011; Tahir *et al*., 2010
Contenido AM (%)	6,1–19,7	Trigo/Maíz (24–26) Amaranto (1–2) Arroz (13–26)	↓ AM ↓ Relación AM/AP → ↑ Hidrólisis	Srichuwong *et al*., 2017; Li *et al*., 2018; Lin *et al*., 2018; Singh *et al*., 2010
Grado de polimerización de AP (DP)	6–12	Amaranto/Trigo (6–12) Maíz/Mijo (12–18)	↑ Cadenas cortas DP (6–12) → ↑ Hidrólisis	Srichuwong *et al*., 2017
Patrón de difracción de rayos X	Tipo A	Cereales Tipo A Tubérculos Tipo B Legumbres Tipo C	↑ Hidrólisis en patrón A que B	Srichuwong *et al*., 2005a Sajilata *et al*., 2006 Li *et al*., 2018

AM: Amilosa; AP: Amilopectina; K_M: Constante de Michaelis-Menten.

Como se observa en la tabla 2, factores característicos del gránulo de quinoa como un tamaño pequeño, un contenido alto en AP con grado de polimerización (6–12) y un patrón A, favorecen la digestibilidad del almidón. Si bien, el estudio del porcentaje de hidrólisis es ampliamente utilizado debido al impacto del índice glucémico en el desarrollo de enfermedades metabólicas, existe también una fracción del almidón que no es digerible. Se ha observado que el incremento del contenido de harina integral de quinoa en formulaciones de pan ha dado lugar a un mayor porcentaje de almidón resistente (fracción no digerible) (Xu, Luo, Yang, Xiao, & Lu, 2019). Esta fracción de almidón resistente llega al intestino grueso, donde es fermentado por acción de la microbiota intestinal dando lugar a un aumento de la producción de AGCC. La generación de AGCC (ej., ácidos propiónico, acético, butírico) puede influenciar de manera beneficiosa en la sensibilidad a la insulina consiguiendo incrementarla a través de cambios en el metabolismo del tejido adiposo y del músculo esquelético (Regmi, van Kempen, Matte, & Zijlstra, 2011; Robertson, Bickerton, Dennis, Vidal, & Frayn, 2005).

Por otro lado, en una matriz alimentaria el almidón interacciona con otros componentes tales como proteínas, lípidos o polifenoles que pueden interferir en su velocidad de hidrólisis. La quinoa contiene un alto porcentaje de polifenoles como los taninos (Filho *et al*., 2017) por lo que la digestibilidad del almidón podría verse

reducida a través de posibles interacciones hidrofóbicas con la AM y fragmentos lineales de AP (Barros, Awika, & Rooney, 2012). Además de la presencia de otros componentes en la matriz alimentaria, la digestibilidad del almidón también va a depender de las prácticas culinarias y, previas a estos, factores como pretratamientos térmicos, pH del medio, secado, entre otros. Un ejemplo de ello es la fracción de almidón dañado en harinas, la cual, es más susceptible a una rápida hidrólisis, a una mayor capacidad para absorber agua y un cambio en las características fisicoquímicas como son las propiedades de gelatinización y de pasta durante las distintas etapas del proceso de panificación (Jukić *et al*., 2019).

1.4.2. Efecto del tratamiento hidrotérmico

El procesamiento de alimentos conduce a cambios estructurales de sus componentes, entre ellos el almidón, lo cual condiciona las características nutricionales del alimento, incluida su digestibilidad (Roopa, & Premaval, 2008). Uno de los fenómenos más comunes que ocurren tras el procesamiento de materias primas amiláceas es la gelatinización del almidón. En las matrices alimentarias de cereales/pseudocereales después de ser procesadas industrialmente, como es el caso del pan, el almidón sufre fenómenos de gelatinización y posteriormente, tras la

cocción/almacenamiento, la retrogradación lo que condiciona su calidad tecnológica y nutricional (Demirkesen, Sumnu, & Sahin, 2013; Ballester-Sánchez, Yalcin, Fernández-Espinar, & Haros, 2019d).

Sin embargo, previo a la gelatinización, el almidón puede sufrir cambios en sus propiedades fisicoquímicas sin destruir su estructura granular a través del proceso llamado 'annealing'. Jacobs, & Delcour, (1998) describe el proceso de '*annealing*' cuando existe un tratamiento hidrotérmico donde el contenido en agua se considera en exceso (> 60 % p/p) o en cantidades intermedias (40–55 % p/p). En ambas condiciones de contenido de agua, a temperaturas por encima de la temperatura de transición vítrea (el paso de un estado termodinámico metaestable, caracterizado por un orden molecular, a un estado gomoso, donde las moléculas pierden su organización), pero por debajo de la temperatura de gelatinización.

El proceso de 'annealing' promueve la reorganización y la perfección de la estructura cristalina lo que provoca mayores temperaturas de gelatinización o grado de cristalinidad. Sin embargo, se ha observado que los gránulos de almidón que sufrieron 'annealing' muestran mayor susceptibilidad a la hidrólisis en comparación con los almidones sin tratamiento hidrotérmico. Esto podría deberse a la mayor facilidad de entrada de enzimas hidrolíticas, como la α-amilasa, a los gránulos de

almidón por un aumento de la porosidad de estos (Nakazawa, & Wang, 2003; O'Brien, & Wang, 2008).

Cuando el almidón se calienta en presencia de agua y se inicia el proceso de gelatinización, su estructura cristalina comienza a romperse y las moléculas de agua se unen mediante enlaces de hidrógeno a los grupos hidroxilo expuestos de AM y AP, provocando un aumento de volumen de los gránulos y mayor solubilidad de sus componentes (Rooney, & Pflugfelder, 1986). La expansión de los gránulos de almidón por la absorción de agua comienza en las zonas intermicelares (menos organizadas y más accesibles) y rompe los enlaces de hidrógeno liberando parte de la AM por lixiviación, por lo que se reduce la birrefringencia y el almidón se vuelve más soluble exponiéndose al ataque enzimático, siendo el grado de gelatinización un factor determinante en la accesibilidad enzimática (Rooney, & Pflugfelder, 1986; Wang *et al*., 2019) (Figura 2). Una vez superada la temperatura de gelatinización, y solo cuando hay exceso de agua, se perderá totalmente su cristalinidad y se formará una pasta viscosa.

Por otro lado, cuando se deja de aplicar altas temperaturas sobre el almidón gelatinizado, se produce el proceso de retrogradación, las moléculas de los gránulos de almidón gelatinizados comienzan a reagruparse en una estructura ordenada (Rooney, & Pflugfelder, 1986). A corto plazo, la reorganización se asocia principalmente a la AM (representado en la figura 3), mientras que a largo plazo la

responsable es la AP (Dobosz *et al*., 2019). Esta retrogradación de la AP se ha correlacionado negativamente con la digestibilidad, cuanto mayor grado de retrogradación, menor es la susceptibilidad enzimática (Lin, Zhang, Zhang, & Wei, 2017).

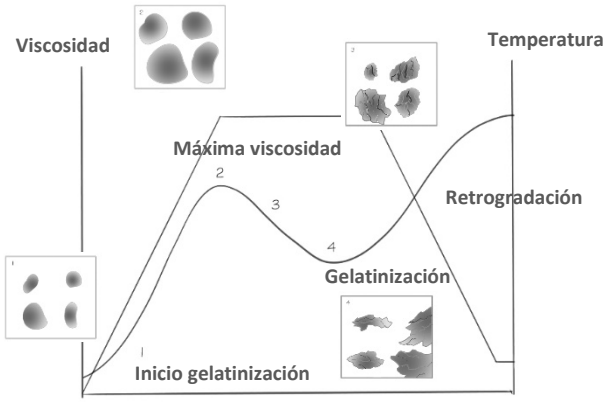

Figura 3: Perfil de viscosidad de una suspensión de almidón por aplicación de un ciclo de calentamiento y enfriamiento en un viscoanalizador rápido (RVA).

Centrándonos en el almidón de quinoa, la fase inicial del proceso de gelatinización y el intervalo de temperaturas durante el cual se van a realizar estos cambios estructurales, van a depender de características como su granulometría pequeña, grado de cristalinidad tipo A, alto contenido en AP y de cadenas cortas (DP 6–12) (Li *et al*., 2018). En concreto, las propiedades térmicas y

reológicas van a determinar la susceptibilidad a estos cambios donde tanto la cantidad de AM/AP como la estructura de la AP se ha demostrado estar significativamente correlacionadas con sus propiedades físicas y tecno-funcionales (Srichuwong *et al*., 2005a; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005b). De hecho, Li, & Zhu (2017a) revelaron mediante la correlación de Pearson, que propiedades relacionadas con la gelatinización, como la capacidad de hinchamiento, el índice de solubilidad en agua o la cristalinidad, se ven afectadas por el perfil de la cadena de AP y el contenido de AM en la quinoa.

Al considerar una matriz alimentaria como el pan, hay que tener presente que existen otros componentes, como son la fibra, las proteínas, los compuestos fenólicos y los minerales, que pueden interactuar con los componentes del almidón y modificar sus propiedades tecno-funcionales. La obtención de mayores temperaturas de gelatinización en muestras de harina de quinoa comparadas con su almidón aislado sugiere esta posible interacción entre el almidón y el resto de los compuestos de la matriz alimentaria (Li, Wang, & Zhu, 2016; Li, & Zhu, 2017b; Srichuwong *et al*., 2017). Por ejemplo, la temperatura máxima de gelatinización de la harina de quinoa se correlaciona negativamente con el contenido de lípidos (Li, & Zhu, 2017b). Esto podría deberse a la presencia del ácido linoleico, presente en gran proporción en la quinoa, donde la formación de complejos

lípidos–amilosa reduce el poder de hinchamiento y solubilidad del almidón retardando la gelatinización y retrogradación del mismo (Ballester *et al*., 2019a; Ai, Hasjim, & Jane, 2013; Li, & Zhu, 2017b). Por otro lado, la mayor presencia de proteínas como las albúminas/globulinas comparadas con glutelinas en la quinoa, al contrario de lo que ocurre en el trigo donde la fracción albúminas/globulinas es la minoritaria, también repercuten en los procesos de cambios térmicos en el almidón (Taylor, & Parker, 2002; Baxter, Blanchard, & Zhao, 2014). Los aumentos observados en la velocidad inicial de absorción de agua en muestras con globulina añadida indican que la adición de esta proteína facilitó la absorción de agua en los gránulos de almidón, al menos en las primeras etapas de cocción. La capacidad de absorción de agua de las albúminas/globulinas facilita el hinchamiento del gránulo del almidón obteniendo temperaturas de gelatinización más bajas al añadir estas proteínas al almidón (Baxter *et al*., 2014). Por ello, la interacción de componentes presentes en la harina con el almidón va a dar lugar a cambios en los parámetros térmicos o de pasta del almidón (Srichuwong *et al*., 2005a; Li, & Zhu, 2017a). Estas diferentes propiedades se han correlacionado con la hidrólisis del almidón y, por tanto, van a repercutir en la digestibilidad del mismo (Li *et al*., 2016; Li, & Zhu, 2017b). Por consiguiente, la sustitución del 25 % de la harina por harina integral de quinoa en la elaboración de productos de

panadería ha provocado cambios en las propiedades térmicas y de pasta, lo que dará lugar al desarrollo de productos con diferentes características fisicoquímicas y distinta digestibilidad (Ballester-Sánchez *et al*., 2019d).

1.4.3. Métodos de estimación de la bioaccesibilidad de glucosa *in vitro*

El estudio de la digestibilidad *in vitro* del almidón ha sido de gran interés debido a la posibilidad de asociarse con la respuesta glucémica, indicador de la respuesta de la glucosa posprandial, utilizado ampliamente hasta día de hoy (Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Graça, Raymundo, & de Sousa, 2021). La realización de estudios *in vitro* con base química para determinar la digestibilidad del almidón tiene ventajas sobre los estudios *in vivo* porque son mucho más económicos, menos complejos y no requieren autorización ética. Con estos estudios se puede determinar la bioaccesibilidad de la glucosa (fracción soluble en el medio gastrointestinal simulado, la cual, está disponible para su absorción) (Minekus *et al*., 2014). Sin embargo, carecen de los procesos fisiológicos de control endocrino y de las interacciones que pueden producirse en el metabolismo epitelial por la absorción de otros nutrientes.

Generalmente, se utilizan dos métodos de estudio como indicadores de la bioaccesibilidad de glucosa: la solubilidad y la dializabilidad. Los métodos de solubilidad (sin ninguna restricción) se correlacionan satisfactoriamente como predictores del índice glucémico (Goñi *et al*., 1997). Sin embargo, no permiten el estudio de los flujos de disponibilidad (liberación y difusión) de glucosa que pueden afectar a la respuesta fisiológica. Con los métodos de dializabilidad (con restricción), donde se incorpora una membrana de diálisis (semipermeable), permiten evaluar la disponibilidad de glucosa (Laparra, & Haros, 2018), asemejándolo al proceso de difusión de la glucosa hacia el epitelio.

En cuanto a la quinoa, la digestibilidad de su almidón se ha estudiado mediante métodos de solubilidad a partir de la hidrólisis de su almidón en muestras de harina (Srichuwong *et al*., 2017; Wu, Wang, Shen, & Qu, 2020), así como de panes con un reemplazo parcial de la harina de trigo por un 5, 10 y 15 % de harina de quinoa (Xu *et al*., 2019). Los resultados apuntan a un efecto positivo en la reducción del índice glucémico comparado con el pan de trigo (Xu *et al*., 2019). Por otro lado, también se han realizado estudios de dializabilidad utilizando la quinoa en productos panarios. Se han determinado mediante tubos de diálisis (10–11 kDa) (Wolter, Hager, Zannini, & Arendt, 2013; Wolter, Hager, Zannini, & Arendt, 2014), así como en sistemas bicamerales donde la disponibilidad de glucosa se valora de modo secuencial a como se produce la digestión por los enzimas gastrointestinales (Laparra, & Haros, 2018). En este último modelo, el cual utiliza membranas de

diálisis (14 kDa), la digestibilidad de panes al 25 % de harina de quinoa, presentan valores de digestibilidad similares al pan de trigo.

Por otro lado, también se ha estudiado la hidrólisis del almidón de quinoa aislado utilizando un método de solubilidad, donde se observó su alta digestibilidad comparada con otros almidones de cereales (Srichuwong *et al*., 2017; tabla 1). Sin embargo, la hidrólisis del almidón aislado de quinoa utilizando métodos de dializabilidad aún no está descrito en la bibliografía.

En la búsqueda de modelos más cercanos a la respuesta fisiológica en el organismo, está adquiriendo importancia la consideración del impacto de los flujos de glucosa en procesos simultáneos de digestión y absorción del almidón (González, González, Zúñiga, Estay, & Troncoso, 2020). En este contexto, los modelos *in vitro* que incorporan distintos cultivos celulares pueden suponer una herramienta adecuada para estimar la potencial respuesta tisular frente a la fracción bioaccesible obtenida del alimento (Laparra, Tako, Glahn, & Miller, 2008). Estos modelos, de modo general, utilizan un sistema bicameral (compartimento dador vs. aceptor) incorporando una membrana de diálisis, la cual, actúa como barrera selectiva excluyendo el contacto de las enzimas digestivas con el cultivo celular. La versatilidad de utilizar un sistema bicameral permite el estudio de las cinéticas de disponibilidad y la respuesta celular a esta (Laparra *et al*., 2008) (Figura 4).

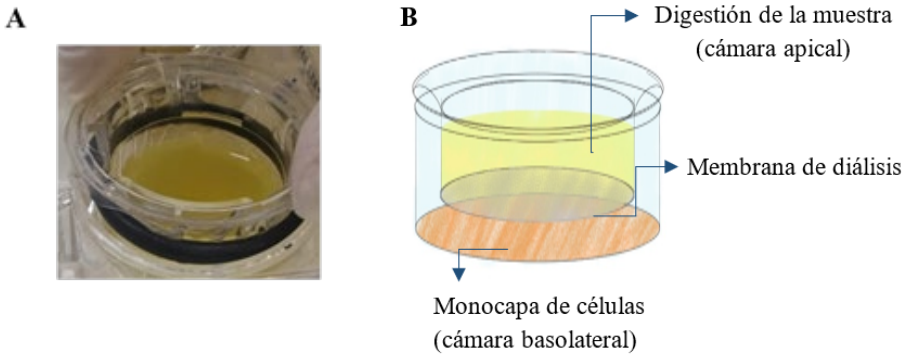

Figura 4: Sistema bicameral (A); representación del diseño experimental de los sistemas *in vitro* que combinan el uso de cultivos celulares con una aproximación química para el estudio de la bioaccesibilidad de nutrientes (B).

1.5. Proteínas: Inhibidores de proteasas

Los cereales contienen distintas fracciones proteicas, las cuales, atendiendo a su solubilidad se pueden clasificar en distintos tipos (Tatham, & Shewry, 2008):

- Albúminas (solubles en agua),
- Globulinas (solubles en solución salina diluida),
- Prolaminas (solubles en soluciones de alcohol) y
- Glutelinas (solubles en ácidos o bases diluidas)

A diferencia de los cereales, tales como el trigo o el arroz donde las principales proteínas son las glutelinas y las prolaminas, representando aproximadamente el 70–80 % y quedándose en tan solo un 20–30 % de albúminas y globulinas, (Tatham, & Shewry, 2008; Amagliani, O'Regan, Keny, & O'Mahony, 2017), en la quinoa y la chía predomina esta última fracción (~ 70–80 % albúminas/globulinas) (Taylor *et al*., 2002; Sandoval-Oliveros, & Paredes-López, 2013). En la quinoa, es destacable el contenido predominante de la globulina 11S llamada también 'chenopodina' con un peso molecular entre 20–39 kDa (Taylor *et al*., 2002; Ballester-Sánchez *et al*., 2019d). Otra globulina característica de la quinoa es la 2S, rica en cisteína, arginina e histidina (Taylor *et al*., 2002). En las semillas de chía la principal fracción proteica es la de las globulinas (52 %) 11S y 7S con un peso molecular de 15 a 50 kDa, aunque también se pueden encontrar las 2S (Sandoval-Oliveros, & Paredes-López, 2013).

De la fracción de albúminas y globulinas, las que presentan actividad inhibidora de proteasas, han recibido una creciente atención, entre ellas, las inhibidoras de amilasa, tripsina/quimotripsina y, en algunos casos, de pepsina (Wisessing, & Choowongkomon, 2012). Estas proteínas inhibidoras se pueden clasificar según el sitio reactivo específico como: inhibidores de cisteína proteasa, inhibidores de metaloide proteasa, inhibidores de aspártico proteasa e inhibidores de serina proteasa (Laskowski, &

Kato, 1980). Dentro de este último grupo, los inhibidores de proteasa tipo serina (STPIs), se engloban distintas familias de acuerdo a sus propiedades estructurales y bioquímicas: i) inhibidores de proteasas serínicas tipo Bowman-Birk, ii) inhibidores de tripsina de soja (usualmente denominados tipo Kunitz), iii) inhibidores de tripsina de mostaza, iv) inhibidores de tripsina/α-amilasa de cereales, v) inhibidores de patata tipo I y tipo II, y vi) inhibidores de calabaza y serpinas (De Leo *et al*., 2002; Clemente *et al*., 2019).

Los STPIs se pueden encontrar en grandes cantidades tanto en legumbres (i.e., lentejas, judías o guisantes), en cereales (i.e., arroz, trigo o cebada), así como pseudocereales (i.e., amaranto) y oleaginosas (i.e., lino) (Laskowski, & Kato, 1980; Srikanth, & Chen, 2016; Tamir *et al*., 1996; Lorenc-Kubis, Kowalska, Pochroń, Żużło, & Wilusz, 2001). Se ha detectado también la presencia de STPIs en las semillas de quinoa (Pesoti *et al*., 2015) y de chía (Turck *et al*., 2019), llegándose a realizar esfuerzos analíticos en la caracterización de la composición de las subunidades proteicas, así como su actividad relativa inhibidora de proteasas (i.e., pancreatina) en comparación con los STPIs presentes en trigo y avena (Laparra, & Haros, 2019a). Los STPIs han sido considerados como factores antinutrientes al ejercer actividad inhibidora sobre las enzimas proteolíticas del sistema digestivo e interferir en la absorción de los nutrientes, lo cual, ha

generado la tendencia de eliminarlos mediante, por ejemplo, la cocción. No obstante, aquellos identificados en granos como el trigo han mostrado una notable resistencia térmica (>100 ºC), al tiempo que se han asociado con una influencia positiva aumentando la severidad de los procesos inflamatorios intestinales (Junker *et al*., 2012). En estos últimos años, esta consideración ha cambiado sobre aquellos STPIs tipo Kunitz, Bowman-birk y derivados de legumbres al considerarse su potencial uso en el control de patologías de base inmunometabólica (i.e., diabetes tipo 2, obesidad) (Carai *et al*., 2009; Obiro, Zhang, & Jiang, 2008). Incluso se ha propuesto la implicación positiva de estos STPIs en procesos biológicos como la apoptosis o el cáncer, debido a su potencial inmunonutricional (Srikanth, & Chen, 2016; Srdić *et al*., 2020; Laparra, Fotschki, & Haros, 2019b; Llopis, Brown, & Saiz, 2020).

A pesar de haberse identificado en distintos cereales y otros alimentos (i.e., tomate, patata), la mayor proporción de estudios en relación con la estructura y actividad de los STPIs se han llevado a cabo con el trigo. Zevallos *et al*., (2017) han identificado contenidos de hasta 10 especies distintas de STPIs en el trigo (i.e., 0.28, 0.53, 0.19, CM16, CM17, CM1, CM2, CM3, CMX1 y CMX3). Entre ellos, se identificó a los compuestos 0.19 y CM3 como los principales responsables de activar la respuesta inmune innata a través del receptor tipo 'Toll' (TLR)-4 (Zevallos *et al*.,

2017; Junker *et al*., 2012). Se ha observado que la ingesta nutricional de estos STPIs provenientes del trigo actúan en el sistema inmune a través de la activación de las células T innatas y macrófagos, así como conducen a cambios en la composición de la microbiota intestinal (Zevallos *et al*., 2017; Pickert *et al*., 2020; Kaliszewska, Martinez, & Laparra, 2016). Esta activación del sistema inmune innato intestinal se ha relacionado con el agravamiento de procesos inflamatorios en otros órganos como el hígado (Ashfaq-Khan *et al*., 2019), las vías respiratorias (Bellinghausen *et al*., 2018) e incluso el sistema nervioso central (Guilherme *et al*., 2020) en ratones, los cuales, pueden repercutir y desencadenar problemas relacionados con el síndrome metabólico.

Hay que señalar que estos compuestos, independientemente de su origen botánico, presentan una elevada estabilidad a los tratamientos térmicos y una digestibilidad relativamente baja por los enzimas gastrointestinales (Junker *et al.,* 2012; Laparra, & Haros, 2019a). En procesos de panificación, donde se pueden alterar la estructura de las proteínas y, por ende, su actividad, se ha observado que se mantiene una actividad proinflamatoria, aunque en menor grado en comparación con su harina (Zevallos *et al*., 2017; Huang *et al*., 2020). En el caso de las albúminas y globulinas provenientes de la quinoa y chía, se ha estimado una termoestabilidad parecida, e incluso mayor en el caso de los

compuestos provenientes de la chía, comparado con el trigo (da Silva *et al*., 2015; Sandoval-Oliveros, & Paredes-López, 2013).

Laparra & Haros (2019b) han analizado la composición y actividad de extractos proteicos enriquecidos con STPIs derivados de quinoa y chía, comparándolos con los descritos en trigo y avena. Las diferencias principales se refieren a la presencia de subunidades homoméricas, como en la *Avena sativa*, así como la presencia de enlaces *N*-terminal glucuronamida en la chía y glucósidos en la quinoa (Laparra, & Haros, 2019a). Por el contrario, en los extractos de trigo se identificaron subunidades heteroméricas. Al considerar su actividad, es notable la menor actividad inhibidora de proteasas de los complejos proteicos derivados de quinoa y chía. La mayor actividad inhibitoria de estos compuestos de quinoa y chía se ha observado en aquellas subunidades < 14 kDa (Srdić *et al*., 2020), siendo en mayor medida a las proteínas 2S (Figura 5) (Laparra & Haros, 2019a; Srdić *et al*., 2020; da Silva *et al*., 2015). Estos estudios también han permitido apuntar a un diferente mecanismo molecular de los STPIs de quinoa y chía, con respecto a los de trigo, en la señalización derivada de su interacción con el receptor TLR4 (Srdić *et al*., 2020).

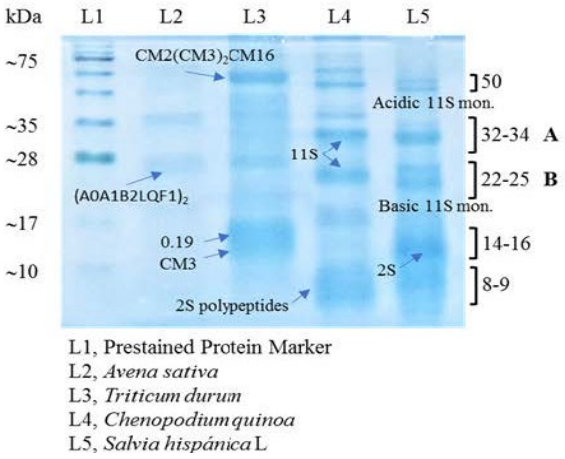

Figura 5: Patrón electroforético (SDS-PAGE, 12 %) obtenidos de la fracción proteica soluble de la avena (L2), el trigo (L3), la quinoa (L4) y la chía (L5) (Srdić *et al*., 2020).

2. Alteraciones de la homeostasis de la glucosa

El control de la homeostasis de la glucosa es un factor clave para la salud debido a la importancia central de esta como fuente de energía siendo, en el caso del cerebro, vital para cubrir sus funciones fisiológicas. Las alteraciones en la utilización de la glucosa y la señalización de la insulina, regulador endocrino de la concentración disponible de glucosa, se pueden observar no solo en enfermedades como el síndrome metabólico, la obesidad o la diabetes tipo 2 (DT2). Además, también desempeñan una función importante en enfermedades neurodegenerativas como el alzhéimer, enfermedades cardiovasculares o enfermedades genéticas como la glucogenosis (Blázquez, Velázquez, Hurtado-Carneiro, & Ruiz-Albusac, 2014; Alberti *et al*., 2009; Weinstein, Steuerwald, De Souza, & Derks, 2018).

El síndrome metabólico fue definido por primera vez en 1998 por la Organización Mundial de la Salud (WHO) y, aunque existen diversas definiciones, todas ellas tienen en común un complejo de factores de riesgo interrelacionados que incluyen: obesidad abdominal, hipertrigliceridemia, hipertensión arterial, reducción de lipoproteínas de alta densidad (HDL) y niveles elevados de glucosa (Alberti *et al*., 2009). En los últimos años, el interés se ha centrado en la posible implicación de la resistencia a la insulina como factor clave en el desarrollo de estas patologías. En la condición de resistencia a insulina, los tejidos presentan una respuesta disminuida para absorber la glucosa circulante ante la

acción de la insulina, circunstancia crítica en estadios previos al desarrollo de diabetes u obesidad, actualmente consideradas las grandes pandemias del siglo XXI (Alberti *et al*., 2009). Según la Federación Internacional de Diabetes (IDF), aproximadamente 468 millones de adultos sufren diabetes (en torno al 90 % son DT2), cifra que se ha multiplicado por 4 en los últimos 25 años (IDF, 2019). Por otro lado, según las últimas estimaciones llevadas a cabo por UNICEF, el número de niños menores de 5 años con sobrepeso en todo el mundo aumentó de 30 a 38 millones entre los años 2000 y 2017 (UNICEF/WHO/World Bank, 2018).

A diferencia de la diabetes o la obesidad donde se caracterizan por ir acompañadas de un estadio de hiperglucemia, existen patologías con el factor inverso, asociándose con problemas de hipoglucemia como es el caso de la glucogenosis. La glucogenosis es un grupo de enfermedades hereditarias, causadas por la falta de una o más enzimas que intervienen en la síntesis o degradación del glucógeno. Se caracterizan por la acumulación de glucógeno en los tejidos dando lugar en algunas de ellas, como es el caso de la glucogenosis tipo I y III, a hipoglucemias severas acompañadas de una disrupción en el metabolismo hepático generando hepatomegalias (Weinstein *et al*., 2018).

Entre las diversas causas ‘exógenas’ que favorecen el desarrollo de las patologías relacionadas con las alteraciones de la homeostasis glucídica, cabe destacar la inactividad física y la dieta. Hábitos dietéticos "occidentales" ricos en carnes rojas, frituras, alimentos procesados altos

en ácidos grasos saturados y trans o cereales refinados que proporcionan carbohidratos de alto índice glucémico, ejercen efectos significativos aumentando la incidencia del síndrome metabólico (Fabiani, Naldini, & Chiavarini, 2019). Concretamente, la ingesta de dietas con elevado contenido en grasas saturadas potencia la aparición de intolerancia a la glucosa a través de un estadio de resistencia a la insulina. Se observa una menor afinidad del receptor de insulina en el hígado, no suprimiendo la producción de glucosa por parte del hígado, así como se reduce la absorción o utilización de glucosa en el músculo esquelético y tejido adiposo, conduciendo todo ello a una hiperinsulinemia compensadora (Lackey *et al*., 2016).

El hígado constituye un órgano clave en el metabolismo sistémico, cuya disfunción metabólica contribuye sustancialmente al desarrollo de resistencia a la insulina y la DT2. Los mecanismos subyacentes a estos procesos no se comprenden del todo, pero implican la acumulación de grasa hepática, alteraciones del metabolismo energético y señales inflamatorias derivadas de varios tipos de células, incluidas las células inmunes. Se ha propuesto que las lipotoxinas, la función mitocondrial, las citocinas y las adipocitocinas desempeñan un papel importante tanto en el desarrollo de la DT2, así como de patologías como la NAFLD (Tilg, Moschen, & Roden, 2016). La presencia de NAFLD constituye un factor agravante del riesgo de desarrollar DT2 y de otras características importantes del síndrome metabólico (principalmente complicaciones cardiovasculares como aterosclerosis e isquemia/infarto de miocardio),

afectándose la morbilidad y mortalidad relacionadas con la disfunción hepática. Si bien, es difícil influir en los factores endógenos del huésped que resultan responsables del desarrollo de NAFLD, como los distintos tipos de genes receptor activado por proliferadores peroxisomales (PPAR) implicados en el metabolismo lipídico (Dongiovanni, & Valenti, 2013), los factores ambientales son predominantes y se pueden abordar con una intención preventiva o terapéutica. Por tanto, el control dietético en los equilibrios homeostáticos lipídicos y de la glucosa es clave para minimizar el riesgo y/o severidad de la hiperglucemia sobre la potencial disfunción hepática.

2.1 Contribución inmunonutricional del eje enterohepático a la homeostasis metabólica

El epitelio intestinal representa la superficie mucosa más extensa del organismo humano, así como el compartimento inmunológico de mayor dimensión. En este contexto, es importante distinguir los tipos de variables que existen en los sistemas homeostáticos. Las variables fisiológicas que se mantienen en un nivel estable, como la glucosa plasmática se denominan 'variables reguladas', aunque, encontramos 'variables controladas' que contribuyen a la estabilidad de las variables reguladas (Cabanac, 2006). Por ejemplo, la concentración de glucosa en sangre es una variable regulada, mientras que la producción de insulina sería una variable controlada para regular la concentración de glucosa.

La mayoría de los procesos fisiológicos solo operan en un rango limitado de condiciones, las cuales, son mantenidas por mecanismos homeostáticos especializados amortiguando las variaciones ambientales y que se ajustan en respuesta a las demandas funcionales y prioridades biológicas del organismo. Solo algunos de estos procesos son susceptibles a alteraciones causadas por el desarrollo de estados disfuncionales, por ejemplo, como la hiperglucemia. Así, el metabolismo de los lípidos y la glucosa puede descontrolarse, dando lugar a dislipidemia, diabetes y obesidad, mientras que el metabolismo de los aminoácidos parece poco susceptible a la desregulación homeostática (Kotas, & Medzhitov, 2015).

Estudios recientes han identificado al receptor inmunológico innato TLR4 intestinal como un factor crítico coordinando la interacción entre la microbiota intestinal y los genes que regulan vías metabólicas importantes en el hospedador (Lu *et al*., 2018). Además, la señalización y activación de respuestas inflamatorias inducidas por TLR4 parece estar sujeta a un control nutricional. La deficiencia de micronutrientes como el hierro se ha asociado con una deriva selectiva hacia una mayor actividad proinflammatoria, derivada de la señalización molecular, subyacente al receptor TLR4 (Wang *et al.,* 2009). Así, la DT2 duplica el riesgo de prevalencia de comorbilidades como la anemia (Gauci, Hunter, Bruce, Davis, & Davis, 2017). Este efecto no se ha asociado con deficiencias en el aporte y biodisponibilidad del micronutriente, sino con el establecimiento de un estado inflamatorio crónico ‘leve’ donde la

producción hepática de mediadores como la hepcidina juega un papel esencial (Aeberli, Hurrell, & Zimmermann, 2009).

La DT2 representa una alteración metabólica cuyo origen puede situarse en las complejas interacciones entre factores genéticos y ambientales y cuyo desarrollo está estrechamente asociado con la obesidad. En el periodo prediabético, la especificidad de la señalización inmune innata que regula la polarización de efectores inmunológicos como los macrófagos, desde su fenotipo M2 (antiinflamatorio) hacia su estado de activación M1 (proinflamatorio), juega un papel crucial en la promoción de la resistencia a insulina (Olefsky, & Glass, 2010). Además, estudios recientes han demostrado un papel crítico de las células linfoides innatas ILC2s, exclusivamente de origen intestinal, en la regulación de la tolerancia a glucosa y resistencia a insulina (Sasaki *et al.,* 2019). Estos hallazgos deberían situarse en su contexto fisiológico para los que se ha determinado la actividad secuencial de los efectores inmunológicos innatos y adaptativos durante el desarrollo fisiológico normal y determinación de la composición de la microbiota intestinal, así como la gestión y homeostasis tisular del organismo (Mao *et al.,* 2018). Así, la bibliografía científica establece una gradación de factores a los que se atribuye la promoción de la resistencia a insulina como: inmunidad innata > inmunidad adaptativa > microbiota.

En un ámbito inmunonutricional, la administración de STPIs se ha mostrado eficaz para conseguir una polarización selectiva de la población monocítica/macrofágica hepática hacia su fenotipo M1 (Junker *et al*.,

2012; Kaliszewska *et al*., 2016; Laparra, & Haros, 2019a; Llopis *et al*., 2020). Estas intervenciones preclínicas evidencian efectos variables, mejorando o agravando, las condiciones proinflamatorias hepáticas en función de la señalización molecular subyacente al receptor TLR4 que se implica en el proceso inflamatorio. Otro aspecto relevante que cabe mencionar es que una gran parte de la investigación previa se ha centrado principalmente en la ingesta y el consumo total de calorías, con un balance positivo continuo, como promotor principal de la obesidad, NAFLD y/o el síndrome metabólico. Sin embargo, estos estudios inmunonutricionales (Junker *et al*., 2012; Kaliszewska *et al*., 2016; Laparra, & Haros 2019a; Llopis *et al*., 2020; Ashfaq-khan *et al*., 2020) apuntan a que su influencia e interacción con el sistema inmunológico intestinal del huésped, así como la composición de los alimentos, independientemente del recuento de calorías, pueden ser determinantes tanto en la salud intestinal como hepática, metabólica y cardiovascular.

La evidencia actual de una asociación de la mejora inmunonutricional de los alimentos con el control de enfermedades metabólicas crónicas ha sido en gran medida inferencial. Así, quedan preguntas clave sin respuesta que requieren un esfuerzo de investigación concertado para armar a la comunidad científica y finalmente a la sociedad con estrategias efectivas de traslación y transferencia.

II

OBJETIVOS

II OBJETIVOS

El objetivo general de la presente tesis doctoral es el estudio de las características tecno-funcionales e impacto fisiológico de las harinas integrales de quinoa y chía o coproductos de su industrialización para ser integradas a la dieta en el ámbito de la alimentación saludable.

Para ello, se ha llevado a cabo los siguientes objetivos específicos:

1. Estudio del almidón de quinoa a través de la determinación de las propiedades tecno-funcionales, efecto del tratamiento hidrotérmico y sus cinéticas de liberación de glucosa comparándolo con otros almidones comerciales. Evaluación de la incorporación de harina de chía en la liberación de glucosa y estudio de la respuesta celular en presencia de insulina.

2. Estudio de los efectos derivados de la administración de formulaciones panarias con harina de quinoa y chía en los desbalances metabólicos causados por el consumo de dietas hipercalóricas.

2.1. Estudio de la respuesta hepática de carácter inmunonutricional en el control de la glucemia en animales prediabéticos.

2.2. Estudio del control de la glucemia en animales con predisposición transgeneracional al acúmulo lipídico hepático y con deficiencia nutricional en hierro.

III

RESULTADOS

Capítulo 1: Potential beneficial effect of hydrothermal treatment of starches from various sources on *in vitro* digestion

Potential beneficial effect of hydrothermal treatment of starches from various sources on *in vitro* digestion

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ABSTRACT

Starches from various botanic origins (maize, quinoa, wheat, potato and rice) were studied. The thermal and pasting properties and their connection with enzyme digestibility were evaluated. Various hydrothermal treatments were applied, taking the starch physical parameters into account, in order to obtain partial and total gelatinisation of the starch structure and determine its influence on enzymatic action. Onset and pasting temperatures of the gelatinisation and pasting processes, respectively, followed the same order in the cereal starches (rice > maize > wheat > quinoa). These results were accompanied by an opposite trend in the percentage of raw starch hydrolysis, with quinoa reaching a level more than 2-fold higher than that of raw maize starch in *in vitro* digestion kinetics. Other technological parameters, such as high peak viscosity or low breakdown, also reflected modifications in the quinoa starch structure which were related to improved digestibility.

However, starch from potato, the only tuber, displayed different characteristics from those of cereal starch, showing greater resistance to digestion. When the starches were pretreated, digestibility increased in all of them compared to their raw counterparts, with the pretreated quinoa and wheat starches showing greater susceptibility to modification of their structure. Although the hydrothermally pretreated maize and rice starches reached about 75% of the hydrolysis index of the corresponding gelatinised starches, raw quinoa had a similar hydrolysis index and quinoa obtained a higher value for total starch hydrolysed. Thus, quinoa starch could be potentially beneficial in the design of more digestible formulations for patients with metabolic disorders such as glycogen storage disease, among others.

Keywords: Glycogen storage disease · Maize starch · Thermal and pasting properties · *In vitro* digestion · Quinoa starch

INTRODUCTION

In recent years, glucose homeostasis has been an important focus of research owing to its physiological involvement in metabolic diseases such as diabetes, obesity and glycogen storage disease (GSD) (Ludwig, 2002; Weinstein, Steuerwald, De Souza, & Derks, 2018). Consequently, several investigations have focused on studying the glycaemic index (GI) of foods and applying various strategies to modify starch digestibility and glucose release in order to manage glucose homeostasis and try to obtain optimal metabolic control (Laparra & Haros, 2018; Li, Gidley, & Dhital, 2019).

The degree of starch gelatinisation is an important determinant for the rate of starch hydrolysis *in vitro* and for the metabolic response *in vivo* (Holm, Lundquist, Björck, Eliasson, & Asp, 1988). Many food processing operations involve alteration of starch structure through thermal treatment, which leads to the starch becoming partially or completely gelatinised, depending on the final product (Delcour et al., 2010). The effects of thermal treatment on the morphological and crystalline structure of starch granules include important changes in physico-chemical properties (Ahmadi-Abhari, Woortman, Oudhuis, Hamer, & Loos, 2013). These changes in starch structure take place in pasting and gelatinisation processes, with swelling and gradual loss of crystallinity until there is total disruption of the starch granule (Horstmann, Lynch, & Arendt, 2017). The nature of these structural changes depends on the starch source, composition, structure and

isolation process, and therefore every starch has a different digestibility (Haros, Blaszczak, Perez, Sadowska, & Rosell, 2006; Ratnayake, & Jackson, 2007; Waigh, Gidley, Komanshek, & Donald, 2000). However, techno-functional parameters can provide information about the crystalline structure of starch and its digestibility (Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005).

The digestibility of starch is an important parameter that affects the severity and clinical manifestations of GSD and other diseases. GSD is a metabolic disorder that affects glycogen metabolism, in which the main clinical manifestation is fasting hypoglycaemia (Weinstein et al., 2018). Since 1984, ingestion of uncooked maize starch (raw) has been used to prevent a fall in glucose concentration overnight in individuals with type I or III GSD (Chen, Cornblath, & Sidbury, 1984). However, the relatively short duration of glucose availability from this dietary source still represents a major disadvantage with regard to the long-term outcome and quality of life of this special group. Also, raw maize starch intake is associated with injurious gastrointestinal symptoms such as abdominal cramps or bloating, which could be partly responsible for colonic fermentation of unused starch (Lee, & Leonard, 1995). Some other starches (i.e., potato, rice, tapioca and arrowroot) have been tested in GSD patients, but these starches displayed significant differences, producing a worse glycaemic response than maize starch (Sidbury, Chen, & Roe, 1986). In recent years, controlled heat-moisture processing of a high-amylopectin-containing maize starch was shown to be effective in

improving maintenance of glucose concentrations, while gastrointestinal symptoms were reduced (Correia et al., 2008). However, not everyone can afford modified starch and many people depend on alternatives that are cheaper and that are easily available. In this connection, the inclusion of "ancient grains" (such as amaranth, quinoa or chia) in cereal bread formulations has been shown in the *in vitro* test to have an effect in delaying glucose release while extending its absorption (Brennan, Menard, Roudaut, & Brennan, 2012; Laparra, & Haros, 2018). Furthermore, these effects were accompanied by increased expression of the peroxisome proliferator-activated receptor (PPAR)-gamma, suggesting an improved insulin resistance that could lead to a significant decrease in glycolysis metabolism in an animal model (Laparra, & Haros, 2018). Thus, starch from ancient grains could have a different digestibility that could help to maintain normoglycaemia longer than standard maize starch.

In view of the above, this study aimed to analyse thermal and pasting properties of starches from various sources – maize, wheat, potato, rice and quinoa – and evaluated the effect of a controlled heat-moisture process – which took their physical parameters into account – on their *in vitro* digestibility. The results were compared with those of the raw (as negative control) and gelatinised (as positive control) starches with the purpose of developing foods/beverages with specific characteristics for people with glucose metabolism disorders.

MATERIALS AND METHODS

Materials and reagents

Commercial maize starch was provided by ACH Food Companies (Argo, USA). Potato starch (C*Gel 300) was purchased from Cargill (Minneapolis, USA). Wheat starch (Natilor) from Chamtor (Pomacle, France). Rice starch (S7260) from Sigma-Aldrich, Belgium. Red quinoa starch was obtained from real Bolivian quinoa (Organic red Quinoa Real©, ANAPQUI (La Paz, Bolivia)) in the laboratory by wet-milling (Ballester-Sánchez, Gil, Fernández-Espinar, & Haros, 2019). The amylose content of starches was determined using enzymatic assay kits and procedures outlined by Megazyme (Megazyme International Ireland Ltd., Wicklow, Ireland). Enzymes were purchased from Sigma-Aldrich: α-amylase (EC 3.2.1.1, A3176-1MU, USA, 16 U/mg), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, 10115, Switzerland, 60.1 U/mg) and pepsin (EC 3.4.23.1, P7000, UK, 480 U/mg).

Pasting properties

To prepare the samples, 3.5 g of starches were weighted and 25 mL of distilled water was added. Pasting properties of the starches were measured using a Rapid Visco Analyser (RVA-4, Newport Scientific, Warriewood, Australia), according to AACC method 76-21.01 (1999). Pasting temperature (P_{temp}), peak time (P_{time}), peak viscosity (PV), hot paste viscosity (HPV), cool paste viscosity (CPV), breakdown (PV-HPV)

and setback (CPV-HPV) were recorded. The experiments were performed in triplicate.

Thermal properties

Gelatinisation and retrogradation properties were determined using differential scanning calorimetry (DSC) (PerkinElmer DSC-7, USA). Indium was used to calibrate the calorimeter (enthalpy of fusion 28.45 J/ g, melting point 156.6 °C). The procedure followed was the method described by Haros et al. (2006), with slight modifications. Ten mg of starch was weighed out and distilled water was added to obtain a water: starch ratio of 3:1 for each sample. The calorimeter scan conditions used were: 25 °C for 1 min and then heating from 25 °C to 120 °C at 10 °C/min. Later, to analyse retrograded starch, the samples were stored in refrigeration for a week and were ran under the same conditions (1 min - 25 °C; from 25 to 120 °C at 10 °C/min). The parameters recorded were: onset temperature (T_o), peak temperature (T_p) conclusion temperature (T_c) and enthalpy of gelatinisation and retrogradation transition (ΔH_G and ΔH_R), respectively. The experiments were performed in triplicate.

Preparation of samples for digestion

Aliquots (100 mg) of the various starch samples were weighed into microcentrifuge tubes and 1 mL of water was added. Raw starches were kept in unheated water for 5 min and were considered the negative control. Pretreatment of the starches was chosen according to their pasting and thermal parameters: maize (70 °C–2 min), quinoa (60 °C–1

min), wheat (60 °C–1 min), potato (70 °C–1 min) and rice (75 °C–2 min). The temperature selected for pretreatment depended on the T_p and T_c of the starch and was such as to achieve partial gelatinisation while avoiding loss of total crystallisation. The P_{temp} and P_{time} values determined previously were taken into account to avoid the formation of paste. Gelatinised starches (GS) were kept in a water bath for 5 min at 100 °C as a positive control.

In vitro starch digestion and GI estimation

The rate of starch hydrolysis was evaluated according to the method described by Goñi, Garcia-Alonso, and Saura-Calixto (1997), with modifications. Briefly, 10 mL of HCl–KCl buffer (pH 1.5) and 400 μL of a solution of pepsin in HCl–KCl buffer (0.1 g/mL) were added to the starches and the samples were placed in a shaking water bath at 37 °C for 1 h. Afterwards, 19.6 mL of Tris-Maleate buffer (pH 6.9) and 1 mL of a solution containing α-amylase in Tris-Maleate buffer (0.01 g/mL) were added and the samples were incubated in the water bath for 2 h. Aliquots were taken at intervals Aliquots were taken at intervals, from 0 to 120 min (0, 20, 40, 60, 90,120 min), and then the enzyme was thermally inactivated during 5 min at 100 °C. After centrifugation (10,000 rpm/10 min), 500 μL of the supernatant was taken from each sample. Then 1.5 mL of sodium acetate buffer (pH 4.75) and 60 μL of a solution of amyloglucosidase in sodium acetate buffer (88 mg/mL) were added and the samples were incubated at 60 °C for 45 min. Glucose, area under the curve (AUC) and hydrolysis index (HI) were determined according to

Laparra, & Haros, 2018. Finally, GI was calculated using the equation GI = 39.71 + 0.549HI (Zabidi, & Aziz, 2009). The hydrolysis kinetics was transformed from a cumulative curve into a linear curve by plotting the reciprocal values of [% starch hydrolysis] and time (Sanz-Penella, Laparra, & Haros, 2014).

Statistical analysis

Multiple ANOVA and Fisher's least significant differences (LSD) were applied to establish statistically significant differences in thermal and pasting properties. The Tukey test was applied to analyse differences in the digestion values. The statistical analyses were performed with Statgraphics Centurion XVI software, and the significance level was established at $P < 0.05$.

RESULTS AND DISCUSSION

Pasting and thermal properties of starches

The determination of pasting parameters revealed differences between the starches, as was expected (Table 1). P_{temp} provides an indication of the minimum temperature required to cook the starch, which could be related to the degree of polymerisation (DP) of amylopectin (Li, & Zhu, 2017; Srichuwong et al., 2017; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005). This parameter decreased following this order: rice > maize > potato ~ wheat > quinoa. Quinoa and wheat are characterised by

a higher proportion of short chains with a DP of 8–12, whereas maize, rice and potato have a high DP of 12–18 (Srichuwong et al., 2005, 2017). The higher proportion of shorter amylopectin chains could affect the crystalline structure (Srichuwong et al., 2017), resulting in a soluble molecule that can be easily digested as it has many end points onto which digestive enzymes can attach, which could have a positive effect on the digestibility of raw starches.

The peak viscosity (PV) parameter indicates the water-binding capacity of starch (Haros et al., 2006). The high PV of potato could possibly be explained, at least partly, by the high content of phosphate ester groups in the amylopectin in this tuber, resulting in repulsion between molecules (Waterschoot, Gomand, Fierens, & Delcour, 2015). Among the cereals, higher PV values were recorded for wheat and quinoa than for maize and rice. These results agree with the conclusions arrived at by Gomand, Lamberts, Visser, & Delcour (2010), who attributed an increase in swelling to short amylopectin chains, whereas long chains prevented this transition. The high viscosity value obtained during the heating process suggests a high water absorption capacity, which has been correlated with a lower resistance to enzymatic digestion (Reddy, Pramila, & Haripriya, 2015). This behaviour could be interesting when formulating foods with specific glycaemic indexes. The breakdown parameter (PV–HPV) can give information about stability under heating conditions. Potato starch showed a very high value, displaying a structural fragility that could lead to easier destruction of the structure

when it is cooked (Haros et al., 2006). Notably, the rice and quinoa starches exhibited a lower breakdown value than maize starch, which suggests a better preserved structure, favouring a lower peak glucose concentration and a slower rate of fall than with conventional maize starch.

During cooling, an important parameter to consider is retrogradation, which is the tendency to restructuration and can be measured through the setback parameter (CPV–HPV). Wheat and potato showed the highest setback viscosities, indicating a low resistance to retrogradation and, as a result, a higher rearrangement. The formation of double helices in this rapid process of restructuration is mainly attributed to amylose, which possesses a larger flexible structure than amylopectin (Van Soest, de Wit, Tournois, & Vliegenthart, 1994). However, the lack of differences in the setback values of the maize and quinoa starches, despite the amylose content determined for maize (amylose 22%) and quinoa (amylose 7%) (data not shown), suggests that other starch characteristics are involved in the retrogradation process.

Table 1: Pasting properties of starch from various sources.

Parameter	Units	Starch				
		Maize	Quinoa	Wheat	Potato	Rice
P_{temp}	°C	75.5 ± 0.5^{c}	66.5 ± 2.1^{a}	69.3 ± 0.0^{b}	69.4 ± 0.0^{b}	82.0 ± 0.6^{d}
P_{time}	min	5.1 ± 0.0^{b}	7.0 ± 0.0^{e}	6.1 ± 0.0^{c}	3.2 ± 0.1^{a}	6.6 ± 0.1^{d}
PV	cP	2906 ± 23^{b}	3380 ± 384^{c}	4607 ± 12^{d}	9751 ± 40^{e}	2385 ± 30^{a}
HPV	cP	1852 ± 23^{ab}	2653 ± 447^{d}	3321 ± 105^{e}	1552 ± 71^{a}	2111 ± 30^{c}
CPV	cP	3089 ± 18^{ab}	3867 ± 474^{c}	5371 ± 87^{d}	3475 ± 49^{bc}	2830 ± 30^{a}
Breakdown	cP	1054 ± 45^{c}	728 ± 63^{b}	1287 ± 93^{d}	8200 ± 111^{e}	274 ± 1^{a}
Setback	cP	1237 ± 41^{b}	1215 ± 28^{b}	2051 ± 18^{c}	1924 ± 121^{c}	720 ± 1^{a}

P_{temp}: Pasting temperature, P_{time}: Peak time, PV: Peak viscosity, HPV: Hot paste viscosity, CPV: Cool paste viscosity, Breakdown: PV-HPV, Setback: CPV-HPV, cP: centipoises.

Mean ± standard deviation, n=3. Values in the same row followed by the same letter are not significantly different ($P < 0.05$).

The gelatinisation parameters were determined by DSC analysis (Table 2). Onset temperature (T_o) showed the same trend as P_{temp}: rice > maize > potato > wheat > quinoa, as was expected. This relationship between gelatinisation and pasting temperatures was also confirmed previously by other researchers (Li, Wang, & Zhu, 2016). Low values in starch gelatinisation and pasting processes might suggest a less crystalline structure, which could result in higher enzymatic susceptibility (Lin et al., 2017; Srichuwong et al., 2017). The gelatinisation enthalpy (ΔH_G) varied from 10 to 12 J/g, except in the case of potato, which had a value of 16 J/g, demonstrating that higher energy was required to disrupt the crystalline structure. The resistance produced by potato may be interpreted as high crystallinity, which could interfere with the accessibility of the enzyme (Shi, Gao, & Liu, 2018). Retrogradation parameters were measured after 7 days at 4 °C, and quinoa starch presented the highest resistance to retrogradation of amylopectin (Table 2). In long-term retrogradation, amylopectin is mainly responsible for reorganisation of structure (van Soest et al., 1994). The presence of short chains in quinoa might contribute to a less compacted starch structure, leading to a starch with low retrogradation, which could be displayed as better digestibility (Lin et al., 2017; Srichuwong et al., 2017). On the other hand, the consumption of retrograded starches may be beneficial for health, owing to the lower depletion of total digestible starch than gelatinised starch (Chung, Lim, & Lim, 2006). Moreover, rearrangement of the crystalline structure could hinder α-amylase action and trigger a slow rate of intestinal digestion which could be reflected in a lower glucose concentration peak *in vivo*.

Table 2: Thermal properties of starch from various sources.

Parameter	Units	Starch				
		Maize	Quinoa	Wheat	Potato	Rice
Gelatinisation						
Onset temperature, T_o	°C	64.9 ± 0.0^{d}	50.7 ± 2.3^{a}	55.1 ± 0.2^{b}	60.8 ± 0.2^{c}	69.7 ± 0.7^{e}
Peak temperature, T_p	°C	69.6 ± 0.1^{c}	58.7 ± 1.6^{a}	60.1 ± 0.1^{a}	65.3 ± 1.1^{b}	76.4 ± 0.5^{d}
Conclusion temperature, T_c	°C	76.0 ± 0.1^{d}	69.3 ± 1.4^{b}	66.5 ± 1.1^{a}	73.4 ± 0.5^{c}	83.1 ± 0.8^{e}
Enthalpy of gelatinisation, ΔH_G	J/g	12.5 ± 0.1^{b}	10.4 ± 0.6^{a}	10.2 ± 0.6^{a}	16.6 ± 1.3^{c}	12.5 ± 0.2^{b}
Retrogradation						
Onset temperature, T_o	°C	44.3 ± 0.1^{c}	36.9 ± 1.0^{a}	43.7 ± 1.0^{c}	44.6 ± 1.2^{c}	41.2 ± 0.1^{b}
Peak temperature, T_p	°C	54.1 ± 1.5^{b}	46.5 ± 0.2^{a}	52.4 ± 1.5^{b}	60.8 ± 0.1^{c}	52.2 ± 2.1^{b}
Conclusion temperature, T_c	°C	63.2 ± 0.8^{bc}	56.3 ± 0.5^{a}	61.3 ± 0.8^{b}	71.5 ± 0.3^{d}	64.4 ± 2.0^{c}
Enthalpy of retrogradation, ΔH_R	J/g	3.5 ± 0.7^{ab}	1.6 ± 0.3^{a}	2.1 ± 0.5^{a}	5.8 ± 0.0^{b}	6.0 ± 2.2^{b}

Mean ± standard deviation, n=3. Values in the same row followed by the same letter are not significantly different ($P < 0.05$).

In vitro starch digestion and GI estimation

In this study, the hydrolysis percentages of various common starches were compared with that of raw maize starch (Table 3). The digestion method used has been proved suitable for establishing variations in susceptibility to enzyme interaction depending on structural differences between the samples considered (Rosin, Lajolo, & Menezes, 2002; Sanz-Penella et al., 2014).

Significant differences ($p < 0.05$) were found in the total (%) starch hydrolysed (TSH_{120}) as a function of the sample considered. Native potato presented the lowest value compared to any other raw starch studied. This is supported by previous studies, which indicate that the lack of peripheral channels in potato starch granules inhibits the penetration of α-amylase, whereas the presence of superficial pores, such as in maize starch, could enable enzymatic action (Dhital, Shrestha, & Gidley, 2010; Lehmann, & Robin, 2007). Moreover, the smaller specific surface area of large granules in potato starch compared to the others cereals may difficult the access and attachment of enzyme (Lehmann, & Robin, 2007; Srichuwong et al., 2005). As indicated at Fig. 1, rice and maize starches had similar hydrolysis, wheat achieved greater hydrolysis and quinoa presented the highest hydrolysis value, reaching around 70% hydrolysis, which is curious, considering that it was uncooked starch. These results are in good agreement with Srichuwong et al. (2017), who investigated starches obtained by various isolation processes and reported a similar trend in hydrolysis relating to short amylopectin chains, but

without giving other digestion parameters. It is highlighted that the amylose content in cereals is generally about 15–30% (Waterschoot et al., 2015) which is corroborated by our cereals starches (maize, 22%; rice, 21%; wheat, 25%). Nevertheless, quinoa presented only about 7% of amylose which could influence in the high digestibility displayed. The lower presence of amylose reported could favour the digestibility due to a higher amylose content has been associated with reduced susceptibility to enzymatic hydrolysis (Chung, Liu, Lee, & Wei, 2011).

In order to determine whether the various sources of starch released the same amount of glucose during digestion, the area under the curve (AUC) and hydrolysis index (HI) were calculated (Table 3). The analyses revealed that the raw starches obtained from quinoa and wheat had significantly higher AUC values than the raw starch obtained from maize. It is important to remember here that major differences would be determined by the structural fragility and short amylopectin proportion, as indicated above. When the various raw starches were tested after thermal processing at 100 °C (GS), there were no significant differences in GI values except for potato GS, which continued to have the lowest GI, owing to the lack of digestibility, as was also observed previously (Shi et al., 2018).

After analysing the raw starches (as negative controls) and the gelatinised starches (as positive controls), the effect of the hydrothermal treatment was investigated, taking into account the effect of the pasting and thermal properties on the enzymatic hydrolysis of starch, in order to

develop food with specific characteristics. The degree of gelatinisation has been reported as one of the main rate-limiting factors in the binding of enzymes to starch for digestion of starches (Wang et al., 2019). The treatment was applied to attain partial gelatinisation of starch in order to evaluate to what extent alterations in starch structure caused by heat-moisture processing affect its digestibility. The pretreatment temperature was selected on the basis of the parameters shown in Tables 1 and 2. The thermal pretreatment of maize and rice starches led to a higher hydrolysis rate than in the case of their raw counterparts, as was observed by Chung et al. (2006) in waxy rice starch subjected to various thermal treatments. Pretreatment of the maize and rice starches, consisting of the application of 70 °C (maize) and 75 °C (rice) for 2 min, led to an HI that was approximately 75% of the HI of the corresponding gelatinised starches. However, the pretreated maize did not hydrolyse totally and did not exceed the hydrolysis values of the raw quinoa. A similar tendency was observed by Ahmadi-Abhari et al. (2013), who reported that wheat starch began to lose crystallinity, and consequently starch digestibility improved, but total hydrolysis was not achieved. In the current investigation, pretreated wheat starch began to lose crystallinity and thus improved its digestibility and reached HI values similar to those obtained for pretreated quinoa starch. The higher hydrolysis observed in the pretreatment of wheat and quinoa in comparison with maize and rice could be due to their low T_p and high PV values, which suggest greater susceptibility to disintegration of their structure (Li et al., 2016).

Table 3: Effect of treatment on *in vitro* digestibility of starch.

Sample	Treatment (°C-min)	TSH_{120} (%)	AUC	HI (%)	GI	Slope (SH/min)
Maize	Raw	34.0 ± 3.4^{bc}	3340 ± 362^{ab}	43.1 ± 4.7^{ab}	63.4 ± 2.6^{ab}	0.4 ± 0.1^{a}
	70 °C-2 min	65.4 ± 1.4^{ef}	6403 ± 54^{d}	82.6 ± 0.7^{d}	85.1 ± 0.4^{d}	3.9 ± 1.2^{abc}
	GS	81.9 ± 5.2^{gh}	8315 ± 642^{e}	107.3 ± 8.3^{e}	98.6 ± 4.6^{e}	8.9 ± 1.0^{de}
Quinoa	Raw	73.8 ± 2.6^{fg}	6352 ± 568^{d}	82.0 ± 7.3^{d}	84.7 ± 4.0^{d}	2.2 ± 0.5^{abc}
	60 °C-1 min	83.7 ± 2.4^{gh}	8766 ± 432^{e}	113.1 ± 5.6^{e}	101.8 ± 3.1^{e}	14.4 ± 3.5^{f}
	GS	91.9 ± 2.7^{h}	8014 ± 200^{e}	103.4 ± 2.6^{e}	96.5 ± 1.4^{e}	3.9 ± 0.4^{abc}
Wheat	Raw	51.6 ± 6.8^{d}	4886 ± 510^{c}	63.0 ± 6.6^{c}	74.3 ± 3.6^{c}	1.1 ± 0.3^{ab}
	60 °C-1 min	82.2 ± 3.7^{gh}	8139 ± 445^{e}	105.0 ± 5.7^{e}	97.4 ± 3.2^{e}	4.5 ± 1.0^{bc}
	GS	81.6 ± 0.2^{gh}	7866 ± 334^{e}	101.5 ± 4.3^{e}	95.4 ± 2.4^{e}	5.1 ± 1.6^{cd}
Potato	Raw	20.7 ± 1.8^{a}	2069 ± 246^{a}	26.7 ± 3.2^{a}	54.4 ± 1.7^{a}	0.4 ± 0.3^{a}
	70°C-1 min	21.8 ± 4.3^{ab}	2464 ± 538^{ab}	31.8 ± 7.5^{ab}	57.2 ± 4.1^{ab}	2.2 ± 0.8^{abc}
	GS	56.1 ± 7.1^{de}	4841 ± 490^{c}	62.5 ± 6.3^{c}	74.0 ± 3.5^{c}	2.4 ± 0.1^{abc}
Rice	Raw	36.6 ± 1.2^{c}	3794 ± 253^{bc}	49.0 ± 3.3^{bc}	66.6 ± 1.8^{bc}	0.6 ± 0.2^{a}
	75 °C-2 min	59.9 ± 6.0^{de}	6213 ± 259^{d}	80.2 ± 3.3^{d}	83.7 ± 1.8^{d}	4.4 ± 1.0^{bc}
	GS	82.7 ± 6.1^{gh}	8411 ± 570^{e}	108.5 ± 7.4^{e}	99.3 ± 4.0^{e}	9.1 ± 1.9^{e}

The data are reported on dry basis. Mean ± standard deviation, n=3. Values in the same column followed by the same letter are not significantly different ($P < 0.05$). TSH_{120}: Total starch hydrolysed at 120 min. AUC: Area under the curve of starch digestion from 0 to 120 min. HI: Hydrolysis index. GI: Glycaemic index. SH: Starch hydrolysed. GS: Gelatinised starch. The slope was calculated using the Lineweaver-Burk's transformation from the cumulative curves.

Data from the hydrolysis parameters were transformed according to Lineweaver-Burk's model in order to obtain approximate values of the kinetic parameters of starch digestion, helping to gain insight into the potential physiological effects (Sanz-Penella et al., 2014). Although the raw quinoa and wheat starches had higher slopes (Table 3), they were accompanied by high hydrolysis, which means that a lower dose would be required. This would help to reduce the digestive inconveniences resulting from the consumption of high amounts of raw maize starch. Furthermore, although the slope values calculated for both raw and gelatinised quinoa starch were similar, the values for gelatinised maize were significantly higher than those of the raw counterpart.

Collectively, these structural changes in quinoa starch may help to maintain glucose concentrations for a longer time and lead to a less rapid rate of fall than in the case of maize starch. Notably, although there are many studies on differences in the techno-functional characteristics of starches and their digestibility, it is not clear how these differences would relate to the rate or efficiency of hydrolysis by pancreatic amylase.

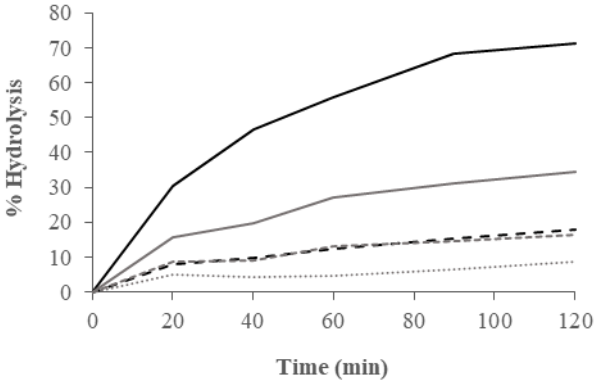

Figure 1. Hydrolysis of raw starches. Symbols: **- - -**, maize starch; ——, quinoa starch; ——, wheat starch; - - -, rice starch; ·····, potato starch.

CONCLUSIONS

To sum up, from this study it can be concluded that it may be possible to modify digestibility by controlling starch properties through variations in temperature or cooking time, which could be useful when designing GI-specific formulations for impaired glucose metabolism. Maize and rice starches showed similar technological characteristics, which were concordant with the lack of differences in digestion. Potato starch showed high resistance to digestibility, whereas quinoa and wheat were more susceptible to enzymatic attack. Furthermore, pasting and thermal parameters for quinoa starch indicated structural changes at granule and molecular level that were reflected in its digestibility. Raw maize starch has been used for years by patients with glycogen storage disease despite the short duration of its effect and the gastrointestinal problems

associated with it. Raw quinoa starch could offer a promising potential for extending normoglycaemia in these patients. The results indicate the starches and their pretreatment, taking into account their physico-chemical characteristics, could be a potential useful dietary source for patients who have an altered glucose metabolism. Knowing these parameters and how enzymatic susceptibility is affected is essential to a better understanding of the changes in starch structure which could be applied to develop specific formulations. This proposal gives information in order to develop simple formulations with cereals/pseudcereals/tubers flours to control the starch digestibility. Taking into account their behaviour according the source, composition, grade of crystallinity and/or structure in the food matrices to control the glucose homeostasis. However, this is a preliminary study which could open the door to future investigations designed to attain a better understanding of the physiological effects *in vivo*.

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Capítulo 2: Kinetic approach to the influence of chia flour on glucose bioaccessibility from hydrothermally treated maize and quinoa starch

Kinetic approach to the influence of chia flour on glucose bioaccessibility from hydrothermally treated maize and quinoa starch

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ABSTRACT

Starch structure and bioactive ingredients play an implicit role in the control of glucose release at intestinal level reducing the risk of inadequate metabolic response(s). This study performs a comparative kinetic approach to glucose release from hydrothermally treated (HT) maize (MS) and quinoa (QS) starch. Besides, chia flour (CF) (20%, w/w) was added to evaluate its influence of on the apparent diffusion coefficients (Dapp) when subjected to simulated gastrointestinal digestion. Hepatocyte cultures were used to monitor mitochondrial enzymes activity (test MTT) to bioaccessible glucose concentrations. With an increasing temperature, Dapp for both QS and its mixtures with CF were kept unaltered, while those for MS were disrupted progressively

affecting glucose bioaccessibility. Principal component analysis revealed differences between maize and quinoa starches, but common features in the corresponding mixtures with CF. Data indicated that quinoa starch helps controlling glucose release and that addition of CF decreased mitochondrial activity in presence of insulin.

Keywords: Starch · Glucose · Bioaccessibility · Quinoa · Maize

INTRODUCTION

Glucose homeostasis (GH) is one of the hallmarks of liver related diseases playing a critical role in the pathogenesis of comorbidities associated to metabolic disorders such as type 2 diabetes (T2D) and obesity. The latter constitute, moreover, due to an excessive glycaemia important risk factors, together with sedentary lifestyle and altered food supply and preferences determining the progression of liver dysfunction (Mohan, Farooq, Deepa, Ravikumar, & Pitchumoni, 2009). Recently, alterations in pancreatic β-cells metabolism in response to hyperglycaemia has led to open a debate concerning T2D as a glycogen storage disease (GSD) of pancreatic β-cells (Ashcroft, Rohm, Clark, & Brereton, 2017). Thus, both hyperglycaemia as well as hypoglycaemia in childhood are associated with increased risk of physiopathological consequences (Gjorgjieva, Mithieux, & Rajas, 2019) at a younger age and later in life.

Several attempts have been made to develop digestion models to evaluate nutrient release from food matrices, including the determination of their bioaccessibility (BA, %) (Minekus et al., 2014; Selma-Gracia, Laparra, & Haros, 2020). Despite differences in the variety of enzymes and other parameters, according to evidence-based gastrointestinal physiology, there are scarce studies evaluating the fluxes of bioaccessible nutrients (Laparra, Tako, Glahn, & Miller, 2008) affecting physiological response(s).

Simulated gastrointestinal digestion models, based on solubility parameters, allow to evaluate food availability to the enzymatic action and hydrolysis approaching glucose release (Selma-Gracia et al., 2020). However, these models did not reveal possible interactions between the nutrients and food ingredients, potentially affecting their influence in response(s) to signals for endocrine control.

Recent studies have demonstrated the benefits of inclusion of chia (*Salvia hispanica* L) flour delaying glucose release and uptake, increasing the expression of biomarkers influencing nutrient fate and energy metabolism (Laparra, & Haros, 2018). Chia's high fiber content (*i.e.*, soluble mucilage) is able to influence glucose availability (Sasaki, & Kohyama, 2012). In addition, chia flour is a good source of immunonutritional agonists; protease inhibitors (STPIs) able to interact with the innate immune 'Toll-like receptor' (TLR)-4 (Hellinger, & Gruber, 2019; Srdić, Ovčina, Fotschki, Haros, & Llopis, 2020). Immunonutritional agonists have been examined as therapeutic agents, in the control of appetite, energy intake, and carbohydrate metabolism (Carai et al., 2009), helping to slow down the digestion of carbohydrates, generally considered to be beneficial for the dietary management of T2D, obesity and GSD (Gjorgjieva et al., 2019; Fabricatore et al., 2011).

The objective of this study is to evaluate kinetic features for glucose release from hydrothermally treated maize and quinoa starch, and the influence of bioactive ingredients provided by CF.

MATERIALS AND METHODS

Materials and reagents

Commercial maize starch was purchased from ACH Food Companies (Argo, USA), red quinoa starch was obtained in the laboratory by wet-milling (Ballester-Sánchez, Gil, Haros, & Fernández-Espinar, 2019) and black CF was provided by Inca's treasure (Ecuador). Reagents were purchased from Sigma-Aldrich: α-amylase (EC 3.2.1.1, A3176-1MU, USA, 16 U/mg), pepsin (EC 3.4.23.1, P7000, UK, 480 U/mg), pancreatin from porcine (EC 232–468-9, P1750, USA, 4XUSP), bile extract porcine (EC 232–369-0, B8631, USA) amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, 10,115, Switzerland, 60.1 U/mg) and insulin from bovine pancreas (EC 234–291-2, I6634, USA, ≥25 USP units per mg).

Samples

Aliquots (100 mg) of maize and quinoa starch were weighted into microcentrifuge tubes. Maize (MS_CF) and quinoa starch (QS_CF) were prepared with 100 mg of starch and mixed with 20

mg defatted CF. Black CF was defatted with *n*-hexane (6 h) under reflux conditions in a Soxhlet equipment (Soxtec™ 2050, Sweden) extracting all the lipid content from black CF (33% g/100 g dry matter). The proportion of black CF was chosen as the maximum percentage allowing adequate solubility conditions. Three different set of samples were prepared with and without CF: raw starches were kept in unheated water for 5 min; the others were subjected to different hydrothermal treatments (HT) to obtain a partial (pretreated) (maize, 70 °C/2 min; quinoa, 60 °C/1 min) and total gelatinisation (maize, 100 °C/5 min; quinoa, 100 °C/ 5 min) (Selma-Gracia et al., 2020). Each set of samples was prepared in triplicate (n = 3) for simulated gastrointestinal digestion.

Simulated gastrointestinal digestion

Three independent set of samples for MS, QS and those mixed with CF were subjected to a simulated gastrointestinal digestion procedure as described elsewhere (Laparra et al., 2008) with slight modifications. An oral phase was included previously to gastric digestion where all samples were incubated in a solution of α-amylase (75 U/mL) for 30 s. The digestion started with a peptic (gastric digestion) step (1 h/37 °C). Then, the intestinal phase of digestion was initiated with a pancreatin-bile extract solution. 2 mL of the gastrointestinal digest was carried out in the upper (donor compartment) chamber and 2 mL of an isotonic saline solution in the bottom (acceptor compartment). The bicameral

system consisted in a molecular weight cut-off dialysis membrane (14 kDa) attached to a plastic insert ring to separate the "gastrointestinal digest" from the acceptor compartment allowing to determine glucose dializability from the bottom chamber considered the bioaccessible fraction for absorption.

Aliquots (250 μL) from the bottom compartment were collected at different intervals (15, 30, 45, 60 min), and isotonic solution was used to replace the volumes removed during sampling. Apparent diffusion coefficients (Dapp) were calculated from the linear slope of the glucose concentration in the bottom chamber according to Laparra et al., (2008). Glucose was quantified by enzymatic kit (D-Glucose Assay Procedure, K- GLUC 07/11, Megazyme).

Cell cultures

The human hepatoblastoma (HB-8065©) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 1 and used in experiments between passages 9–11. Cell cultures were grown in Eagle's Minimum Essential Medium (EMEM Glutamax, Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were maintained at 37 °C in 5% CO_2, 95% air and the culture medium were changed every two days.

Mitochondrial Enzymes Activity (MTT Assay)

Metabolic responses were evaluated by monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) conversion (TOX1, Sigma-Aldrich) according to a commercial kit (Srdić et al., 2020). For experimental studies cells were seeded at a density of 3×10^5 cells/well onto 12 well plates (Costar, Cambridge, MA, USA) and EMEM was replaced by 1 mL of minimum essential media (MEM) without fetal bovine serum. Two different sets of cultures were prepared: i) untreated cell cultures, and ii) treated with insulin (10 ng/mL) (Sefried, Häring, Weigert, & Eckstein, 2018). In order to detect early metabolic response(s) intestinal digestion, the bicameral system fitted with the dialysis membrane was applied onto cells. Cell seeded plates were returned to the incubator for additional for 30 min.

Principal Component and Statistical Analyses

The principal component analysis (PCA) was performed using Statgraphics© Plus (version 5.1). Multiple ANOVA and Fisher's least significant differences (LSD) were applied to establish statistically significant differences. The statistical analyses were analysed with Statgraphics Centurion XVl software, and the significance level was established at $P < 0.05$.

RESULTS AND DISCUSSION

Starch Digestibility

Starch hydrolysis was estimated from soluble glucose concentrations at endpoint of the digestion period (Table 1). After simulated gastrointestinal digestion, raw MS displayed significantly lower digestibility than raw QS, which could be attributed to the lower amylose/amylopectin ratios in QS (Selma-Gracia et al., 2020; Srichuwong et al., 2017). Besides, the digestibility percentage for QS is estimated around 60%, which results higher than that calculated (39.8%) from other solubility methods (Laparra, & Haros, 2018). Digestibility values were increased according to the HTs applied only for MS, however, this increase was reflected in significant decreases in bioaccessible glucose (BA, %). Meanwhile, BA (%) remained constant in all hydrothermally treated QS samples suggesting the highest digestibility of raw QS. This result is supported by reduced pasting and onset temperature as well as peak viscosity values indicative of the great susceptibility to enzymatic activity of QS (Selma-Gracia et al., 2020). Despite not observing statistically significant ($P > 0.05$) differences in starch digestibility in MS and QS mixed with CF, all HTs displayed a downward trend in BA (%). In this vein, consumption of wheat bread-containing chia seeds promoted a lower postprandial glycaemia in comparison with bread without seeds (Ho et al., 2013).

Evaluation of Fluxes of Glucose

Total basal glucose concentrations for MS and QS and the effect of HT on those are shown in Fig. 1. To show clearly the fit of the data points to a 2-parameter exponential distribution, data were plotted against the theoretical curves.

HT of MS significantly increased total dialyzable glucose concentration. In fact, glucose for MS_100 was 6-fold higher than MS ($P > 0.05$); however, Dapp values resulted not statistically different. From data for QS, it was calculated a clear inverse relationship between total basal glucose concentrations and Dapp. These data suggest different interactions between starch structure and composition on glucose dialyzability probably due to differences in the length between amylose/amylopectin chains in quinoa starch and/or their accessibility to digestive enzymes. The inclusion of CF slightly decreased glucose concentrations in the acceptor compartment as supported by reduced Dapp values. The decreased Dapp after addition of CF is concordant with several *in vivo* studies, which have reported a better control on carbohydrate metabolism (Menga et al., 2017).

The contribution of chia seeds to dietary soluble fiber through its mucilage content has been suggested to contribute slowing down food glucose release (Reyes-Caudillo, Tecante, & Valdivia-López, 2008). However, the lack of significant differences in the amounts of glucose extracted with the aliquots at different time points between samples mixed or not with CF does not support a potential role for the mucilage reducing glucose dializability (Fig. 1 E-F). It could be expected that this effect must be revealed at a higher magnitude during earlier digestion times when the free glucose to mucilage ratio is low, and thereby it is unlikely that this interaction could manifest in a linear correlation (Fig. 1E-F). Notably, the advanced $t_{1/2}$ for both MS_CF (11.5%) and QS_CF (6.6%) in presence of CF could be due not only to the mucilage content. Menga et al., (2017) showed that chia (whole) seeds displayed 2-fold-higher slowly digestible starch fraction of rice flour in comparison to the isolated mucilage.

Table 1: Effect of inclusion of chia flour in glucose release. Values are expressed as mean ± standard deviation (n=3)

Sample	Treatment	Soluble glucose (mg)		Bioaccessibility (%)	Dapp (cm^2/seg) ($\cdot x10^{-2}$)
		before digestion	after digestion		
Raw	MS	0.89 ± 0.16^{a}	2.90 ± 0.21^{a}	7.6 ± 1.9^{d}	1.46 ± 0.29^{b}
	QS	2.94 ± 0.20^{c}	8.38 ± 0.18^{f}	4.5 ± 0.5^{ab}	2.84 ± 0.37^{g}
Pretreated	MS_70	1.95 ± 0.33^{b}	4.61 ± 0.20^{b}	6.1 ± 0.6^{c}	1.67 ± 0.17^{bcd}
	QS_60	5.74 ± 0.64^{de}	8.41 ± 0.23^{f}	4.4 ± 0.1^{ab}	2.27 ± 0.23^{f}
Gelatinised	MS_100	5.76 ± 0.80^{de}	7.72 ± 0.55^{de}	3.7 ± 0.2^{a}	1.59 ± 0.19^{bc}
	QS_100	5.62 ± 0.21^{d}	7.22 ± 0.23^{d}	4.0 ± 0.5^{ab}	1.53 ± 0.18^{bc}
Raw	MS_Ch	0.91 ± 0.15^{a}	3.43 ± 0.60^{a}	5.3 ± 2.0^{bc}	1.09 ± 0.20^{a}
	QS_Ch	3.07 ± 0.33^{c}	8.33 ± 0.18^{f}	3.7 ± 0.4^{a}	1.99 ± 0.19^{def}
Pretreated	MS_70_Ch	1.97 ± 0.45^{b}	5.41 ± 0.06^{c}	4.6 ± 0.3^{abc}	1.61 ± 0.08^{bc}
	QS_60_Ch	5.47 ± 0.86^{d}	8.37 ± 0.07^{f}	3.6 ± 0.0^{ab}	2.20 ± 0.10^{ef}
Gelatinised	MS_100_Ch	6.67 ± 1.20^{ef}	8.15 ± 0.71^{ef}	2.4 ± 1.6^{a}	1.81 ± 0.35^{cde}
	QS_100_Ch	7.28 ± 0.51^{f}	7.70 ± 0.15^{de}	3.7 ± 0.9^{a}	1.56 ± 0.05^{bc}

Superscript letters indicate statistical differences between samples within the same column ($P < 0.05$). MS: maize starch; MS_70: maize starch at 70 °C; MS_100: maize starch at 100 °C; MS_CF: maize starch with chia flour; MS_70_CF: maize starch with chia flour at 70 °C; MS_100_CF: maize starch with chia flour at 100 °C; QS: quinoa starch; QS_60: quinoa starch at 60 °C; QS_100: quinoa starch at 100 °C; QS_CF: quinoa starch with chia flour; QS_60_CF: quinoa starch with chia flour at 60 °C; QS_100_CF: quinoa starch with chia flour at 100 °C; Bioaccessibility = (total glucose in the acceptor compartment / total glucose after digestion at 60 min) x•100; Dapp: apparent diffusion coefficient

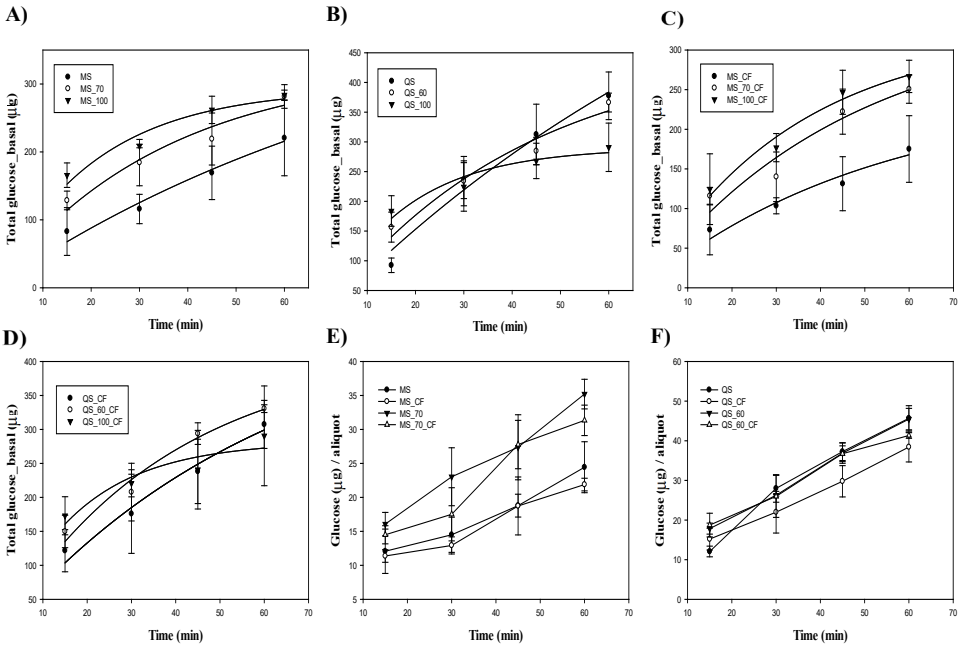

Fig. 1 (A-D) Kinetics of basal glucose accumulations in the acceptor compartment from maize (MS) and quinoa (QS) starch digestion and the influence of chia flour (CF). Raw starches: maize (MS), quinoa (QS), maize with chia flour (MS_CF) and quinoa with chia flour (QS_ CF). (E-F) Amount of glucose extracted with the aliquots in acceptor compartment (bioaccessible) during simulated gastrointestinal digestion Results are expressed as mean ± standard deviation (n = 3). Hydrothermal treatment: i) Maize (MS_70) and quinoa (QS_60) pretreated samples (maize 70 °C/2 min and quinoa 60 °C/1 min), ii) Maize (MS_100) and quinoa (QS_100) gelatinized samples (maize/quinoa 100 °C/5 min), mixed or not with chia. $t_{1/2}$, time that takes for reaching half the maximum total basal glucose concentration.

Principal Component Analysis (PCA)

To synthesize the information for identifying patterns in such a way to highlight similarities as well as differences between the analyzed samples data obtained from the kinetic analysis were subjected to PCA (Fig. 2A).

Different parameters to estimate glucose dializability from MS and QS starch (*i.e.*, amount of glucose extracted with the aliquot, cumulative glucose in basal media and Dapp) were statistically associated. Cumulative glucose concentrations were determined as responsible for the major weight of PC#1 (Eigenvalue, 3.93), meanwhile, the lower weight of PC#2 (Eigenvalue, 0.061) included the other groups of parameters calculated. The analysis clearly discriminated between the digestibility of MS and QS and those in presence of CF, which demonstrates the influence of the bioactive components found in CF on glucose BA changes. Moderate differences in PCA were detected in MS and QS in presence of CF, compared to their non-added counterparts. This is in accordance with previous studies, where immunonutritional agonists found in CF (Laparra, & Haros, 2019) have proved their metabolic modulatory affecting glucose utilization and mitochondrial function (Srdić et al., 2020).

As an approach to estimate the influence of STPIs from CF, we performed a Lineweaver-Bürke's transformation of the bioaccessible glucose concentrations (Fig. 2B-C). Notably, addition of CF differentially increased V_{max} values from MS (by 34%) and QS (by 62%) (Fig. 2D). The latter observations also transpire in the way of a direct effect on enzyme activity rather than the mucilage-mediated physico-chemical modulation of glucose dialyzability.

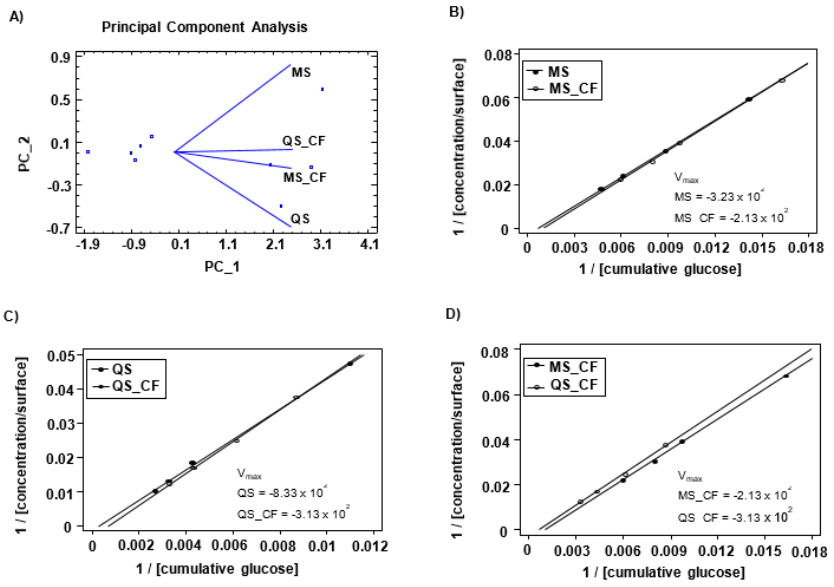

Fig. 2 (A) Principal component analysis of the kinetic parameters of glucose dializability from raw maize (MS) and quinoa (QS) starch mixed or not with chia flour (CF). (B-D) Lineweaver-Bürke's transformation from glucose concentration in the acceptor compartment. The average of three replications was represented and maximum velocity (V_{max}) (inverse of y-intercept value) was calculated from the different raw starch formulations.

Hepatic Metabolic Response to Bioaccessible Glucose

Glucose undergoes poor biotransformation at intestinal level, while hepatocytes perform regulatory response(s) on glucose uptake (Sefried et al., 2018; Dengler, & Gäbel, 2019), as well as liver function is affected by immunonutritional agonists (Laparra, Laparra, & Haros, 2019) (Fig. 3). Raw MS and QS samples were used for metabolic assays because of the marked differences caused by HT.

Significant differences were quantified in total bioaccessible glucose from MS and QS (Fig. 3A). However, mitochondrial metabolic responses did not mirror these differences in glucose concentration when insulin was added (Fig. 3B). Addition of CF provided 0.92 ± 0.02 μg bioaccessible immunonutritional agonists (Laparra, & Haros, 2019). When HepG2 cells were exposed to insulin (10 ng/mL) a significant inhibitory response was observed in those samples mixed with CF in comparison with MS and QS. Collectively, these data could suggest the participation of cellular molecular mechanisms influencing glucose utilization and insulin signaling. Interaction of immunonutritional agonists provided by chia with the innate immune 'Toll-like' receptor (TLR)-4 with an inhibitory effect on key enzymes in the glycolytic process can explain the observed effects (Srdić et al., 2020).

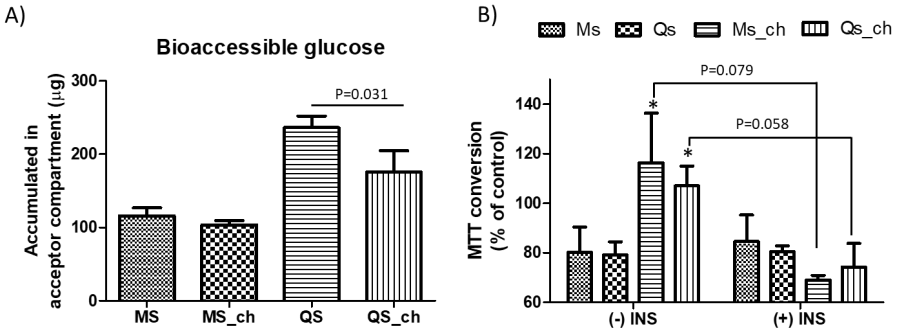

Fig. 3 (A) Total bioaccessible glucose from raw maize (MS) and quinoa (QS) starch mixed or not with chia flour (CF). (B) MTT conversion changes by HepG2 cells exposed ((+) INS) or not ((−) INS) to insulin (INS). Results are expressed as mean ± standard deviation (n = 2). *Indicates statistically significant ($P < 0.05$) differences in relation to treatment.

The increased mitochondrial activities of cell cultures in presence of CF, without insulin exposure, is concordant with its stimulatory capacity on the expression of peroxisome proliferator-activated receptor gamma (PPARγ) (Laparra, & Haros, 2018) inducing the 6-phosphofructo-2-kinase activity (Guo et al., 2013). However, insulin is a physiologic homeostatic regulator of hepatocytes to the innate immune TLR4, which dominance promotes a deficient insulin signaling (Jia et al., 2014). From a molecular perspective, immunonutritional agonists delay TLR4 retrieval to the plasmatic membrane, thus interfering its functional activity (Srdić et al., 2020). Here, STPIs from CF could be thought as responsible for the inhibitory effects reducing MTT conversion

values by activation of the IRS1/PI3K/AKT pathway (Dengler, & Gäbel, 2019). Overall, chia's influence on glucose metabolism highlights its potential promoting a more adequate preservation insulin-induced glycogen storage or gluconeogenesis in order to prevent, or at least to restrain, cell stress during disturbed glucose metabolism pathologies.

CONCLUSIONS

Determination of glucose Dapp allows defining both qualitative and quantitative differences between MS and QS starch. Notably, hydrothermal pretreatment of QS had negative effects on Dapp for glucose. Despite differences in glucose kinetics of MS and QS, not significantly different MTT values were quantified in presence or not of insulin. However, inclusion of CF displayed an opposite trend, indicating that, apart from mucilage, chia's compounds can interact in cellular response.

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Capítulo 3: Inclusion of *Salvia hispanica* L. and *Chenopodium quinoa* into bread formulations improves metabolic imbalances derived from a high-fat intake in hyperglycaemic mice

Inclusion of *Salvia hispanica* L. and *Chenopodium quinoa* into bread formulations improves metabolic imbalances derived from a high-fat intake in hyperglycaemic mice

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ABSTRACT

High-energy intake causes imbalances in nutrient homeostasis contributing to a high prevalence of metabolic chronic diseases. The extent to what metabolic imbalances can be ameliorated by the inclusion of immunonutritional ingredients obtained from flours favouring nutrient and calorie management remains poorly understood. Herein, it is demonstrated that partial replacement of wheat flour (WB) with that from *Chenopodium quinoa* varieties [red (RQ, 25% w/w) and white (WQ, 25% w/w)] as well as from *Salvia hispanica* L., [whole (Ch, 20% w/w) and semi-defatted (Ch_D, 20% w/w)] in bread formulations ameliorates the metabolic and inflammation consequences of high-fat diet consumption in hyperglycaemic animals. Feeding animals with

bread formulations replacing wheat flour effectively reduced insulin resistance (by 2-fold, HOMAir). The reduction in starch content did not appear as a determinant of controlling HOMAir. Only animals fed with RQ and Ch diet displayed increased plasma levels of triglycerides, which significantly contributed to mitigate HFD-induced hepatic lipid peroxidation. The latter was increased in animals receiving Ch_D diet, where PUFAs were eliminated from chia's flour. Feeding with WQ and Ch samples caused an upward trend in hepatic TNF-α and IL-6 levels. Despite similarities between immunonutritional agonists in animals fed with RQ and WQ, IL-17 levels were quantified higher for animals fed with WQ. All bread formulations except Ch_D samples significantly increased the hepatic granulocyte–monocyte colony stimulation factor levels. These results indicated that replacement of wheat flour with that from quinoa and chia improved the metabolic imbalances in hyperglycaemic animals.

INTRODUCTION

Western lifestyle (*i.e.*, food choice and lack of physical activity) has led to an intense and continuous increase in the prevalence of metabolic diseases such as obesity, type 2 diabetes (T2D) (Bhupathiraju, & Hu, 2016) and other major features of metabolic syndrome. In early stages of metabolic disorders, there is an increased risk of developing T2D associated with alterations in insulin resistance (Sung, Jeong, Wild, & Byrne, 2012). Hyperglycaemia also disrupts physiological lipid handling and management causing dysfunctional alterations in hepatocytes. (Williams, Shackel, Gorrell, McLennan, & Twigg, 2013). Of note, recent data have suggested that non-alcoholic fatty liver disease (NAFLD) poses a high risk for the development of T2D, placing the liver in the centre of nutritional studies (Linder et al., 2014). In this context, a significant bulk of evidence indicates the close relationship between innate immune signalling and hepatic metabolism (Bai, & Li, 2019), worsening or improving the consequences of excessive nutrient availability.

Diet has acquired great importance due to its physiological involvement in the metabolic modulation, where nutritional strategies can help either prevent or ameliorate metabolic alterations (Kolb, & Martin, 2017). Prospective nutritional evaluations clearly established a direct relationship between consumption of white bread and the risk of becoming

overweight/obese (de la Fuente-Arrillaga et al., 2014). Besides, research efforts have evidenced the potential of replacing wheat flour with that obtained from Latin-American crops (*Chenopodium quinoa* and *Salvia hispanica*) in bread formulations reducing glycaemic index as well as the hepatic inflammatory milieu (Laparra, & Haros, 2016; Laparra, & Haros, 2018). These studies evidenced the positive effects of increasing the expression of hepatic immunometabolic transcription factors such as PPAR-γ, helping to control glycaemic index and nutrient fate in energy metabolism. Recent research has also evidenced the positive effects of immunonutritional protease inhibitors found in *C. quinoa* and *S. hispanica* to increase the hepatic $F4/80^{+}$ cell population (Laparra, Fotschki, & Haros, 2019a), which exerts a key regulatory function on hepatic cholesteryl ester transfer protein raising high-density lipoprotein (HDL)-cholesterol (van der Tuin et al., 2018). Additionally, *S. hispanica* is recognised as a rich source of polyunsaturated fatty acids that may play an important role in the management of insulin resistance (Lepretti, Martucciello, Burgos Aceves, Putti, & Lionetti, 2018). The promotion of a favourable immunometabolic environment may significantly contribute to the prevention and treatment of T2D. These are novel perspectives connecting the altered 'gut-liver' milieu to risk factors for immunometabolic imbalances influencing T2D severity and progression. However, current evidence for an

association of bread formulations replacing wheat flour with that from Latin-American crops with metabolic and inflammation perturbations caused by high-fat diet (HFD) in glucose homeostasis has largely been inferential.

Research efforts have been made to develop animal models that reproduce the transition from pre-diabetes to diabetes, which occurs mainly associated with the apparition of hyperglycaemia to emulate the long-term complications of T2D (Barrière et al., 2018; Fernández-Espinar, Gil, Segura-Campos, & Haros, 2016). The repeated administration of low doses of streptozotocin to mimic an early prediabetic (hyperinsulinemic) phase with modest dysglycaemia failed to reproduce the insulin resistance axis typically seen in T2D (Ballester-Sánchez, Millán-Linares, Fernández-Espinar, & Haros, 2019a). Nevertheless, overfeeding a high-fat diet to animals made them develop insulin resistance, where hyperglycaemia is associated with hypertriglyceridemia (Iglesias-Puig, & Haros, 2013). Lessons from preclinical long-term (56 weeks) models of T2D reveal three distinct phases: the earliest effects starting from 2 weeks characterized, among other, by hyperglycaemia and insulin resistance (phase 1) (Barrière et al., 2018). Longer periods of treatment cause a decline in insulin production (phase 2, 18–42 weeks) and decrease the fasting and post-IPGTT glucose levels (phase 3, 42–56 weeks). Taken together, these studies seem to suggest that translational value of

animal models can be further enhanced when attaining a better understanding of the role of environmental factors that influence the onset, severity and progression of the early stages of the disease.

The objective of this study was to evaluate the influence of wheat flour replacement with that from *C. quinoa* (*i.e.*, white and red) and *S. hispanica* seeds (*i.e.*, whole and semi-defatted) in bread formulations on metabolic consequences of HFD consumption by hyperglycaemic animals. Inclusion of flours from *C. quinoa*, white and red varieties, as well as *S. hispanica*, whole and defatted, was based on different scenarios; (i) to evaluate the effects derived from replacement of wheat immunonutritional agonists with those provided by *C. quinoa* and *S. hispanica*, and (ii) to evaluate the interaction of immunonutritional agonists with different starch levels in the presence of polyunsaturated fatty acids (PUFAs) provided by *S. hispanica*, influencing insulin resistance development.

MATERIALS AND METHODS

Bread samples

Red and white quinoa seeds (Organic quinoa Real©) from ANAPQUI, La Paz, Bolivia, were purchased from Ekologikoak (Bizkaia, Spain). Whole and semi-defatted chia flours were provided by Primaria Premium Raw Materials Company (Valencia, Spain). Wheat flour was purchased from a local market. Five bread formulations were prepared containing different proportions of flours to adjust the starch and lipid contents (Table 1) (Fernández-Espinar et al., 2016; Ballester-Sánchez et al., 2019a): red quinoa (RQ) at 25%, white quinoa (WQ) at 25%, chia (Ch) at 20%, and semi-defatted chia (Ch_D) at 20%, and they were compared to wheat bread (WB) formulations (Iglesias-Puig, & Haros, 2013). These formulations were chosen to provide similar contents of the immunonutritional agonists (Laparra, & Haros, 2019b) within the bread.

Immunonutritional agonists in flours: bioaccessibility

Aliquots (0.5 g) of flours were processed, extracted and sequentially filtered through a 50–30 kDa membrane (Amicon®) (Laparra, & Haros, 2019b). Then, protease inhibitory activity was quantified in the <30 kDa fraction (Laparra, & Haros, 2019b).

Flours used for bread formulation were subjected to simulated gastrointestinal digestion (Laparra, & Haros, 2018) and

bioaccessible immunonutritional agonists were monitored by HPLC-RP-DAD (Laparra, & Haros, 2019b). Briefly, flours were subjected to a two-step gastric (pepsin, 1 h, 37 °C) and intestinal (pancreatin/bile extract, 1 h, 37 °C) digestion (Laparra, & Haros, 2018). After addition of the pancreatin–bile extract solution, the volume was made up to 10 mL with an isotonic saline solution and 2 mL of the gastrointestinal digestion was carried out in the upper (donor compartment) chamber of a bicameral system. The bottom (acceptor compartment) of the system was filled up with 2 mL of an isotonic saline solution.

The bioaccessible protein profile (Laparra, & Haros, 2019b) was obtained using a 1260 Agilent HPLC system equipped with a quaternary pump and a photodiode array detector set at 280 nm (Agilent Technologies, Waldbronn, Germany). The separation (0.8 mL min^{-1}) was performed using a Poroshell 120 (7.5 cm × 4.6 mm) C18 column (Agilent).

Animals

In this study, 5-week-old male C57BL/6 mice with an average weight of 15–18 g were obtained from the Centro de Investigaciones Biológicas (CIB-CSIC) in Madrid, Spain. Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of CSIC (Consejo Superior de Investigaciones

Científicas) and the protocol was approved by its Ethics Committee (Ethic code, Proex 080/19).

Experimental design

All animals were injected intraperitoneally with streptozotocin (STZ) (two doses of 25 mg kg^{-1} in two successive days) to induce hyperglycaemia (Laparra, Díez-Municio, Moreno, & Herrero, 2015). Then animals were put under a high-fat diet (HFD) (de Magalhães et al., 2019) (EF AIN93G modified, Ssniff Spezialdiäten GmbH, Soest, Germany) for 3 weeks. Mice were maintained in a controlled environment of temperature (21–23 °C) and humidity (55%) under a 12 h:12 h (light : darkness) cycle with free access to food and water. Animals were distributed in individual groups (n = 6 per group) according to the formulation of the bread administered. Bread formulations were administered 3 times per week (14 mg per day per animal) during the period of study. The amount of bread administered was established according to the daily nutritional recommendation for bread consumption of 150 g per day per 70 kg body weight. The food intake and body weight of each group were monitored every 2 days. After treatment, mice were killed by cervical dislocation. Animals treated with STZ and put under a HFD, but not receiving bread formulations were used as controls.

Biochemical parameters

Blood samples were centrifuged (6000g per 10 min) to get clear supernatants. Insulin and glucose concentrations were determined in plasma samples. Insulin (RAB0817-1KT, Sigma- Aldrich, Darmstadt, GER), glucose (MAK263-1KT, Sigma- Aldrich, Darmstadt, GER) and triglycerides (Cayman Chemical, n° 10010303, Michigan, USA) were measured using commercial kits. Insulin resistance (IR) was defined as the homeostatic model assessment of insulin resistance (HOMAir) value, and its behaviour was studied according to the treatment received. HOMAir was calculated according to the following formula (de Magalhães et al., 2019): [insulin ($\mu U\ mL^{-1}$) × glycaemia ($mg\ dL^{-1}$)]/405.

Determination of hepatic triglycerides and malondialdehyde levels

Liver sections were kept in Krebs's buffer (1 mL), containing the Complete Protease Inhibitor Cocktail (Sigma), until analysis. Samples were thawed and homogenised using a TissueRuptor (Qiagen). Then, the homogenates were centrifuged (10 000g per 15 min) to get clear supernatants. Hepatic triglyceride determination was performed using a commercial kit (Cayman Chemical, n° 10010303, Michigan, USA) according to the manufacturer's instructions. Malondialdehyde (MDA) was

determined in hepatic homogenates using a thiobarbituric acid–reactive substance (TBARS) HPLC method as described elsewhere (Young et al., 1999). The samples (50 µL aliquots) were chromatographed with a linear gradient of water and acetonitrile with 0.1 % triflouroacetic acid (0–60 %, 17 min). Four malondialdehyde standards were included (0.025–0.25 µM) in each TBARS procedure and HPLC analysis, and the concentration of MDA in the samples was calculated from a standard curve. The HPLC analysis was performed using an Agilent 1260 system (Germany) equipped with a diode array detector with a Poroshell C18 (5 µm 2.7 × 50 mm) (Agilent) at 532 nm. The total protein content in plasma and hepatic homogenates was determined by the Pierce®-BCA method (ThermoFisher®, Rockford, USA) to normalise the results between the different samples.

Hepatic inflammatory markers

Innate immune 'Toll-like' receptor (TLR)-4 was determined by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). Validated Gene Expression Assays for murine TLR4 (forward 5′-TGG GAA CAC ACG GTT GGA AA-3′, reverse 5′-ACA GCA AGT TGT AGC ACT ACT GA-3′) was purchased from Applied Biosystems (Foqter City, CA, USA). qRT-PCR was performed with 500 ng of cDNA from liver sections, using the Universal PCR Master Mix (Applied

Biosystems, ThermoFisher®). Quantitative values were calculated by the $2^{-\Delta Ct}$ method (Laparra, & Haros, 2019b).

The cytokine profile was quantified in liver samples homogenised in an isotonic solution using a TissueRuptor (Qiagen) and sonicated. Tumor necrosis factor (TNF)-α (cat. no. 32673019, Friesoythe; GER), granulocyte macrophage colony-stimulating factor (GM-CSF) (cat. no. 32673129, Friesoythe; GER), interleukin-6 (IL-6) (cat. no. 32670069, Friesoythe; Germany) and interleukin-17 (IL-17) (cat. no. 32670179, Friesoythe; GER) (ImmunoTools) were determined by ELISAs according to the manufacturer's instructions. The results of the ELISA assays were normalised according to the total protein content (Pierce®-BCA).

Statistical analyses

Statistical analyses were performed using the Statgraphics Centurion XVI software. For normally distributed data, ANOVA and the Student *t* test were applied. Statistical significance was established at $P < 0.05$ for all comparisons.

RESULTS

Immunonutritional compounds in bread formulations and high-fat diets

According to their origin, white or red *C. quinoa*, reverse phase-HPLC-DAD analyses did not reveal significant differences between bioaccessible protease inhibitors displaying immunonutritional agonist activity (Laparra et al., 2019a; Laparra, & Haros, 2019b). Previous research showed that immunonutritionally active fraction from *C. quinoa* and *S. hispanica* provides serine-type protease inhibitors within complexes with a certain homology between different crops (Laparra, & Haros, 2019b). Immunonutritional agonists were identified as glycoproteins with an N-terminal glucuronamide linkage in *S. hispanica*, while they were glucosides in *C. quinoa*. The most protease inhibitory activity was identified in complexes of low molecular weight (<30 kDa). In this study, there were quantified significant variations in the proportion of immunonutritional compounds quantified in a low-molecular-weight (<30 kDa) protein fraction from *C. quinoa* and *S. hispanica* (Table 2). Despite variations in the total amount of immunonutritional agonists, *C. quinoa* provided a similar proportion of those within the <30 kDa fraction than that of *T. durum*. However, that fraction from *S. hispanica* was up to 4.2-

fold higher. Immunonutritional agonists displayed significant differences in relation to their bioaccessibility (Table 2).

The levels of both carbohydrates and fat were taken into consideration to put animals under a high-fat diet. The carbohydrate level of the diet used (43%) was elevated up to the highest level commonly used in the literature according to the experimental model (STZ-treated animals) (Barrière et al., 2018; Laparra et al., 2015). The fat content was established at the highest percentage to be representative of a HFD (42%) as reported previously (30–50%) (Gheibi, Kashfi, & Ghasemi, 2017). According to the literature, both HFD and 'very' HFD (58–60%) develop insulin resistance in animals. Moreover, to target early events responsible for the onset and progression of immunometabolic changes, the study period was fitted to the earlier phase (2–18 weeks) reported previously (Barrière et al., 2018).

Table 1: Proximal composition of raw materials used to obtain flours.

Components (g per 100g d.m.)	Flours				
	Wheat[a]	Red quinoa[a]	White quinoa[a]	Chia[b]	Defatted chia[b]
Starch	66.2 ± 1.3	62.6 ± 1.1	61.8 ± 1.7	1.7 ± 0.4	1.8 ± 0.2
Proteins	12.4 ± 0.1	12.8 ± 0.7	13.0 ± 0.7	21.4 ± 1.7	29.4 ± 0.2
Lipids	1.1 ± 0.1	5.3 ± 0.2	5.4 ± 0.6	34.4 ± 0.4	7.6 ± 0.3
Ash	0.48 ± 0.08	2.32 ± 0.04	2.37 ± 0.02	4.77 ± 0.04	6.28 ± 0.03

The data are reported on dry basis. Values are expressed as mean ± standard deviation (n = 3). d.m. dry matter. [a]Ballester-Sánchez et al.. 2019; [b]Fernández-Espinar et al., 2016

Table 2: Content of immunonutritional agonists quantified in flours from different botanical origin and their estimated bioaccessibility and protease inhibitory activity of fraction <30 kDa. Results are expressed as mean ± SD (n = 4).

Botanical origin	Molecular size (mg protein per g flour)			Bioaccessibility[a]	Prot. inh activity
	>50 kDa	>30 kDa	<30 kDa	(μg min^{-1})	(%)
Triticum durum	0.099 ± 0.002[a]	0.022 ± 0.007[a]	0.011 ± 0.003[a]	0.41 ± 0.03	-2.20 ± 0.08[a]
***Chenopodium quinoa*[b]**	0.029 ± 0.001[b]	0.017 ± 0.002[a]	0.013 ± 0.005[a]	0.99 ± 0.08	-0.60 ± 0.08[b]
Salvia hispanica	0.147 ± 0.006[c]	0.082 ± 0.021[b]	0.046 ± 0.001[b]	0.62 ± 0.04	-0.83 ± 0.05[b]

[a] Bioaccessibility of immunonutritional agonists is expressed as the rate of appearance in the basal compartment representing the proportion of those available for absorption or to exert bioactive effects. [b] Analysis of the different varieties, white vs. red, did not reveal significant differences for the extractability of the immunonutritional agonists. a–c Different superscript letters indicate statistically significant differences within the same column.

Changes in body weight

Animals fed with RQ, WQ and Ch_D samples exhibited a negative trend in body weight (BW) gain (Fig. 1) relative to WB, but only administration of RQ samples had a statistically significant effect (Fig. 1A). Notably, the observed differences in HFD-induced BW gain were not due to reduced food intake. There were no significant differences in daily food (energy) intake between the different groups of treatment (90.2 kJ d^{-1}) (215.6 kcal d^{-1}) over the study period. Although not significant ($p > 0.05$), due to data dispersion, an opposite trend could be found in the mean values for body weight gain between animals administered with Ch or Ch_D formulations. Morphometric comparison of the hepatosomatic ratios (liver/ BW) revealed a positive trend in all groups of treatment in relation to WB. Only animals receiving RQ samples exhibited a significant ($P = 0.03$) increase relative to WB (Fig. 1B).

Glycaemic parameters

Feeding animals with RQ, WQ and Ch samples significantly decreased serum glucose concentrations (Fig. 2A) in relation to those receiving WB. The significant reduction in starch provided by Ch and Ch_D samples does not appear as a major contributor controlling hyperglycaemia in comparison to RQ and WQ samples. Animals administered with RQ and Ch samples

displayed a 2-fold lower glycaemia than animals administered with WB. Only animals fed with Ch_D samples did not display significantly decreased glucose concentrations. All groups of treatment displayed a positive trend decreasing insulin levels in comparison to WB (Fig. 2B). This effect on insulin regulation reached statistical significance in those animals administered with WQ and Ch_D samples. Notably, all groups of treatment displayed reduced HOMAir values (Fig. 2C), evidencing the improved insulin resistance. HOMAir values were close to #3, even lower for RQ and Ch sample-fed animals, which is considered the limit to establish an insulin resistance.

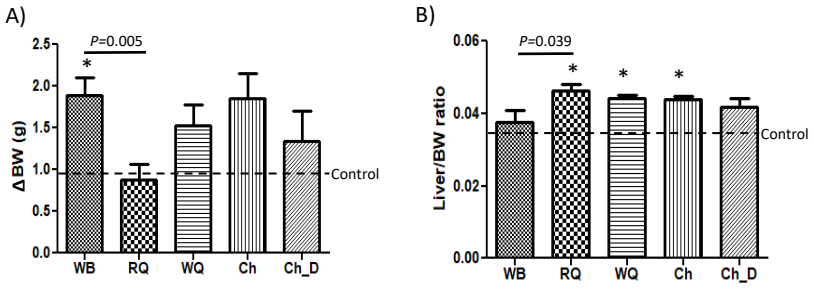

Fig. 1 Variations in body weight gain (A) and liver-to-body-weight ratio (B) in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to controls.

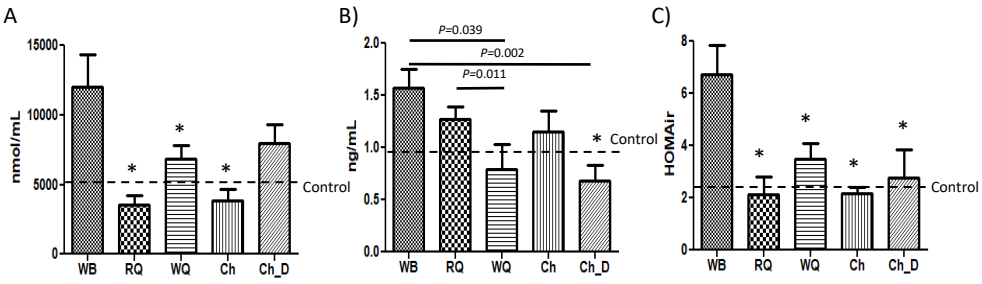

Fig. 2 Biochemical parameters (plasma glucose, A; insulin, B and HOMAir, C) in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to WB.

Lipid parameters

Plasma triglyceride concentrations (Fig. 3A) were increased ($p < 0.05$) only in animals administered with Ch_D samples, but an increasing trend was also observed in those receiving RQ and Ch samples. Interestingly, highest mean triglyceride concentrations are associated with those animals exhibiting the lowest glycaemia (Fig. 2A). Otherwise, significant differences were not quantified in hepatic triglyceride concentrations between different groups of treatments (Fig. 3B). However, all groups exceed normal hepatic triglyceride contents genetically determined in mice (Lin, Yue, Chen, & Schonfeld, 2005).

Hepatic levels of malondialdehyde (MDA), a reactive metabolite derived from lipid peroxidation, were quantified to evaluate the influence of bread formulations in the control of HFD-induced excessive oxidative stress (Fig. 3C). Feeding with RQ, WQ and Ch samples significantly reduced the degree of lipid peroxidation in relation to WB. Between the quinoa groups, a significant difference ($P < 0.05$) was quantified, where RQ samples exhibited a more positive effect reducing the hepatic MDA levels. Notably, animals fed with Ch_D samples showed 1.5-fold higher MDA levels than its whole counterpart, from where a protector role can be hypothesised for chia's fat in the hepatic lipid peroxidation.

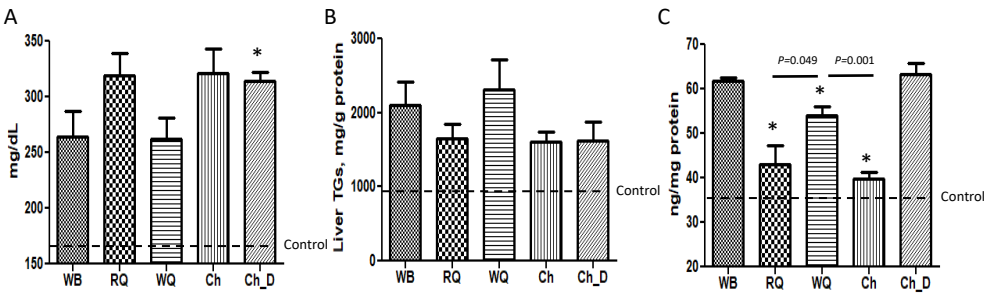

Fig. 3 Plasma triglycerides (A), hepatic triglycerides (B) and peroxidised lipids (C) in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to WB.

Hepatic Inflammatory markers

Hepatic expression (mRNA) levels of TLR4 increased compared to WB, except for Ch_D that obtained similar values (Fig. 4A). Animals fed with WQ and Ch samples displayed a slight upward trend in TNF-α levels compared to WB (Fig. 4B). Changes in IL-6 concentrations (Fig. 4C) mirrored those of TNF-α in animals fed with WQ and Ch, and a significant difference was quantified ($P < 0.05$) between the concentrations in animals fed with RQ and WQ samples. This behaviour was similar in IL-17 levels (Fig. 4D), suggesting that WQ samples could exert a greater influence in the hepatic inflammatory processes in comparison to RQ samples. In relation to variations in GM-CSF (Fig. 4E), only animals fed with Ch_D samples showed significantly ($P < 0.05$) reduced levels of the cytokine in relation to WB. Besides, feeding with WQ samples promoted upward trends in GM-CSF values suggestive of a more prominent anti-inflammatory innate immune response. This myeloid population contributing to ameliorate the hepatic metabolic stress.

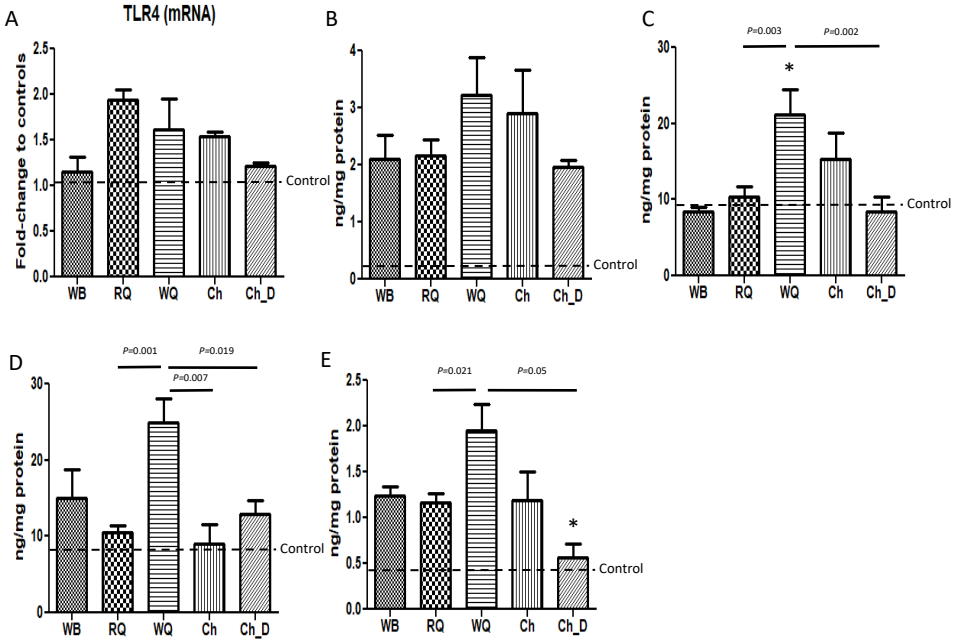

Fig. 4 Hepatic inflammatory markers: (A) Toll-like' receptor (TLR)-4; (B) tumour necrosis factor (TNF)-α; (C) interleukin (IL)-6; (D) IL-17; and (E) granulocyte–monocyte colony stimulating factor (GM-CSF), in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to WB.

DISCUSSION

This study shows that replacement of wheat flour with those obtained from different varieties of *C. quinoa* (*i.e.*, white and red) and processed seeds (*i.e.*, whole and semi-defatted) of *S. hispanica* in bread formulations provides beneficial effects ameliorating the immunometabolic conditions derived from high-energy intake. These effects appeared modifiable in hyperglycaemic animals fed with a HFD during 3 weeks likely reproducing early features of T2D/metabolic syndrome (Srinivasan, Viswanad, Asrat, Kaul, & Ramarao, 2005).

According to the proximate composition of flours, similar starch contents in WB and those from *C. quinoa*, WQ and RQ, samples make unlikely that better controlled variations in the plasma glucose concentrations could be attributed to starch (Table 1). Moreover, the statistical significance between insulin levels when animals receive RQ *vs*. WQ samples allows hypothesizing that these changes are not completely derived from glycaemia and may imply the participation of immunonutritional agonists found in *C. quinoa* (Laparra, & Haros, 2019b). Variations in the plasma glucose concentrations were inversely associated with the protease inhibitory activity, but directly to the higher bioaccessibility estimated for immunonutritional agonists from wheat (*T. durum*) and quinoa (*C. quinoa*) flours (Table 2). In wheat flours, these compounds have been found as part of homodimeric and

heterotetrameric complexes, while monomeric units are majorly present in flours from quinoa and chia (Laparra, & Haros, 2019b; Srdić, Ovčina, Fotschki, Haros, & Laparra, 2020). Major structural differences rely on the lack of glycoconjugate prosthetic groups in immunonutritional agonists from wheat, but carriage of an N-terminal glucuronamide linkage in *S. hispanica* and glucosides in *C. quinoa* (Laparra, & Haros, 2019b). Pilot studies revealed slight, but significant, differences in the coefficients of diffusion for the immunonutritional agonists from RQ and WQ samples (*data not shown*), where the slowest kinetic might be associated with a better control over insulin production (Fig. 1B). Immunonutritional agonists found in the flours have been proved to have a significant potential to down-regulate the expression of key enzymes (*i.e.*, GAPDH) involved in the glycolysis processes according to the following gradation (Srdić et al., 2020): wheat, 34.9%; quinoa, 44.2% and chia, 51.5%. These effects occur via their immunonutritional role interacting with the innate immune 'Toll-like' receptor (TLR)-4 (Srdić et al., 2020; Junker et al., 2012) (Fig. 4A). Accordingly, the lower immunonutritional potential of RQ samples is supported by downward trends in the hepatic inflammatory milieu, TNFα (Fig. 4B) and IL-6 (Fig. 4C), which is dependent on TLR4 engagement of adaptor molecules and downstream molecular signalling.

To approach the comprehensive effect of n-3 PUFAs, chia flour was defatted attaining a reduction in the lipid fraction up to 70% (Table 1). An increase in the protein fraction was observed, which could be attributed, at least in part, to the conversion factor used to estimate crude protein from total nitrogen (Fernández-Espinar et al., 2016; González-Luna et al., 2016). Otherwise, methods targeting peptides containing three or more amino acid residues (Valverde, Orona-Tamayo, Hernández-Pérez, & Paredes-López, 2016) are affected to a lesser extent by protein compositional differences to provide greater concentration accuracy. Accordingly, significant lower protein contents in defatted chia flours have been reported, 22.7 ± 0.7 g per 100 g, (Valverde et al., 2016) in comparison to those using the conversion of nitrogen (Fernández-Espinar et al., 2016; González-Luna et al., 2016), 29.4–31.7 g per 100 g. Defatting causes a significant reduction (by 39.7 %) in the α-amylase inhibitory activity of chia (Segura-Campos, Martínez-Leo, Fernández-Espinar, Gil, & Haros, 2016) attributed to the immunonutritional compounds. These effects can explain the upward trend in glucose concentrations in animals fed with Ch_D samples in relation to their counterparts receiving Ch samples (Fig. 2A). Collectively, significant impairments can be hypothesized in the tertiary structure of the compounds responsible for the immunonutritional activity due to the loss of capability tointeract with TLR4 (Fig. 4A). Previous data suggest

that n-3 PUFA may improve postprandial hyperglycaemia as well as insulin secretion ability and hypertriglyceridemia, with impaired glucose metabolism (Sawada et al., 2016; O'Mahoney et al., 2018). Neither duration nor dosage appears to explain the observed heterogeneity in response to n-3 PUFAs (O'Mahoney et al., 2018). Herein, the significant reduction in the lipid fraction could be concordant with the variation observed in plasma glucose; however, it cannot explain the improved control on insulin levels (Fig. 2B and C). This observation transpires in the sense of supporting the role of immunonutritional compounds.

Immunonutritional agonists from quinoa and chia have been shown to significantly down-regulate the expression of the fatty acid translocase (*i.e.*, CD36) (Laparra et al., 2019a), thereby modulating the breadth of signalling for TLR4 (Srdić et al., 2020). In the last few years, different studies pointed out the significant effect of regulation of the TLR4/NF-κB-pathway on obesity-associated imbalances in biochemical parameters (*i.e.*, triglycerides) (van der Tuin et al., 2018; Laparra et al., 2015; Chen et al., 2017). Commonly, the reduction in plasma triglyceride concentrations was derived either from inhibition of its downstream molecular signalling or delaying the intracellular retrieval of the receptor allowing signal transduction. In this scenario, proteolytic cleavage and shedding of TLR4 has been demonstrated as the mechanism to prevent the liver from

developing insulin resistance (Uchimura et al., 2014). These observations agree with the findings relative to the important regulatory and sequential role of innate and adaptive immunity to establish tissue lipid homeostasis (Mao et al., 2018). When considering the influence of n3-PUFAs, data did not support potential positive effects of PUFAs regulating the plasma triglyceride concentrations, in accordance with the lack of correlation between the effects and level of n3-PUFA intake. Health effects of n-3 PUFAs are partly mediated by their oxidized metabolites, *i.e.*, eicosanoids and other oxylipins. Herein, TLR4 has been identified as a mediator able to induce epoxy-oxylipin products, promoting the protective effects of soluble epoxide hydrolase (Thomson et al., 2015). This protective effect appears quite well reflected in the reduced MDA levels quantified in animals fed with Ch samples in comparison to those receiving Ch_D samples. While important for the clearance of these molecules from the circulation, CD36-dependent signalling has also been implicated in the pro-inflammatory effects of modified endogenous ligands of TLR4 (Stewart et al., 2010).

Herein, the extent to what RQ and WQ samples affect MDA levels (Fig. 3C) appears inversely associated with the level of TLR4 transcripts (Fig. 4A), which is commonly associated with the protein content. These differences are revealed by the upward trends in TNFα and IL-6 production (Fig. 4B and C). Collectively,

lower mRNA levels of TLR4 with increased cytokine production may interpret the experimental data, showing faster kinetics of TLR4 mRNA translation and protein expression, which aggravate glycaemia-induced metabolic stress. However, the role of TNF-α and IL-6, which can elicit either positive or negative effects, on metabolic control and insulin sensitivity is still under debate (Lechleitner, Koch, Herold, Dzien, & Hoppichler, 2000; Carey, & Febbraio, 2004; Sadagurski et al., 2010). The lack of significant differences in the phenolic content between RQ and WQ samples (Ballester-Sánchez, Gil, Haros, & Fernández-Espinar, 2019b) transpires in the sense of a protein-mediated effect as a unique promoter of different TLR4 transcript levels. Moreover, neither the isolated polyphenols profile nor total antioxidant capacity (DPPH method) estimated for *S. hispanica* flours, semi-defatted or not, could be associated with the extractable or bioaccessible total antioxidant capacity estimated for these fractions (Pellegrini et al., 2017).

TLR4 mRNA levels together with the trend for both cytokines quantified in animals receiving Ch samples are tempting to suggest similarities between immunonutritional agonists in animals receiving WQ and Ch samples. However, it should not be ruled out that immunonutritional agonists also promote physiological variations in lipid-dependent kinases (*i.e.*, protein kinase C–PKC) (Srdić et al., 2020). These kinases have shown

wideranging roles in signal transduction and modulation of insulin action (Schmitz-Peiffer, & Biden, 2008). Accordingly, variations in MDA levels between animals fed with WQ and Ch samples are explained (Fig. 3A–C), at least in part, by lower PKC expression levels favoured by Ch samples helping to ameliorate the effects of fat oversupply. The decreased hepatic IL-17 levels (Fig. 4D) also support positive effects on the high-fat diet-induced metabolic stress by inhibiting fatty acid β-oxidation (Shen et al., 2017). With this in mind, the negative balance in GM-CSF levels (Fig. 4E) attained by feeding with Ch samples allows suggesting a better controlled HFD- induced impaired insulin sensitisation (Fig. 2A and B) as reflected in lower HOMAir (Fig. 2C). Overall, the lack of these inflammatory response(s) helps supporting the partial loss of bioactivity by immunonutritional agonists during defatting as suggested previously.

CONCLUSIONS

The replacement of wheat flour with that from *C. quinoa* (*i.e.*, white and red) or *S. hispanica* (*i.e.*, whole and semi-defatted) in bread formulations ameliorates the severity of metabolic consequences of high-fat diet consumption in hyperglycaemic animals. A relative short STZ/HFD model (3 weeks) allows us to monitor both hyperglycaemia and hypertriglyceridemia as well as

insulin sensitization and resistance development. Herein, protease inhibitors displaying immunonutritional activity provided by flours rather than other nutrients, such as PUFAs, significantly contributed to a higher extent to the beneficial outcomes. These results indicated that the immunonutritional potential of different bread formulations rather than calorie intake or starch content appears as a major determinant of the control of glucose homeostasis. This opens new avenues on food formulation, influencing innate immunity to shape nutrient homeostasis. Further research with human trials is needed to determine the magnitude of these beneficial effects.

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Capítulo 4: Immunonutritional bioactives from *Chenopodium quinoa* and *Salvia hispanica* L. flour positively modulate insulin resistance and preserve alterations in peripheral myeloid population

Immunonutritional bioactives from *Chenopodium quinoa* and *Salvia hispanica* L. flour positively modulate insulin resistance and preserve alterations in peripheral myeloid population

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ABSTRACT

Innate immunity plays a determinant role in high fat diet (HFD)-induced insulin resistance. This study compares the effects of immunonutritional bioactives from *Chenopodium quinoa* (WQ) or *Salvia hispanica* L. (Ch) when used to partially replace wheat flour (WB) into bread formulations. These flours were chosen to condition starch and lipid content in the products as well as because their immunonutritional activity. To be administered with different bread formulations, HFD-fed C57BL/6J mice were distributed in different groups: (i) wild type, (ii)

displaying inherited disturbances in glucose homeostasis, and (iii) displaying dietary iron-mediated impairment of the innate immune TLR4/TRAM/TRIF pathway. We analyze the effects of the products on glycaemia and insulin resistance (HOMA-IR), plasmatic triglycerides, intestinal and hepatic gene expression and variations of myeloid (MY), and lymphoid (LY) cells population in peripheral blood. Our results show that feeding animals with WQ and Ch formulations influenced the expression of lipogenic and coronary risk markers, thus attaining a better control of hepatic lipid accumulation. WQ and Ch products also improved glucose homeostasis compared to WB, normalizing the HOMA-IR in animals with an altered glucose and lipid metabolism. These positive effects were associated with positive variations in the peripheral myeloid cells population.

Keywords: Insulin resistance · Innate immunity · Myeloid population · Obesity

INTRODUCTION

Nowadays, more than 1600 million people worldwide are overweight or obese. According to the World Health Organization (WHO), this global health problem affects all groups of the population and this number will continue increasing by >140% in 2050. Obesity, together with other chronic diseases (i.e., type 2 diabetes–T2D, hypertension, dyslipidemia, physical inactivity, chronic kidney and liver diseases, and smoking), contributes an additional risk factor to cardiometabolic health, while hyperglycaemia confers a substantial independent risk for the adverse physiological outcomes (Gupta et al., 2020). For example, gestational hyperglycaemia and obesity are independently associated with adverse outcomes during pregnancy (Gupta et al., 2020; Catalano et al., 2012). Worldwide hyperglycaemia kills some 3.4 million people a year (WHO). Beyond alleviating glucose levels, the greatest benefits for disease prevention appear to be derived from improving the 'glycemic control', while the normalization of glycaemia results in a more modest reduction of the effects (Riddle et al., 2018).

Non-alcoholic fatty liver disease (NAFLD) incurs a high risk for the development of T2D and other major features of the metabolic syndrome (Buzzetti, Pinzani, & Tsochatzis, 2016). Metabolic imbalances, including those affecting glucose homeostasis, arise from complex interactions between genetic and environmental factors (Lu et al., 2018). While the host's endogenous factors are rather difficult to influence, the environmental (i.e., dietary) factors are predominant and addressable

from a preventive or therapeutic approach. Besides, emerging evidence supports the metabolic (re)programming of defined components of the innate immune system (i.e., innate immune both myeloid and lymphoid cells), which act as key mediators to induce obesity (Sasaki et al., 2019; Petersen, Bilkei-Gorzo, Govaere, & Härtlova, 2020). Furthermore, significant changes in the subpopulations of lymphocytes have been reported in young adults with metabolic syndrome (Rodríguez, Gonzalez, Aguilar-Salinas, & Nájera-Medina, 2018). Notably, intestinal innate immune 'Toll-like' receptor (TLR)-4 is a well-known element, which has been identified as an essential regulator of insulin resistance and accumulation of macrophages within the adipose tissue (Lu et al., 2018). These immune changes are critical mechanism(s) to normalize the distinctive stamp of obesity in the glucose homeostasis dysregulation. In this context, immunonutritional strategies can play an important role in controlling the severity and progression of the disease. However, whether the use of bread formulation prepared by replacing wheat flour (Selma-Gracia, Haros, & Laparra, 2020) modulates myeloid and lymphoid populations and glycaemic control remains elusive.

The question of how endogenous (i.e., transgenerational inheritance) (Chamorro-García et al., 2013) and environmental factors (i.e., diet) (Barrière et al., 2018) influence early stages and the onset of alterations in lipid and glucose homeostasis has promoted the development of different preclinical models. The exposure of non-pregnant female mice to the obesogenic tributyltin (TBT) enabled a transgenerational

inheritance of disturbances in glucose homeostasis (Chamorro-García et al., 2013) and inhibition of the insulin receptor expression (Li et al., 2017). Furthermore, it caused a permanently metabolic (re)programming towards the hepatic fat accumulation and weight gain, particularly in conditions of caloric excess (Chamorro-García et al., 2013). Besides, it was demonstrated that there was a development of hypoglycemia associated with hypertriglyceridemia and a transitory insulin resistance in animals fed a high fat diet (HFD) (Barrière et al., 2018). These long term (56 weeks) preclinical models of T2D revealed that an elevated hyperglycaemia associates with early insulin resistance after 2 weeks under HFD feeding. Metabolic alterations caused by HFD that result in and are associated with hepatic steatosis implies a dysmetabolic hepatocellular iron uptake (Dongiovanni et al., 2015). In addition to this, HFD-induced activation of the TLR4/MyD88 pathway leads to an inhibited macrophage proliferation, which associates with macrophage infiltration into adipose tissue (Griffin et al., 2018). The TLR4/MyD88-dependent signaling likely contributes to promote hepatic inflammation (Yang et al., 2017), whereas the production of type I interferons via TLR4/TRIF-dependent signaling appears to exert protective roles in the metabolic dysfunction (Wieser et al., 2016). This raises interest in evaluating to what extent modulating the immunonutritional potential of food formulations can influence changes in the proportions of peripheral myeloid immune cells in individuals with alterations in the glucose homeostasis.

Bread formulations enriched with *Chenopodium quinoa* and *Salvia hispanica* L. flours provide an effective alternative to improving metabolic imbalances derived from a HFD intake in hyperglycaemic mice (Selma-Gracia et al., 2020). These effects were attributed to immunonutritional bioactives (protease inhibitors found in the low molecular weight albumins/globulin fractions) that interact with TLR4 (Laparra, & Haros, 2019; Llopis, Brown, & Saiz, 2020; Srdić, Ovčina, Fotschki, Haros, & Llopis, 2020; Laparra, Fotschki, & Haros, 2019. Notwithstanding, this interaction appears different to that exerted by immunonutritional bioactives from wheat (Junker et al., 2012). At the molecular level, compounds derived from *C. quinoa* and *Salvia hispanica* L. display glycoside and glucuronide groups bound to the amino acid backbone, while those from wheat (*Triticum durum*) lack glycosidic prosthetic groups (Laparra, & Haros, 2019). Proteome analyses on human-like macrophages shed some light on the biological activity of protease inhibitors from *C. quinoa* and *S. hispanica* L, supporting their correlation with TLR4/TRIF signaling (Srdić et al., 2020) that contrasts with TLR4/MyD88 of wheat (Junker et al., 2012).

In this study, we hypothesize that partial replacement of wheat flour by that of *C. quinoa* and *S. hispanica* L. will improve the immunonutritional potential of bread formulations. More specifically, such substitution will positively contribute to ameliorate alterations of glucose and lipid homeostasis through the modulation of the innate immune potential within the 'gut-liver' axis. To this end, different

preclinical models that reproduce major features of the obesogenic and hyperglycemic conditions mimicking the human disease are used. These results can provide novel points of view with significant implications for the ongoing debate about developing appropriate interventions to maximize the beneficial effects and immunometabolic control of obesity.

MATERIALS AND METHODS

Bread Samples

Distinct bread formulations were prepared containing different proportions of flour from white quinoa (WQ) at 25% and chia (Ch) at 20% and were compared to wheat bread (WB) (Iglesias-Puig, & Haros, 2013). The different proportions of *C. quinoa* and *S. hispanica* flour were chosen to normalize the protein content in the products because as immunonutritional bioactives derive from it. The chemical composition of *C. quinoa*- and *S. hispanica* L-containing bread formulations is shown in Table 1.

Table 1. Proximal composition of *C. quinoa*- and *S. hispanica* L-containing bread formulations administered in the study.

Component g/100g d.m.	**Bread**		
	Wheat [23]	**White quinoa [23]**	**Chia [24,25]**
Starch	66.2 ± 1.3	61.8 ± 1.7	53.3 ± 0.1
Proteins	12.4 ± 0.1	13.2 ± 2.5	14.1 ± 0.5
Lipids	1.1 ± 0.1	2.2 ± 0.1	7.8 ± 0.1
Ash	0.5 ± 0.1	1.5 ± 0.0	1.3 ± 0.0
Iron (μmol/g) [26]	0.4 ± 0.1	0.6 ± 0.0	0.7 ± 0.0
$InsP_6$ (μmol/g) [26]	n.d.	2.0 ± 0.0	1.7 ± 0.0

Values are expressed as mean ± standard deviation (n = 3). d.m. dry matter; n.d. not detected; InsP6, phytic acid. [23]: Ballester et al., 2019; [24]: Fernández-Espinar et al., 2016; [25]: Miranda-Ramos et al., 2020; [26]: Laparra et al., 2016.

Animals

C57BL/6 mice with 6 weeks of age were obtained from the Centro de Investigaciones Biológicas (CIB-CSIC) in Madrid, Spain. Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of CSIC (Consejo Superior de Investigaciones Científicas) and the protocol was approved by its Ethics Committee (Proex No.080/19).

Mice were maintained under a controlled environment of temperature (21–23 ˚C) and humidity (55 %) and a 12 h:12 h (light:dark) cycle with food and water ad libitum. After treatment, mice were sacrificed by cervical dislocation.

Experimental Design

Different models were used to reproduce the main metabolic and immunological alterations in the functionality of the "gut-liver" axis and were of great relevance in the influence of glucose homeostasis (Figure 1). All bread formulations were administered (14 mg/day/animal) to the different animal models; three times per week for 3 weeks to model 1 (Figure 1A) and for 2 consecutive days to model 2 (Figure 1B).

The amount of bread administered was established according to the daily nutritional recommendation for bread consumption (i.e., 150 g/day/70 kg body weight) that was previously proved to be effective controlling glucose homeostasis (Selma-Gracia et al., 2020). Food intake and changes in body weight of each group were monitored every 2 days. Livers were removed and weighed to calculate the whole body to liver weight (hepatosomatic index). After treatment, mice were killed by cervical dislocation. Animals put on a HFD but not receiving bread formulations werer used as controls.

Biochemical Parameters

Blood samples were centrifuged (6000 g/10 min) to get clear supernatants. Glucose, insulin, and triglycerides concentrations were determined in plasma samples. Glucose was determined by glucose kit (KA1648, Abnova, Taoyuan, Taiwan), insulin by ELISA kit (RAB0817-1KT, Sigma-Aldrich, Darmstadt, GER) and triglycerides with a commercial kit (Cayman) (n◦ 10010303). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) value was used to define insulin resistance according to the following formula: [insulin (μIU/mL)* glycaemia (mg/dL)]/405 (Matthews et al., 1985).

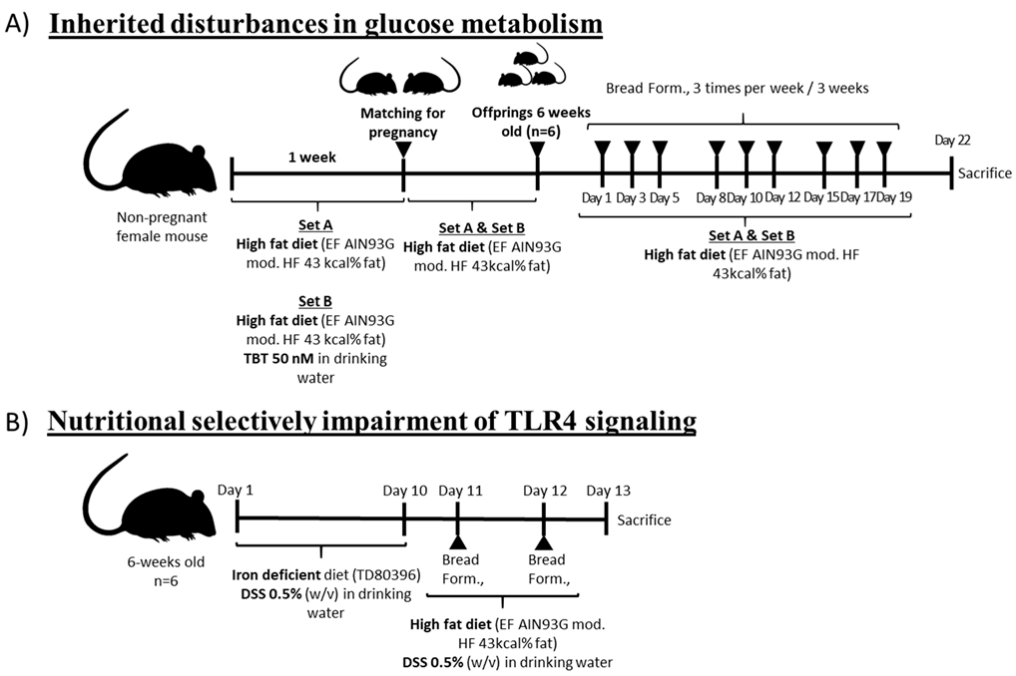

Figure 1. Schematic representation of the two experimental models used to mimic early disturbances in glucose homeostasis and the administration pattern for the bread formulations (bread form: wheat-, *Chenopodium quinoa*- and *Salvia hispanica*- based bread formulations. Model 1 (A): Male animals (n = 6/group) were split into two groups depending on their origin; (i) pregnant female mice under standard conditions (F0_A) or (ii) pregnant female mice exposed (F0_B) to the obesogenic tributyltin TBT 50 nM (w/v) via drinking water to develop a state of obesity (Chamorro-García et al., 2013). After, both F1_A and F1_B generations were kept on a HFD until reaching 6 weeks of age. Model 2 (B): Male mice (n = 6/group, 6 weeks-old) from pregnant females under standard conditions were kept on an iron-deficient diet (AIN93G modified, Ssniff Spezialdiäten GmbH, Soest, Germany), exposed to 0.5%, and administered with dextran sulfate sodium (DSS) (w/v) via drinking water (Wang et al., 2012) for 10 days. After, animals were put on a HFD and continued to be exposed to 0.5% DSS for an additional 2 days.

Hemogram

Complete blood count was performed on an automated hemocytometer (Abacus Junior Vet, ELECTROMEDINTER SL) to calculate total red blood cells (RBC) and leukocyte counts (WBC) as well as the lymphocytes (LY), myeloid (MY) (macrophages, monocytes), and granulocytes (GR) (granulocytes, eosinophils, neutrophils) population percentages.

Transcripts of Hepatic Lipogenic and Coronary Risk Markers and Macrophage Identifiers

Validated Gene Expression Assays for murine fatty acid synthase (FASN) (forward 5′-TTC CCA CCA AGT GTG GGT AT -3′, reverse 5′-TGG GAC CTT CAG CTT GCT TC -3′), sterol regulatory element-binding protein 1 (SREBP1a) (forward 5′- TCA AAA CCG CTG TGT CCA GT -3′, reverse 5′- GAC GTC TCA ACC CGC TAG G -3′), prostaglandin- endoperoxide synthase 2 (PTGS2) (forward 5′- AAA AGA GAA CGT GAG AGG GCA -3′, reverse 5′- TCA AAC TGG GAA CGG GTG AC -3′), arachidonate 15-lipoxygenase (ALOX15) (forward 5′- TCC CAT TCT AGG GGA GAG GG -3′, reverse 5′- CCT TGA CCA GCT CAG TAG GC -3′), CD68 (forward 5′- AGA AGT GCA ATG GTG GGT CT-3′, reverse 5′- TGG GGC TTA AAG AGG GCA AG -3′), CD206 (forward 5′- TGC AAG CTT GTA GGA AGG AGG -3′, reverse 5′- GAT TAG AGT GGT GAG CAG GC -3′) and β-actin (forward 5′-GGC TCC TAG CAC CAT GAA GAT CAA -3′, reverse 5′- AGC TCA

GTA ACA GTC CGC CTA GAA -3′) was purchased from Applied Biosystems (Foqter City, CA, USA). Qrt-PCR was performed with 500 ng of cDNA from liver sections, using the Universal PCR Master Mix (Applied Biosystems, Foster, CA, USA) Quantitative values were calculated by using the $2^{-\Delta Ct}$ method (Selma-Gracia et al., 2020).

Statistical Analyses

The results are presented as the mean and standard error of the mean (SEM). The statistical analysis between the different groups of treatment within a same experimental model was conducted using one-way analysis of variance (ANOVA) and the Kruskal-Wallis post hoc test by ranks. Because of the different conditions used to obtain the animals for the different models, the comparison between groups of animals receiving the same bread formulation was performed by comparison of the means. Statistical analyses were performed with the software Statgraphics Centurion XVI and significance was established at $p < 0.05$ for all comparisons.

RESULTS AND DISCUSSION

Immunonutritional Bioactives

This study evaluates the modulatory role of the inclusion of *C. quinoa* and *S. hispanica* L. into *T. aestivum*-based bread formulations on the HFD-induced immunometabolic effects in conditions of caloric excess as

well as hepatic steatosis, which is elicited by prenatal exposure to the obesogenic TBT (Chamorro-García et al., 2013). Partial replacement of wheat flour by that from *C. quinoa* or *S. hispanica* L. into bread formulations was evaluated as an immunonutritional strategy to provide glycoside- and glucuronide-carrying proteins (2S seed storage protein), which have shown to display immunonutritional potential (Figure 2). Here, is shown the molecular weight of the molecular backbone as well as the glycoside (Figure 2A) and glucuronide (Figure 2B) linkage as deduced from the RP-HPLC-Ms/Ms (Laparra, & Haros, 2019) analyses on protein bands. These glycoside and glucuronide groups were not found to be associated with the wheat-derived immunonutritional bioactives (Laparra, & Haros, 2019). Interestingly, the effects observed could not only be directly associated to these structural features but to their capacity to interact with TLR4 (discussed below). The inclusion of *C. quinoa* or *S. hispanica* L. flours modified the content of bioactive 2S globulins according to the following gradation: 2.21 ± 0.04 mg/g (WB) = 1.74 ± 0.32 mg/g (WQ) < 8.19 ± 0.11 mg/g (Ch). Focusing on the control of glucose homeostasis, the particular composition of the products help to provide additional information on how glycaemia results affected by the different carbohydrate and lipid changes (Table 1) (Selma-Gracia et al., 2020).

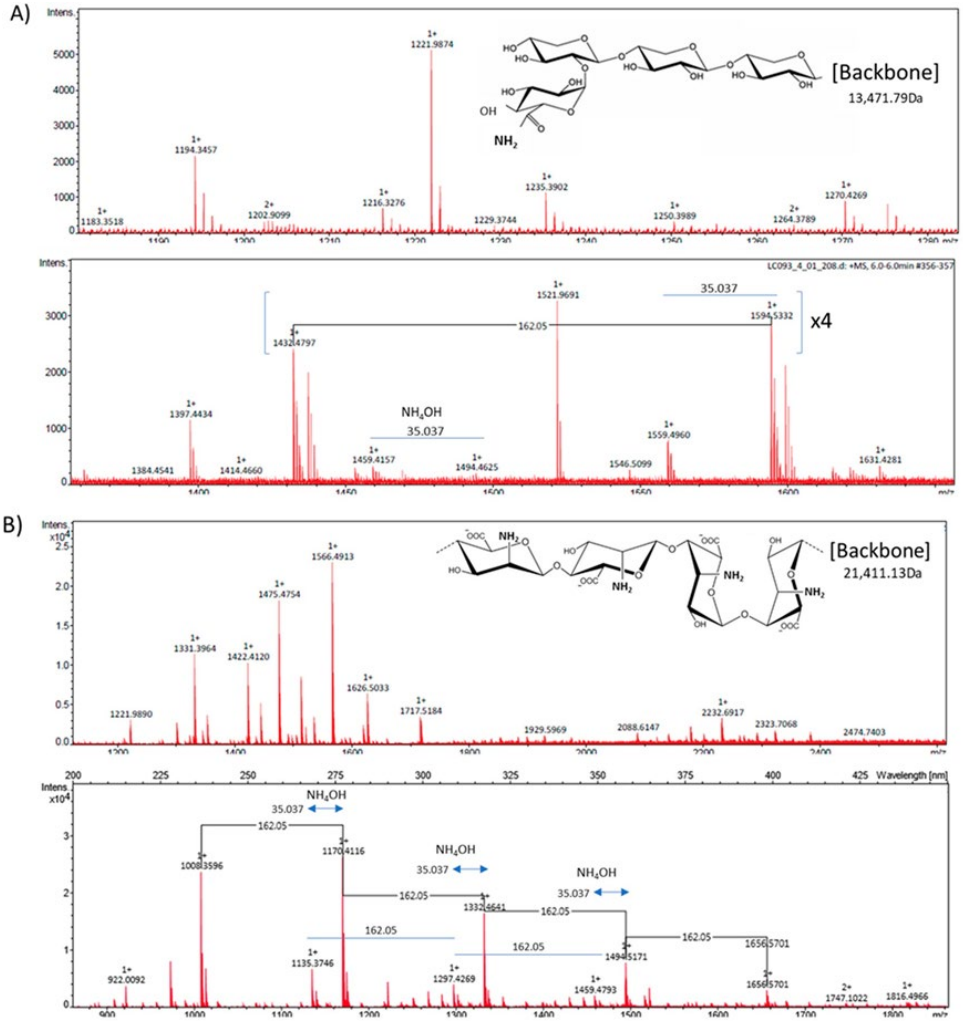

Figure 2. Mass spectrometry '*m/z*' signals from the bioactive fraction obtained from *C. quinoa* (**A**) and *S. hispanica* L (**B**) (Laparra, & Haros, 2019; Srdić et al., 2020).

Immunnutritonal Influence of Obesogenic Effects

To determine whether the different products affect hepatic lipid homeostasis, C57BL/6J mice were put on a 43% HFD (Figure 3), as established in previous studies (Selma-Gracia et al., 2020). Significant differences in the daily food (energy) intake between wild type or TBT-exposed mice in the different groups of treatment (30.0 kcal/day) (143.7 kJ/day) were not quantified. Feeding WB, only TBT-treated animals increased the body weight (BW) gain. However, contrasting patterns were observed for WQ and Ch (Figure 3A). Despite the model, animals receiving WQ increased BW, whereas those fed with Ch maintained a BW similar to that observed in the control group. These effects are attributed to bioactive ingredients in bread formulations, since it was previously shown that F1 animals directly exposed in utero to TBT display low effects on BW gain in eight-week-old animals (Chamorro-García et al., 2013). Wild type and TBT-exposed animals fed HFD and administered with WQ showed a similar hepatosomatic (liver to BW) index of the animals compared to controls (Figure 3B). Otherwise, mice fed with WB and Ch showed higher liver/BW ratios. It is important to point out here that these variations were associated with changes in the liver weight for WB (1.30 ± 0.24 g), whereas in those administered with Ch (1.09 ± 0.13 g) and WQ (1.06 ± 0.05 g) it remained unaltered. As shown, iron deficiency favored higher values of the hepatosomatic index in all groups of treatment (Figure 3C), without causing differences in liver weight. In agreement with the TBT-derived obesogenic effects,

animals coming from pregnancy females exposed to TBT displayed elevated triglycerides (TGs) levels (Figure 3D) that support alterations in hepatic lipid homeostasis. Plasmatic TGs levels in TBT-exposed animals ranged between 11.1 ± 6.6 and 45.8 ± 16.4%; higher than their respective counterparts. To further evaluate hepatic response(s), we quantified the relative variation of hepatic transcripts (mRNA) for lipogenic (i.e., FASN and SREBP1a) as well as coronary risk (i.e., ALOX15 and PTGS2) markers (Figure 3E–H). Rt-qPCR analyses of hepatic tissue revealed opposite patterns in the expression of FASN as a function of the treatment and feeding. In perinatal TBT-exposed mice, feeding WQ up-regulated FASN gene expression, whereas animals fed with WB and Ch exhibited down-regulation on this parameter. Data for SREBP1a showed similar trends for both models with significant higher expression levels in animals fed WQ. When considering the effects in the expression of ALOX15 and PTGS2, those were sharply increased in animals fed with WQ and Ch, but only slightly affected in those receiving WB. In this regard, it can be hypothesized that the administration of bread formulations containing *C. quinoa* or *S. hispanica* L. flour could help controlling the HFD/TBT-induced disturbances in lipid homeostasis. However, administration of WB seems to impair the physiological control of these biomarkers mostly in TBT-exposed animals.

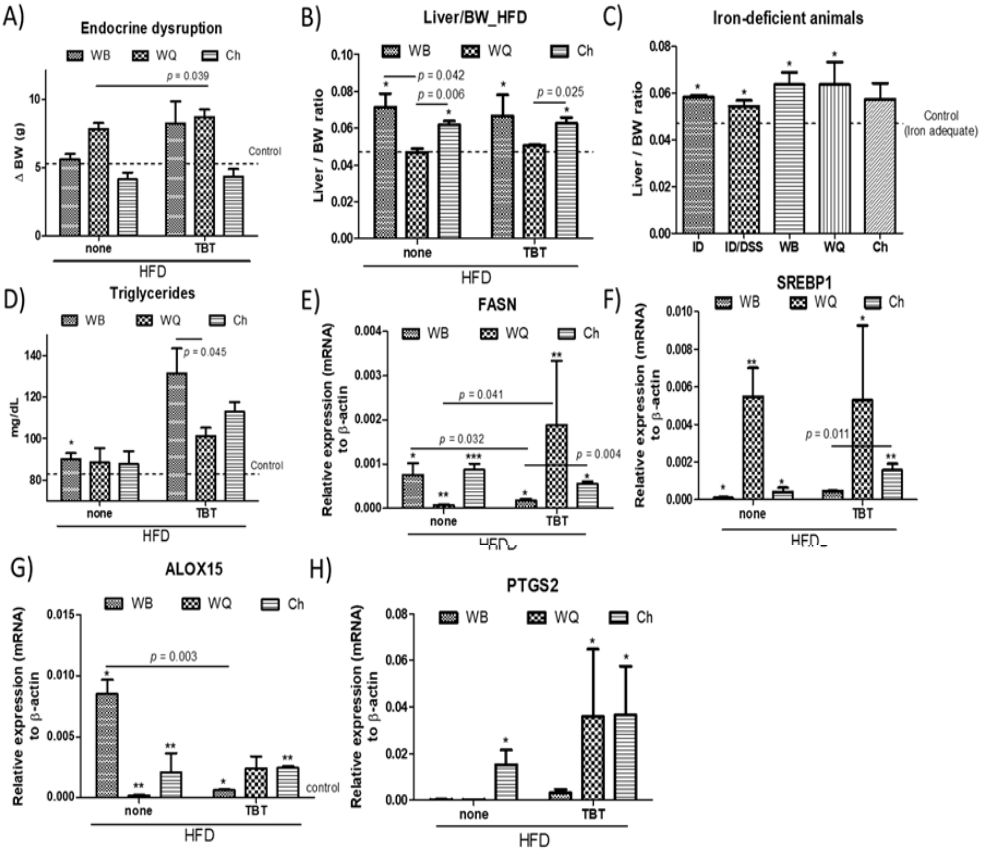

Figure 3. Effects of bread formulations-WB, wheat bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread-on body weight (BW) gain (**A**), changes in the hepatosomatic index (Liver/BW ratio) in wild type and tributyltin-exposed mice (**B**) as well as iron deficient mice (**C**), plasmatic triglyceride concentrations (**D**), and transcript levels (mRNA) of lipogenic markers-fatty acid synthase (FASN) (**E**) and sterol regulatory element-binding protein 1 (SREBP1a) (**F**), and coronary risk markers-arachidonate 15-lipoxygenase (ALOX15) (**G**) and prostaglandin-endoperoxide synthase (PTGS2) (**H**). Results are expressed as mean ± mean standard error (n = 3–6). Untreated controls are represented by the dotted line. *, **, *** indicates statistical differences between animals put under the same experimental model.

Carbohydrates and polyunsaturated fatty acids (PUFA) are identified as positive (Sul, Latasa, Moon, & Kim, 2000) and negative (Teran-Garcia et al., 2007), respectively, regulators of FASN transcripts. This dietary regulation fails to explain the changes observed in the FASN mRNA levels, and the apparent decrease of lipogenic markers in animals fed with WQ. TBT exposure raises the possibility of TBT-mediated alterations in molecules regulating glucose levels as well as fatty acid breakdown, for example, decreasing adiponectin (Li et al., 2017; Liu et al.., 2012). FASN activity is required to promote cholesterol synthesis to facilitate TLR4 signal transduction and proinflammatory macrophage activation (Carroll et al., 2018). One of the most well-established transcription factors affecting FASN is SREBP; SREBP1 promotes fatty acid synthesis, while SREBP2 is more specific to cholesterol synthesis (Fhu, & Ali, 2020). SREBP1 amplifies autophagy (Hu et al., 2019) that can regulate the hepatocellular lipid accumulation by its selective degradation. Accordingly, increased ALOX15 expression levels in animals fed WQ and Ch may suggest that mediators such as lipoxin, resolvin, and protectin, among other metabolites, could contribute to ameliorate hepatic inflammation and insulin resistance (López-Vicario et al., 2016). Furthermore, PTGS2 expression levels support protective effects against diet-induced steatosis by increased transcripts of hepatic cyclooxygenase (COX)-2 expression (Chan, Liao, & Hsieh, 2019). Collectively, these data suggest a potential increase of phagocytic conditions in animals fed with the different bread formulations (i.e., WQ

>> Ch > WB) that may interpret the results from animals fed with WQ and Ch as a better control of the lipid accumulation.

Control of Alterations in Glucose Homeostasis

Hepatic lipogenesis results from insulin stimulation via lipogenic gene expression. To ascertain the possible association between changes in lipid homeostasis and alterations in insulin resistance, we measured plasmatic glucose concentrations and insulin levels to determine the HOMA-IR (Figure 4). This approach allowed to observe significant HOMA-IR increases in the HFD-groups administered with WB and Ch in relation to controls (Figure 4A). Feeding WQ and Ch products significantly reduced HOMA-IR relative to WB in perinatal TBT-exposed animals on a HFD. The effect on HOMA-IR values was similar, while inducing different trends in hyperglycaemic animals (streptozotocin-induced) fed with WQ and Ch on a HFD (Selma-Gracia et al., 2020). Glucose concentrations in both preclinical models were equal when feeding all bread formulations, showing increased values in relation to the control mice (Figure 4B). However, only animals fed with WQ and Ch displayed increased insulin levels. After removing the TBT stress, the animals could recover glucose homeostasis control via insulin receptor signal and insulin levels (Li et al., 2017). Here, both WQ and Ch bread formulations enabled a better control on insulin production (WQ > Ch >> WB) in perinatal TBT-exposed animals (Figure 4C). When comparing the effects of *C. quinoa*- and *S. hispanica* L-containing bread formulations on the relative variations in insulin production to 'non-

treated' animals on the HFD, a similar reduction of insulin production in both hyperglycemic (streptozotocin-induced) (Selma-Gracia et al., 2020) and perinatal TBT-exposed animals was observed. Taken together, variations in HOMA-IR seem to occur in TBT-exposed animals as an apparently improved insulin sensitivity (Figure 4B,C), as downward insulin levels seem to suggest. This is also supported by the absence of differences in postprandial glycaemia despite the significantly reduced carbohydrate content in Ch formulation, as well as the negligible effect of dietary polyunsaturated fatty acids from Ch in comparison to animals fed with WQ. Thus, changes in the immunonutritional bioactive protein fraction (Selma-Gracia et al., 2020) seems to significantly take control on glucose homeostasis independently to the disease stage.

Because insulin resistance relates to intestinal epithelial TLR4 expression and activity (Lu et al., 2018), we examined whether the different bread formulations influenced glucose homeostasis in animals displaying a nutritional impairment of TLR4 signaling. Low iron levels selectively impair TLR4 signaling in macrophages through the TLR4/TRAM/TRIF pathway (Wang et al., 2009) earch efforts identified the TRIF-dependent TLR signaling as a preventive factor in hepatic inflammation and diet-induced lipid accumulation (Yang et al., 2017; Chen et al., 2017). Feeding with the different products, glucose concentrations in iron-deficient mice (ID, Haemoglobin, 11.1 ± 0.8 g/dL) were significantly reduced (Figure 4D): WB, 46%; WQ, 63% and Ch, 50% in relation to ID animals fed only with HFD, and WB, 52%; WQ,

68% and Ch, 58% to perinatal TBT-exposed mice. Animals receiving bread formulations exhibited significant lower BW values relative to those not receiving an additional feeding to the HFD, although this difference lacked statistical significance between the different products (Figure 4F). These observations transpire in the sense that dietary starch amount contributes but does not determine glycaemia, which seemed to depend, to a significant extent, on innate immune signals that stem at the intestinal level. Experimental data may allow to interpret a differential engagement of the TLR4/TRIF signaling by WQ and Ch (Figure 2), in line with a previous report (Srdić et al., 2020). Notably, the structural differences between bioactives provided by *C. quinoa* and *S. hispanica* L flours in comparison to wheat support their differential capacity to induce innate immunity via TLR4/MyD88-dependent signaling. This suggestion also aligns with the different FASN expression levels in TBT-exposed animals, as only TLR4/MyD88-dependent signaling impairs adiponectin signaling, contributing to insulin resistance (Benomar et al., 2016).

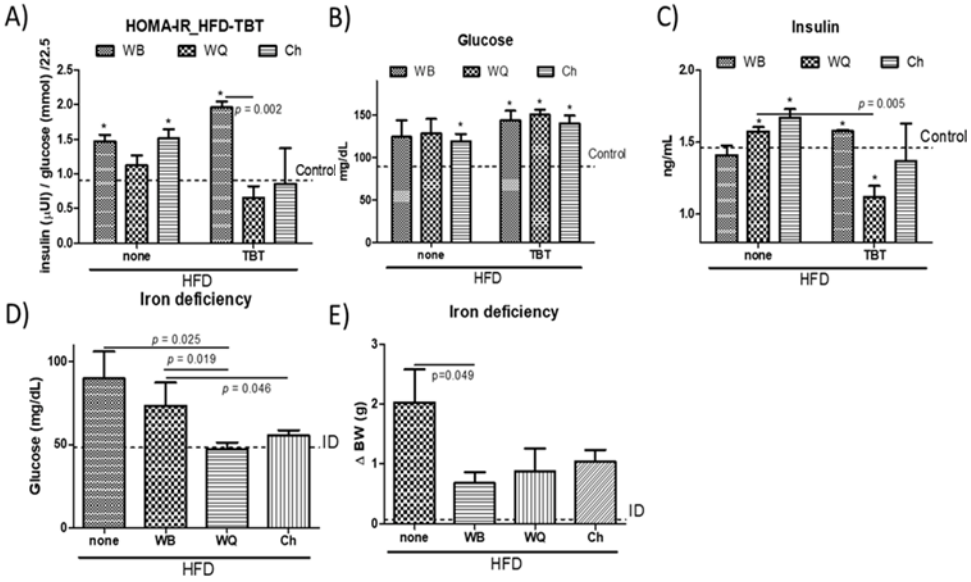

Figure 4. Biochemical parameters (HOMA-IR, **A**; glucose, **B** and insulin, **C**) in wild type and tributyltin (TBT)-exposed mice, glucose levels in iron-deficient (ID) (D) animals and body weight gain in ID animals (E), all fed with a high-fat diet and administered with different bread formulations: WB, wheat bread; WQ, white quinoa flour (25%)–containing bread and Ch, chia flour (20%)–containing bread. Results are expressed as mean ± mean standard error (n = 6). Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to controls.

Variations on Peripheral Immune Populations

The effects on immune cells and hepatocytes can modulate metabolism in T2D and obesity conditions and, reciprocally, the nutritional status influences immune homeostasis. The alterations in peripheral leukocyte populations in different animal groups are shown in Tables 2 and 3. Wild type and TBT-exposed mice that were fed a HFD did not show differences in the RBC or WBC of the animals. WB administration caused downward trends in peripheral MY cells of wild type animals but significant increases in GR percentages in TBT-exposed animals. These alterations were not observed in those mice who were feed with WQ or Ch formulations. In contrast, upward trends in the MY population were observed through feeding with these samples. The variations relative to controls calculated in peripheral MY and LY populations in mice that were fed with the different products are shown in Figure 5. In animals without perinatal TBT exposure (Figure 5A), opposite significant variations in the MY population after feeding WB or WQ and Ch formulations were observed. These variations only reached statistical significance between animals fed WB and WQ. Meanwhile, variations in the LY population were no longer observed. By contrast, in perinatal TBT-exposed animals, MY variations were narrowed, losing statistical significance between the different groups of treatment (Figure 5B). Otherwise, feeding with WQ and Ch caused significantly increased LY in relation to WB. We do not have direct evidence to explain the increases in the LY population. In this sense, a possible explanation

could be that the expansion of lymphoid cells and disturbances of hepatic glucose homeostasis may occur as sequential events derived from losses in metabolic control by myeloid cells. When considering iron-deficient mice, feeding both WQ and Ch products promoted clear increases in the MY population, which points out the significant influence of TLR4/TRIF signaling in these changes. However, innate immune signature in MY reached statistical significance only in animals fed with Ch, while changes in the LY population were not observed (Figure 5C).

Table 2. Total red blood cells (RBC) and leukocyte (WBC) counts, and lymphoid (LY), myeloid (MY), and granulocytes (GR) percentages (%) in peripheral blood of wild type animals and those perinatally-exposed to tributyltin. Wheat bread (WB), white quinoa bread (WQ) and chia bread (Ch). Results are expressed as mean ± standard deviation (n = 5–6). Different superscript letters (a–c) indicate statistical differences ($p < 0.05$).

Treatment		WB		WQ		Ch	
Parameter	**Control**	**None**	**TBT**	**None**	**TBT**	**None**	**TBT**
RBC (x10^9/L)	8.8 ± 0.5 ^{a}	8.5 ± 0.1 ^{a}	8.6 ± 0.4 ^{a}	8.7 ± 0.3 ^{a}	8.6 ± 1.3 ^{a}	7.6 ± 1.5 ^{a}	7.4 ± 1.6 ^{a}
WBC (x10^9/L)	4.1 ± 0.9 ^{a}	3.4 ± 1.1 ^{a}	7.0 ± 1.2 ^{b}	3.7 ± 1.1 ^{a}	5.3 ± 1.2 ^{a}	3.8 ± 1.1 ^{a}	4.9 ± 2.8 ^{a}
LY (%)	91.7 ± 5.3 ^{a}	92.5 ± 3.5 ^{a}	84.9 ± 5.1 ^{a}	89.8 ± 4.6 ^{a}	93.2 ± 2.9 ^{a}	93.2 ± 3.5 ^{a}	94.6 ± 1.6 ^{a}
MY (%)	3.3 ± 1.5 ^{a}	2.9 ± 1.1 ^{a}	3.1 ± 0.3 ^{a}	4.0 ± 0.8 ^{a}	3.4 ± 1.4 ^{a}	3.7 ± 1.3 ^{a}	4.5 ± 1.7 ^{a}
GR (%)	4.9 ± 1.8 ^{a}	2.9 ± 1.1 ^{a}	12.0 ± 4.8 ^{b}	2.4 ± 1.9 ^{a}	3.4 ± 1.6 ^{a}	3.3 ± 1.6 ^{a}	1.1 ± 0.7 ^{c}

Table 3. Total red blood cells (RBC) and leukocyte (WBC) counts, and lymphoid (LY), myeloid (MY), and granulocytes (GR) percentages (%) in peripheral blood of iron-deficient mice. Wheat bread (WB), white quinoa bread (WQ) and chia bread (Ch).

Treatment		WB	WQ	Ch
Parameter	**Control**			
RBC (x10^9/L)	6.6 ± 0.8 ^{a}	7.3 ± 0.8 ^{a}	6.5 ± 0.9 ^{a}	6.5 ± 0.7 ^{a}
WBC (x10^9/L)	2.3 ± 0.6 ^{a}	3.1 ± 1.1 ^{a}	3.4 ± 1.3 ^{a}	3.6 ± 1.8 ^{a}
LY (%)	95.9 ± 1.4 ^{b}	93.5 ± 1.4 ^{ab}	93.7 ± 3.8 ^{ab}	90.8 ± 7.2 ^{a}
MY (%)	3.8 ± 0.1 ^{a}	3.3 ± 0.7 ^{a}	4.0 ± 0.9 ^{a}	4.0 ± 0.2 ^{a}
GR (%)	2.9 ± 0.6 ^{ab}	2.8 ± 1.5 ^{ab}	1.5 ± 0.8 ^{a}	3.8 ± 2.2 ^{b}

Results are expressed as mean ± standard deviation (n = 5–6). Different superscript letters (a–c) indicate statistical differences ($p < 0.05$).

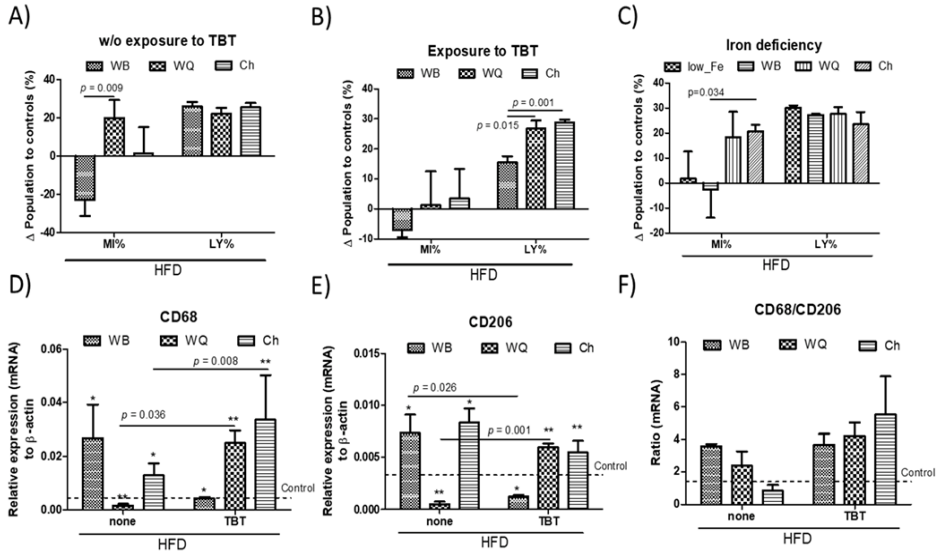

Figure 5. Peripheral relative variations of myeloid (MY) and lymphoid (LY) populations in wild type (**A**) and tributyltin (TBT)-exposed mice (**B**) as well as in iron-deficient (**C**) animals. Hepatic rt-qPCR analysis (mRNA) of selected macrophage marker genes; CD68 (**D**), CD206 (**E**) and their relative variation (**F**). Animals were fed with different bread formulations-WB, wheat bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread. Results are expressed as mean ± mean standard error (n = 6). Untreated controls are represented by the dotted line. *, ** indicates statistical differences between animals put under the same experimental model.

Differences in immune stimulation can promote the modulation of cell-mediated immunity within the gut-liver axis, modulating the proportion of immune cells infiltrating into liver, and thereby HFD-induced insulin resistance. Hence, rt-qPCR analysis allowed to evaluate

the relative mRNA levels of selected macrophage marker genes (Figure 5D–F). Since HFD-induced insulin resistance is commonly accompanied with chronic inflammation, the mRNA levels of CD68 (M1 phenotype) and CD206 (M2 phenotype) were measured (Figure 5D,E). In HFD-animals, the results show that feeding with WQ and Ch significantly down-regulated the CD68 gene expression in relation to WB. However, animals with perinatal TBT exposure displayed rather different results, showing a significant up-regulation of the CD68 transcripts in animals fed with WQ and Ch. In contrast, WB significantly decreased those transcripts in relation to their counterparts without TBT exposure. By comparison, CD206 transcripts exhibited similar trends but at a lower level than CD68. Experimental data show that both WB and Ch products up-regulate hepatic CD68 and CD206 mRNA levels, respectively, indicating that feeding does not block macrophage infiltration. These results allowed to calculate relative variations between macrophage's M1/M2 phenotypes higher than those in controls (Figure 5F), which were not observed in those animals fed with Ch without perinatal TBT exposure.

Myeloid cells (i.e., monocytes and macrophages) dominantly express the TLR4 receptor in relation to lymphoid (i.e., ILCs and Th cells) populations. Taken together, data transpire in the sense of a peripheral myeloid trafficking/polarization as a key cell-mediated coordination process between hepatic metabolic lipid alterations and long-term, adaptive immune responses. This is in line with the generally accepted

alterations in the macrophage metabolism contributing to impair liver dysfunction (Oates et al., 2019). Increased myelopoiesis and M1 polarization may represent an integrated mechanism to control circulating nutritional fatty acids induced TLR4-mediated activation of macrophages (Shi et al., 2006) ending on and inhibited proliferation (Griffin et al., 2018) and malfunction in the lympho-myeloid populations (Liu et al., 2018). Furthermore, proliferation/survival and cytokine production of ILC2s, active contributors to diet-induced obesity (Sasaki et al., 2019), is suppressed by IFN-γ and, to a lesser extent, by IL-27, which expression in human macrophages occurs in an IFNα mediated fashion (Pirhonen, Sirén, Julkunen, & Matikainen, 2007). This could be an example of the important role of diet influencing reciprocal interaction between innate immunity and the metabolic system.

These data are consistent with previous studies demonstrating a role for innate lymphoid cells determining insulin secretion and glycaemia (Sasaki et al., 2019). For example, alterations in the myeloid cell populations have been identified as potentially relevant effectors in the metabolic control of glucose homeostasis. However, the underlying innate immune signaling allowing myeloid control of liver metabolism is unclear. Because WQ and Ch provide immunonutritional agonists are able to interact with the innate immune TLR4, intestinal TLR4 may act as a key trigger of the immunometabolic processes. Notably, the immunonutritionally-induced resistance to obesity in animals developing a transgenerational increased liver fat accumulation, in which insulin

resistance also occurs, was rescued by feeding with WQ and Ch but not with WB. These findings support that intestinal TLR4 signaling was likely important in the control of glycaemia and insulin resistance. Collectively, data evidenced a role for immunonutritional agonists from *C. quinoa* and *S. hispanica* in intestinal innate immunity in the control of glucose homeostasis.

CONCLUSIONS

In summary, the reciprocal contributions of lymphoid cells populations and lipid metabolism to immunonutritional outcomes of the products cannot be excluded. Indeed, lipid metabolism is important for the expansion and function of the lymphoid cell population, enhancing TLR4-mediated proinflammatory signaling (Kim et al., 2019). Fatty acid synthase- dependent MyD88 palmitoylation is necessary for TLR4-induced inflammation (Carroll et al., 2018); however, both WQ and Ch may interact with TLR4 triggering MyD88-independent signaling in myeloid cells (Srdić et al., 2020). Thereby, WQ and Ch products improved glucose homeostasis in comparison to WB, this is consistent with previous studies demonstrating that it associates with early increased peripheral myeloid cells population (Nishimura et al., 2009; De Furia et al., 2013). Collectively, it is likely that immunonutritional agonists may contribute to maintaining the function of hepatic myeloid populations [i.e., monocytes and macrophages], which act as critical

effectors determining the function of innate lymphoid cells (i.e., Tregs and ILCs) in the regulation of glucose homeostasis in mice on a HFD.

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Institutional Review Board Statement: Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of CSIC (Consejo Superior de Investigaciones Científicas) and the protocol was approved by its Ethics Committee (Proex 080/19).

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Capítulo 5: La incorporación de harina de *Chenopodium quinoa* y *Salvia hispanica* L. en formulaciones de pan contribuye al control glucémico mejorando el control inmunonutricional de la homeostasis del hierro

La incorporación de harina de *Chenopodium quinoa* y *Salvia hispanica* L. en formulaciones de pan contribuye al control glucémico mejorando el control inmunonutricional de la homeostasis del hierro

Las alteraciones en la homeostasis de la glucosa (i.e., diabetes tipo 2 – DT2) se han relacionado con patologías asociadas a deficiencias en micronutrientes, cuyo origen está asociado al desarrollo de un estado 'leve' de inflamación intestinal (Gauci, Hunter, Bruce, Davis, & Davis, 2017). Se desconocen los mecanismos moleculares concretos que subyacen en la respuesta metabólica a la deficiencia de hierro, si bien, se ha observado que la mayor expresión de genes implicados en los procesos de lipogénesis, así como una menor oxidación lipídica hepática, puede contribuir a la hiperglucemia en respuesta a la deficiencia de hierro en la dieta (Davis *et al*., 2012). Al igual que ocurre en la DT2, la anemia también es una manifestación común en pacientes con determinados estados de glucogenosis, principalmente Ia y Ib, aunque estas afecciones presentan una fisiopatología diferente al estado diabético (Wang *et al*., 2012). Además, la obesidad representa otra de las condiciones fisiopatológicas en que se favorecen los fenómenos de gluconeogénesis hepática y resistencia a insulina, estrechamente asociados con alteraciones en la homeostasis del hierro que agravan la enfermedad (Vecchi *et al*., 2014).

El mantenimiento de la homeostasis del hierro corporal requiere la coordinación de múltiples mecanismos reguladores del metabolismo del micronutriente. Así, la deficiencia en hierro agrava los procesos inflamatorios mediados por el receptor inmune innato tipo 'Toll' (TLR)-4 cuya expresión intestinal constituye uno de los factores críticos en el control de la resistencia a insulina (Lu *et al*., 2018). La activación de este receptor se ha observado que es inducido por estímulos inflamatorios cuya expresión está regulada principalmente por la vía de señalización TLR4/MyD88.

En términos de estudio de la biodisponibilidad del hierro, el sistema combinado de digestión gastrointestinal simulada con cultivos de la línea celular Caco-2 (Figura 1) ha demostrado ser útil en estudios de absorción del micronutriente a partir de matrices panarias (Sanz-Penella, Laparra, Sanz, & Haros, 2012). Este modelo *in vitro* muestra una alta correlación a los estudios con humanos a través de la formación de ferritina (Glahn, Lee, Yeung, Goldman, & Miller, 1998). Sin embargo, a pesar de las ventajas en relación con su simplicidad, reproducibilidad y economicidad en la absorción de hierro por las células Caco-2, no están integrados los procesos inflamatorios y el control de estos por el sistema de fagocitos mononucleares (SFM), compuesto por monocitos, macrófagos y sus células precursoras que desempeña un papel crucial en el mantenimiento de la homeostasis del hierro.

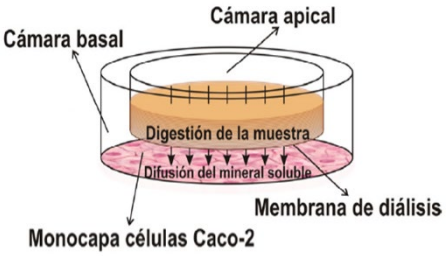

Figura 1: Esquema del sistema bicameral. En la parte apical se encuentra la muestra digerida y en la parte basolateral, separada por la membrana de diálisis, las células Caco-2 (Sanz Penella, 2012).

Pese a que existen una serie de diferencias fisiológicas en la absorción de hierro entre ratones con respecto a los humanos (ej. son capaces de sintetizar el ácido ascórbico), resultan un modelo válido para el estudio de las interacciones inmunonutricionales derivadas de los ingredientes alimentarios como los que aportan los productos panarios que incluyen quinoa y chía. En el presente estudio se utilizó un modelo preclínico nutricional que favorece la señalización del receptor TLR4 hacia su vertiente más inflamatoria (favoreciendo la vía Myd88 al inhibir selectivamente la vía TRAM/TRIF), repercutiendo en la señalización de la inmunidad innata y alterando la barrera epitelial intestinal (Wang *et al*., 2009) (Figura 2). Este modelo exhibe respuestas comparables de aquellos factores reguladores de la homeostasis del micronutriente, lo cual, nos permite estudiar su influencia en la respuesta sistémica en la homeostasis de glucosa (Selma-Gracia, Megušar, Haros, & Llopis, 2021). Así, se pretende valorar el potencial de los ingredientes

inmunonutricionales que contienen las formulaciones de pan en la homeostasis del hierro y el control de la glucosa ante una ingesta alta de energía en la dieta.

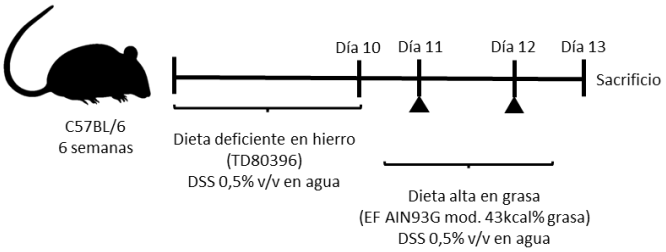

Figura 2. Modelo de deficiencia en hierro. Se utilizan ratones con una dieta deficiente en hierro durante 10 días en combinación con la administración de dextrano sulfato de sodio (DSS). Tras este periodo de tiempo, se cambia la dieta deficiente en hierro por una dieta alta en grasa sola o en combinación con las diferentes formulaciones panarias: pan de trigo, pan de quinoa blanca (25 %), pan de quinoa roja (25 %), pan de chía entera (20 %) y pan de chía semidesgrasada (20 %) durante dos días.

La influencia del reemplazo parcial de harina de trigo por la de quinoa y chía en los productos panarios en la biodisponibilidad de hierro se evaluó, en una primera aproximación, utilizando la línea celular Caco-2. En la figura 3A, se muestra la concentración de ferritina en células Caco-2 expuestas (18 h) a la fracción soluble derivada de la digestión gastrointestinal simulada de los productos panarios. La formación de ferritina en las células Caco-2 se incrementó entre un 4–14 % dónde se observa unos valores significativamente mayores en el caso del pan de

trigo comparado con los panes elaborados con harina de quinoa y chía entera. Curiosamente, el desgrasado de la harina de chía favoreció una mayor (>50 %) absorción del micronutriente comparado con su análogo sin desgrasar.

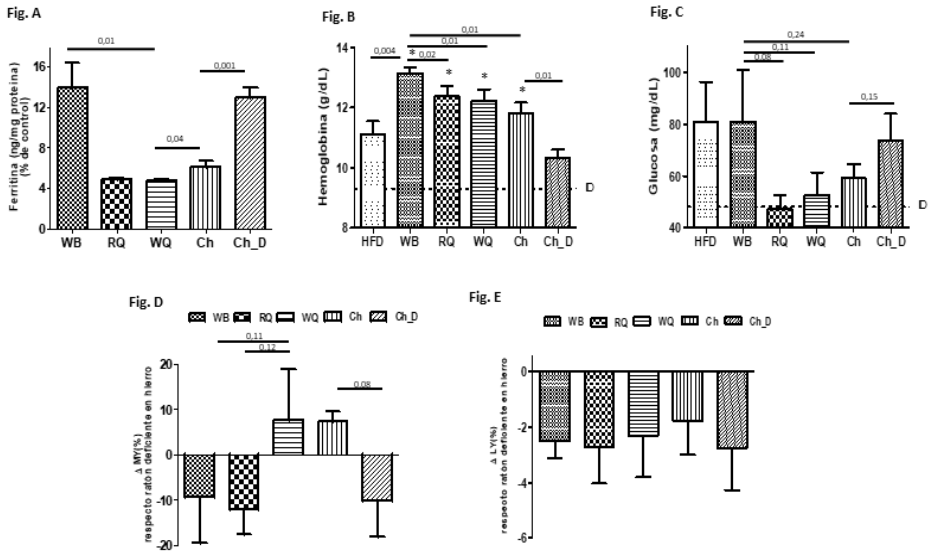

Figura 3. Contenido de ferritina en células Caco-2 expuestas a la fracción bioaccesible (A) obtenida de en panes de trigo (WB) quinoa roja (RQ), quinoa blanca (WQ), chía (Ch) y chía semidesgrasada (Ch_D). Efecto de la ingesta de estos panes en un modelo deficiente en hierro, alimentado con una dieta alta en grasa (HFD, 42%) sobre los niveles de hemoglobina (B); glucosa (C); poblaciones de células periféricas mieloides (MY) (D); linfoides (LY) (E). El grupo de ratones alimentados solo con una dieta deficiente en hierro (ID) está representado por la línea de puntos. Los resultados se muestran como media ± error estándar medio. *Indica diferencias estadísticamente significativas (p <0,05) en relación con el grupo ID.

En las figuras 3B–3E se muestran los valores fisiológicos cuantificados en el modelo animal. La formación de hemoglobina (Hb) (Figura 3B) aumentó en todos los grupos respecto al grupo deficiente en hierro (ID). Sin embargo, aquellos animales que solo recibieron la dieta hipercalórica, al igual que pan a base de harina de chía semidesgrasada, no resultaron significativamente mayores comparados con los ratones ID. Entre los grupos administrados con las distintas formulaciones panarias, al grupo que se le administró pan de trigo presentó valores de Hb significativamente elevados a los grupos de quinoa y chía (sin desgrasar).

Las diferencias en la absorción del micronutriente, a partir de las muestras de pan con quinoa y chía en relación con el pan de trigo, aparecen minimizadas en comparación con las detectadas en el modelo de células Caco-2 (Figura 3A-B). Es destacable que la biodisponibilidad del micronutriente desde los productos con harina de chía semidesgrasada se invierte con respecto a aquellos que incluyen harina no desgrasada.

Es importante señalar que solo aquellos animales administrados con los productos que incluyen la harina de quinoa y chía presentan concentraciones de glucosa sistémica significativamente inferiores a aquellos que reciben el pan de trigo y la dieta hipercalórica (Figura 3C). Al valorar la respuesta adaptativa del SFM, únicamente los grupos administrados con los productos de WQ y Ch (no desgrasada) presentan variaciones positivas en la proporción de células mieloides periféricas (Figura 3D). Estas diferencias pueden atribuirse, al menos en parte, a la

diferente señalización molecular asociada a los ingredientes inmunonutricionales (i.e., inhibidores de proteasas tipo serina: STPIs) con respecto al trigo (Junker *et al*., 2012; Srdić, Ovčina, Fotschki, Haros, & Llopis, 2020; Selma-Gracia *et al*., 2021). Esta diferente señalización molecular se ve reflejada en variaciones positivas en la población mieloide, donde la administración de estos panes es capaz de remontar el efecto inducido por la dieta grasa en la proliferación mieloide (Selma-Gracia *et al*., 2021). A su vez, este efecto nos hace presuponer una mayor utilización del hierro dietético.

Los STPIs derivados del trigo se han asociado a una señalización mediada por TLR4 que implica la molécula adaptadora MyDD88 y una mayor respuesta inflamatoria (Junker *et al*., 2012). Por otro lado, los componentes aportados por la harina de quinoa y chía interaccionan con el receptor promoviendo su señalización a través de la molécula TRIF, lo cual, supone una menor activación de los procesos inflamatorios (Srdić *et al*., 2020). Esto toma especial relevancia dado que la activación inducida por HFD de la vía TLR4/MyD88 conduce a una inhibición de la proliferación de macrófagos, la cual, cursa con la infiltración de macrófagos en el tejido adiposo agravando los procesos metabólicos (Griffin *et al*., 2018). En todos los casos se descartó la generación de procesos inmunogénicos de las células linfoides basándonos en las variaciones negativas de la población linfocitaria (Figura 3E).

En conjunto, los resultados evidencian la coordinación inmunonutricional determinando la absorción de nutrientes, así como el control de la homeostasis del micronutriente viéndose reflejado en un mejor control de la glucemia. Esto podría tener consecuencias importantes minimizando las consecuencias fisiopatológicas y severidad de la DT2, obesidad e incluso glucogenosis.

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IV

DISCUSIÓN GENERAL

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En el presente trabajo de Tesis Doctoral, la discusión general de los resultados sigue el orden establecido en el apartado de "*Objetivos*".

Objetivo 1) Estudio del almidón de quinoa a través de la determinación de las propiedades tecno-funcionales, efecto del tratamiento hidrotérmico y sus cinéticas de liberación de glucosa comparándolo con otros almidones comerciales. Evaluación de la incorporación de harina de chía en la liberación de glucosa y estudio de la respuesta celular en presencia de insulina.

El almidón de quinoa presenta diferencias en cuanto a sus características tecnológicas con respecto a los almidones de arroz y maíz, si bien, estas resultan bastante similares a las del almidón de trigo (Capítulo 1). Es destacable su pico de viscosidad (PV), el cual, se sitúa intermedio a los calculados para trigo y maíz, difiriendo su capacidad de absorción de agua significativamente de los mismos. Además, el almidón de quinoa presenta la menor Tª de gelatinización permitiendo estimar una baja cristalinidad y, por tanto, una menor compactación de su estructura. En conjunto, estas características favorecen una mayor digestibilidad del almidón de quinoa en relación con otros almidones (i.e., maíz, trigo, patata, arroz). Así, a partir de la hidrólisis enzimática del

mismo se pueden calcular parámetros como el área bajo la curva (AUC) y velocidad de hidrólisis, los cuales fueron mayores en comparación con el resto de almidones.

Por otro lado, el efecto del tratamiento térmico en el almidón de quinoa, en condiciones de pretratamiento (superiores a la Tª de inicio, pero inferiores a la Tª final de gelatinización, para obtener una gelatinización parcial), favorece la digestibilidad, así como la velocidad de hidrólisis del mismo. Sin embargo, cuando el almidón está completamente gelatinizado, aunque la digestibilidad aumenta, se consigue reducir significativamente la velocidad del proceso con respecto al pretratamiento, resultando similar al almidón crudo (el cual no se ha sometido a tratamiento térmico). Esta mayor reducción en la velocidad de hidrólisis del almidón comparado con el pretratamiento se podría asociar con una liberación ralentizada de insulina *in vivo* (Seal *et al*., 2003). En conjunto, el almidón de quinoa podría ser potencialmente beneficioso en el diseño de formulaciones más digeribles para pacientes con trastornos metabólicos que afectan a la homeostasis de la glucosa, entre otros, como la glucogenosis (tipo I o III).

El estudio más detallado de la bioaccesibilidad de glucosa desde el almidón de quinoa pone de manifiesto la similitud del coeficiente de difusión aparente (Dapp) calculado para el almidón de quinoa y maíz gelatinizados (Capítulo 2). Aquí es remarcable que el valor calculado para el almidón de quinoa (gelatinizado)

resulta similar al del maíz 'crudo', actual tratamiento nocturno para los pacientes de glucogenosis (Chen, Cornblath, & Sidbury, 1984), aportando una mayor cantidad de glucosa disponible para su absorción, principal problema al que hacen frente los pacientes de glucogenosis. Ello avala, con una aproximación fisiológica (Laparra *et al*., 2008), el potencial beneficio de la inclusión del almidón de quinoa en el tratamiento de estos pacientes.

La contribución de las semillas de chía desgrasada a la fibra dietética soluble a través de su contenido de mucílagos se valoró como una estrategia adicional para contribuir a ralentizar la liberación y absorción de glucosa desde los almidones. Sin embargo, la falta de diferencias significativas en la cantidad de glucosa extraída, así como en los Dapp calculados, no nos permite avalar un papel relevante del mucílago en la bioaccesibilidad de la glucosa desde el almidón de quinoa o maíz.

Hasta el momento no ha sido abordada la respuesta metabólica hepática frente a la fracción bioaccesible de glucosa obtenida del almidón de quinoa. El empleo de la línea celular HepG2 (ATCC, HB8965®) como modelo de parénquima hepático ha permitido, en una primera aproximación, valorar el efecto de la presencia de insulina, así como su posible interacción con los ingredientes inmunomoduladores proporcionados por la harina de chía en el estrés metabólico mitocondrial. La diferente bioaccesibilidad de la glucosa desde los almidones de quinoa y maíz no se presenta

como la responsable de las diferencias en la respuesta celular. Al añadir la harina de chía se obtuvieron efectos contrapuestos dependiendo de la presencia o no de insulina. Ello sugiere la presencia de componentes bioactivos en la chía que interfieren en la utilización de la glucosa y la señalización de la insulina. Desde una perspectiva molecular, hipotetizamos que los efectos observados podrían estar motivados por la contribución de la harina de chía con el aporte de inhibidores de proteasas tipo serina (STPIs) (Laparra, & Haros, 2019a), a través de la señalización del receptor inmune innato tipo 'Toll' (TLR)-4 (Srdić, Ovčina, Fotschki, Haros, & Llopis, 2020; Jia *et al*., 2014). En los estudios descartamos el posible efecto derivado de los ácidos grasos omega-3/6, tanto sobre el receptor TLR4 como en la sensibilidad a insulina, al utilizar chía desgrasada (Liu *et al*., 2013). En conjunto, se intenta aproximar el riesgo de desarrollar 'resistencia a insulina' por los pacientes de glucogenosis tipo I debido al fallo mitocondrial hepático causado por las alteraciones del metabolismo de carbohidratos (Rossi *et al*., 2018). La mezcla de almidón de quinoa con harina de chía podría ser potencialmente beneficiosa en el control y/o prevención de los trastornos metabólicos en pacientes de glucogenosis tipo I así como también en la diabetes o la obesidad (Takamura *et al*., 2008).

Objetivo 2) Estudio de los efectos derivados de la administración de formulaciones panarias con harina de quinoa y chía en los desbalances metabólicos causados por el consumo de dietas hipercalóricas.

2.1. Estudio de la respuesta hepática de carácter inmunonutricional en el control de la glucemia en animales prediabéticos.

Las distintas formulaciones utilizadas de los productos panarios, dado su diferente contenido en almidón y fracción lipídica, permiten inferir la potencial implicación individual de estas fracciones alimentarias, así como de la actividad de su fracción proteica en los efectos observados.

La utilización de animales prediabéticos (baja dosis, 25 mg/kg, de estreptozotocina) (Laparra, Díez-Municio, Moreno, & Herrero, 2015) ha permitido evaluar las consecuencias metabólicas del consumo de una dieta alta en grasa por animales hiperglucémicos para detectar los posibles beneficios de la reformulación de los productos panarios en el control de esta afección (Capítulo 3). Hay que señalar que el periodo de intervención (3 semanas) está dirigido a las etapas tempranas (2–18 semanas) responsables de la aparición y progresión de las posteriores complicaciones crónicas que ocurren en la DT2 (Barrière *et al*., 2018).

El reemplazo parcial de la harina de trigo por la de variedades (i.e., roja y blanca) de quinoa, así como de chía tanto entera como desgrasada ejerce efectos positivos, reduciendo la gravedad de las alteraciones metabólicas derivadas del consumo de una dieta alta en grasa por animales hiperglucémicos. Estos efectos se ven reflejados en una reducción significativa del índice HOMAir (siglas en inglés del modelo homeostático para evaluar la resistencia a la insulina) y, por tanto, de la resistencia a insulina. Al comparar el efecto de la administración del producto con quinoa, roja y blanca, con el derivado del producto con chía se observa que el contenido de almidón, no resulta un factor determinante en la resistencia a la insulina. Por otro lado, al considerar el bajo grado de peroxidación lipídica hepática cuantificado en los animales administrados con el producto de chía, podría sugerir la importancia de los omega-3/6 en el control de estos. Sin embargo, la similitud del grado de peroxidación lipídica hepática en el grupo administrado con quinoa roja con el de chía hace que se replantee esta posibilidad. Ambas formulaciones, desde un punto de vista inmunonutricional, favorecen un mejor perfil en la producción de citoquinas hepáticas implicadas en el control de la masa grasa corporal (i.e., IL-6) (Wallenius *et al*., 2002) e inhibición de la β-oxidación de ácidos grasos (i.e., IL-17) (Shen *et al*., 2017). Estos efectos pueden asociarse con la mayor contribución de la chía en cuanto a

agonistas inmunonutricionales del receptor TLR4 como los STPIs. Es conocido el papel que desempeña este receptor en el desarrollo de resistencia a la insulina (Lu *et al*., 2018), así como en la inducción de enzimas implicados en procesos lipogénicos tanto en modelos múridos como en humanos (Hong *et al*., 2020; Todoric *et al*., 2020).

2.2. Estudio del control de la glucemia en animales con predisposición transgeneracional al acúmulo lipídico hepático y con deficiencia nutricional en hierro.

La utilización de un modelo preclínico de ratones hembra no gestantes expuestas al obesógeno tributilo de estaño ha permitido valorar los efectos de los productos panarios en animales que adquieren una herencia transgeneracional con alteraciones en la homeostasis de la glucosa y predisposición a la acumulación lipídica hepática (Capítulo 4) (Chamorro-García *et al*., 2013). Existe evidencia de la relevancia de la nutrición prenatal y posnatal, aumentando o reduciendo, el riesgo y severidad en el desarrollo de NAFLD (Lynch, Chan, & Drake, 2017).

El reemplazo parcial de la harina de trigo permitió modificar el potencial inmunonutricional de los productos panarios, derivado tanto de la actividad como cantidad en el aporte de STPIs presentes en la fracción de globulinas 2–11S, conforme a la siguiente gradación: pan de trigo, 2,21 mg/g ~ pan con harina de

quinoa, 1,74 mg/g < pan con harina de chía, 8,19 mg/g. La administración de estos productos resultó efectiva en el control de la expresión de biomarcadores lipogénicos (i.e., FASN, SREBP1a) y de riesgo coronario (i.e., ALOX15, PTGS2) en el hígado. Al comparar los efectos derivados de las formulaciones con quinoa blanca y chía, es evidente el mayor efecto de la formulación de quinoa, lo cual, apunta directamente a diferencias estructurales entre los ingredientes inmunonutricionales que condicionarían su interacción con TLR4 (Srdić *et al*., 2020). Sin embargo, a pesar de las diferencias en los biomarcadores hepáticos, tanto la quinoa como la chía se asociaron con un mejor control de HOMAir. A su vez, esta reducción en el índice HOMAir parece relacionarse con una variación positiva de la población mieloide en sangre periférica, a diferencia del trigo que obtiene una variación negativa respecto a ratones controles sin tratar.

La utilización de un modelo preclínico de deficiencia nutricional en hierro ha permitido aproximar, desde un punto de vista molecular, la influencia de los productos panarios en las vías de señalización de los procesos inflamatorios subyacentes al receptor TLR4. TLR4 tiene dos vías distintas de señalización: la que requiere la proteína adaptadora MyD88, y la MyD88-independiente, la vía TRIF (Chen *et al*., 2017). Hasta el momento de redacción de esta Tesis, no se ha estudiado un potencial mecanismo de acción de los agonistas inmunonutricionales de la

quinoa y chía utilizando matrices alimentarias reales para su aporte. Los resultados experimentales derivados de los estudios llevados a cabo han permitido apuntar a la activación, por los productos con harina de quinoa y chía, de la señalización de TLR4 a través de la vía TRIF, la cual, aparece como un factor preventivo en la inflamación hepática y la acumulación de lípidos inducida por la dieta (Chen *et al*., 2017). Los efectos positivos de estos productos con respecto al de trigo avalan la señalización de los agonistas inmunonutricionales proporcionados por el trigo a través de la molécula adaptadora MyD88 agravando los procesos inflamatorios. Esto remarca los potenciales beneficios de la inclusión de harinas de quinoa y chía en productos panarios en la prevención de procesos inmunometabólicos, por ejemplo, como la malabsorción y la inflamación. Estos efectos se producen tanto en el ámbito intestinal como hepático durante el desarrollo de NAFLD favoreciendo la deficiencia nutricional en hierro.

Ante una deficiencia de este micronutriente, el control de la homeostasis del hierro es un factor clave para no agravar las patologías metabólicas. Se observaron que las formulaciones panarias aumentaron los niveles de hemoglobina, excepto en el grupo de chía semidesgrasada, respecto a los ratones alimentados solo con dieta alta en grasa (Capítulo 5). Sin embargo, a pesar del incremento de hemoglobina, solo la quinoa y la chía (sin desgrasar) mostraron niveles significativamente inferiores de

glucemia comparada con el grupo de dieta alta en grasa. Estos valores fueron acompañados de una tendencia positiva en las células mieloides respecto al grupo deficiente en hierro, a diferencia del pan de trigo. En el caso del pan de chía con harina semidesgrasada, siguió la misma variación negativa que el trigo, resultando en valores similares en la glucemia. Por tanto, la conservación de una tendencia positiva en la proporción de las mieloides sugiere un mejor control de la homeostasis de la glucosa.

V

CONCLUSIONES

V CONCLUSIONES

Tras la realización de la presente investigación, las conclusiones alcanzadas fueron las siguientes:

1) El almidón de quinoa presenta un comportamiento distinto a otros almidones de cereales comunes como el trigo o el maíz, siendo mayor su hidrólisis y susceptibilidad a la gelatinización en tratamientos hidrotérmicos.
2) La harina de chía no afecta la cinética de la liberación de glucosa desde el almidón de quinoa, si bien, favorece un mayor control en la respuesta metabólica y susceptibilidad a insulina de los cultivos celulares de hepatocitos.
3) El reemplazo parcial de la harina de trigo por la harina de quinoa o chía, entera o semidesgrasada, favorece a un mejor control de las alteraciones metabólicas relativas a la homeostasis de glucosa, así como la severidad de las mismas, derivadas del consumo de una dieta hipercalórica en animales prediabéticos. En esta mejora en los desbalances metabólicos, toma importancia el aporte de componentes inmunonutricionales frente al contenido en almidón y aporte de ácidos grasos poliinsaturados. Sin embargo, a efectos de marcadores inflamatorios y hepáticos, se desarrollan en menor magnitud en el alimento

que contiene chía desgrasada en relación con su homólogo con la harina entera.

4) Los efectos de la inclusión de harina de quinoa o chía en las formulaciones panarias sobre el control de la homeostasis de la glucosa parecen asociadas a variaciones positivas de la población mieloide en el torrente circulatorio periférico.
5) El potencial inmunonutricional de la harina de quinoa blanca y chía entera con respecto a la de trigo contribuye al control glucémico mejorando la homeostasis del hierro. Este mejor control de la quinoa y chía se asocia con variaciones positivas en la proporción de células mieloides en el torrente circulatorio periférico.

VI

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VII

ANEXO

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Potential beneficial effect of hydrothermal treatment of starches from various sources on *in vitro* digestion

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Quinoa starch

ABSTRACT

Starches from various botanic origins (maize, quinoa, wheat, potato and rice) were studied. The thermal and pasting properties and their connection with enzyme digestibility were evaluated. Various hydrothermal treatments were applied, taking the starch physical parameters into account, in order to obtain partial and total gelatinisation of the starch structure and determine its influence on enzymatic action. Onset and pasting temperatures of the gelatinisation and pasting processes, respectively, followed the same order in the cereal starches (rice > maize > wheat > quinoa). These results were accompanied by an opposite trend in the percentage of raw starch hydrolysis, with quinoa reaching a level more than 2-fold higher than that of raw maize starch in *in vitro* digestion kinetics. Other technological parameters, such as high peak viscosity or low breakdown, also reflected modifications in the quinoa starch structure which were related to improved digestibility. However, starch from potato, the only tuber, displayed different characteristics from those of cereal starch, showing greater resistance to digestion. When the starches were pretreated, digestibility increased in all of them compared to their raw counterparts, with the pretreated quinoa and wheat starches showing greater susceptibility to modification of their structure. Although the hydrothermally pretreated maize and rice starches reached about 75% of the hydrolysis index of the corresponding gelatinised starches, raw quinoa had a similar hydrolysis index and quinoa obtained a higher value for total starch hydrolysed. Thus, quinoa starch could be potentially beneficial in the design of more digestible formulations for patients with metabolic disorders such as glycogen storage disease, among others.

1. Introduction

In recent years, glucose homeostasis has been an important focus of research owing to its physiological involvement in metabolic diseases such as diabetes, obesity and glycogen storage disease (GSD) (Ludwig, 2002; Weinstein, Steuerwald, De Souza, & Derks, 2018). Consequently, several investigations have focused on studying the glycaemic index (GI) of foods and applying various strategies to modify starch digestibility and glucose release in order to manage glucose homeostasis and try to obtain optimal metabolic control (Laparra & Haros, 2018; Li, Gidley, & Dhital, 2019).

The degree of starch gelatinisation is an important determinant for the rate of starch hydrolysis *in vitro* and for the metabolic response *in vivo* (Holm, Lundquist, Björck, Eliasson, & Asp, 1988). Many food processing operations involve alteration of starch structure through thermal treatment, which leads to the starch becoming partially or completely gelatinised, depending on the final product (Delcour et al., 2010). The effects of thermal treatment on the morphological and crystalline structure of starch granules include important changes in physico-chemical properties (Ahmadi-Abhari, Woortman, Oudhuis, Hamer, & Loos, 2013). These changes in starch structure take place in pasting and gelatinisation processes, with swelling and gradual loss of crystallinity until there is total disruption of the starch granule (Horstmann, Lynch, & Arendt, 2017). The nature of these structural changes depends on the starch source, composition, structure and isolation process, and therefore every starch has a different digestibility (Haros, Blaszczak, Perez, Sadowska, & Rosell, 2006; Ratnayake & Jackson, 2007; Waigh, Gidley, Komanshek, & Donald, 2000). However, techno-functional parameters can provide information about the crystalline structure of starch and its digestibility (Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005).

The digestibility of starch is an important parameter that affects the

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severity and clinical manifestations of GSD and other diseases. GSD is a metabolic disorder that affects glycogen metabolism, in which the main clinical manifestation is fasting hypoglycaemia (Weinstein et al., 2018). Since 1984, ingestion of uncooked maize starch (raw) has been used to prevent a fall in glucose concentration overnight in individuals with type I or III GSD (Chen, Cornblath, & Sidbury, 1984). However, the relatively short duration of glucose availability from this dietary source still represents a major disadvantage with regard to the long-term outcome and quality of life of this special group. Also, raw maize starch intake is associated with injurious gastrointestinal symptoms such as abdominal cramps or bloating, which could be partly responsible for colonic fermentation of unused starch (Lee & Leonard, 1995). Some other starches (i.e., potato, rice, tapioca and arrowroot) have been tested in GSD patients, but these starches displayed significant differences, producing a worse glycaemic response than maize starch (Sidbury, Chen, & Roe, 1986). In recent years, controlled heat-moisture processing of a high-amylopectin-containing maize starch was shown to be effective in improving maintenance of glucose concentrations, while gastrointestinal symptoms were reduced (Correia et al., 2008). However, not everyone can afford modified starch and many people depend on alternatives that are cheaper and that are easily available. In this connection, the inclusion of "ancient grains" (such as amaranth, quinoa or chia) in cereal bread formulations has been shown in the *in vitro* test to have an effect in delaying glucose release while extending its absorption (Brennan, Menard, Roudaut, & Brennan, 2012; Laparra and Haros, 2018). Furthermore, these effects were accompanied by increased expression of the peroxisome proliferator-activated receptor (PPAR)-gamma, suggesting an improved insulin resistance that could lead to a significant decrease in glycolysis metabolism in an animal model (Laparra and Haros, 2018). Thus, starch from ancient grains could have a different digestibility that could help to maintain normoglycaemia longer than standard maize starch.

In view of the above, this study aimed to analyse thermal and pasting properties of starches from various sources – maize, wheat, potato, rice and quinoa – and evaluated the effect of a controlled heat-moisture process – which took their physical parameters into account – on their *in vitro* digestibility. The results were compared with those of the raw (as negative control) and gelatinised (as positive control) starches with the purpose of developing foods/beverages with specific characteristics for people with glucose metabolism disorders.

2. Materials and methods

2.1. Materials and reagents

Commercial maize starch was provided by ACH Food Companies (Argo, USA). Potato starch (C*Gel 300) was purchased from Cargill (Minneapolis, USA). Wheat starch (Natilor) from Chamtor (Pomacle, France). Rice starch (S7260) from Sigma-Aldrich, Belgium. Red quinoa starch was obtained from real Bolivian quinoa (Organic red Quinoa Real©, ANAPQUI (La Paz, Bolivia) in the laboratory by wet-milling (Ballester-Sánchez, Gil, Fernández-Espinar, & Haros, 2019). The amylose content of starches was determined using enzymatic assay kits and procedures outlined by Megazyme (Megazyme International Ireland Ltd., Wicklow, Ireland). Enzymes were purchased from Sigma-Aldrich: α-amylase (EC 3.2.1.1, A3176-1MU, USA, 16 U/mg), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, 10115, Switzerland, 60.1 U/mg) and pepsin (EC 3.4.23.1, P7000, UK, 480 U/mg).

2.2. Pasting properties

To prepare the samples, 3.5 g of starches were weighted and 25 mL of distilled water was added. Pasting properties of the starches were measured using a Rapid Visco Analyser (RVA-4, Newport Scientific, Warriewood, Australia), according to AACC method 76-21.01 (1999). Pasting temperature (P_{temp}), peak time (P_{time}), peak viscosity (PV), hot paste viscosity (HPV), cool paste viscosity (CPV), breakdown (PV-HPV) and setback (CPV-HPV) were recorded. The experiments were performed in triplicate.

2.3. Thermal properties

Gelatinisation and retrogradation properties were determined using differential scanning calorimetry (DSC) (PerkinElmer DSC-7, USA). Indium was used to calibrate the calorimeter (enthalpy of fusion 28.45 J/g, melting point 156.6 °C). The procedure followed was the method described by Haros et al. (2006), with slight modifications. Ten mg of starch was weighed out and distilled water was added to obtain a water: starch ratio of 3:1 for each sample. The calorimeter scan conditions used were: 25 °C for 1 min and then heating from 25 °C to 120 °C at 10 °C/min. Later, to analyse retrograded starch, the samples were stored in refrigeration for a week and were ran under the same conditions (1 min - 25 °C; from 25 to 120 °C at 10 °C/min). The parameters recorded were: onset temperature (T_o), peak temperature (T_p) conclusion temperature (T_c) and enthalpy of gelatinisation and retrogradation transition (ΔH_G and ΔH_R), respectively. The experiments were performed in triplicate.

2.4. Preparation of samples for digestion

Aliquots (100 mg) of the various starch samples were weighed into microcentrifuge tubes and 1 mL of water was added. Raw starches were kept in unheated water for 5 min and were considered the negative control. Pretreatment of the starches was chosen according to their pasting and thermal parameters: maize (70 °C–2 min), quinoa (60 °C–1 min), wheat (60 °C–1 min), potato (70 °C–1 min) and rice (75 °C–2 min). The temperature selected for pretreatment depended on the T_p and T_c of the starch and was such as to achieve partial gelatinisation while avoiding loss of total crystallisation. The P_{temp} and P_{time} values determined previously were taken into account to avoid the formation of paste. Gelatinised starches (GS) were kept in a water bath for 5 min at 100 °C as a positive control.

2.5. In vitro starch digestion and GI estimation

The rate of starch hydrolysis was evaluated according to the method described by Goñi, Garcia-Alonso, and Saura-Calixto (1997), with modifications. Briefly, 10 mL of HCl–KCl buffer (pH 1.5) and 400 μL of a solution of pepsin in HCl–KCl buffer (0.1 g/mL) were added to the starches and the samples were placed in a shaking water bath at 37 °C for 1 h. Afterwards, 19.6 mL of Tris-Maleate buffer (pH 6.9) and 1 mL of a solution containing α-amylase in Tris-Maleate buffer (0.01 g/mL) were added and the samples were incubated in the water bath for 2 h. Aliquots were taken at intervals Aliquots were taken at intervals, from 0 to 120 min (0, 20, 40, 60, 90,120 min), and then the enzyme was thermally inactivated during 5 min at 100 °C. After centrifugation (10,000 rpm/10 min), 500 μL of the supernatant was taken from each sample. Then 1.5 mL of sodium acetate buffer (pH 4.75) and 60 μL of a solution of amyloglucosidase in sodium acetate buffer (88 mg/mL) were added and the samples were incubated at 60 °C for 45 min. Glucose, area under the curve (AUC) and hydrolysis index (HI) were determined according to Laparra and Haros, 2018. Finally, GI was calculated using the equation $GI = 39.71 + 0.549HI$ (Zabidi & Aziz, 2009). The hydrolysis kinetics was transformed from a cumulative curve into a linear curve by plotting the reciprocal values of [% starch hydrolysis] and time (Sanz-Penella, Laparra, & Haros, 2014).

2.6. Statistical analysis

Multiple ANOVA and Fisher's least significant differences (LSD) were applied to establish statistically significant differences in thermal and pasting properties. The Tukey test was applied to analyse differences in the digestion values. The statistical analyses were performed with

Statgraphics Centurion XVl software, and the significance level was established at $P < 0.05$.

3. Results and discussion

3.1. Pasting and thermal properties of starches

The determination of pasting parameters revealed differences between the starches, as was expected (Table 1). P_{temp} provides an indication of the minimum temperature required to cook the starch, which could be related to the degree of polymerisation (DP) of amylopectin (Li & Zhu, 2017; Srichuwong et al., 2017; Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005). This parameter decreased following this order: rice > maize > potato ~ wheat > quinoa. Quinoa and wheat are characterised by a higher proportion of short chains with a DP of 8–12, whereas maize, rice and potato have a high DP of 12–18 (Srichuwong et al., 2005, 2017). The higher proportion of shorter amylopectin chains could affect the crystalline structure (Srichuwong et al., 2017), resulting in a soluble molecule that can be easily digested as it has many end points onto which digestive enzymes can attach, which could have a positive effect on the digestibility of raw starches.

The peak viscosity (PV) parameter indicates the water-binding capacity of starch (Haros et al., 2006). The high PV of potato could possibly be explained, at least partly, by the high content of phosphate ester groups in the amylopectin in this tuber, resulting in repulsion between molecules (Waterschoot, Gomand, Fierens, & Delcour, 2015). Among the cereals, higher PV values were recorded for wheat and quinoa than for maize and rice. These results agree with the conclusions arrived at by Gomand, Lamberts, Visser, and Delcour (2010), who attributed an increase in swelling to short amylopectin chains, whereas long chains prevented this transition. The high viscosity value obtained during the heating process suggests a high water absorption capacity, which has been correlated with a lower resistance to enzymatic digestion (Reddy, Pramila, & Haripriya, 2015). This behaviour could be interesting when formulating foods with specific glycaemic indexes. The breakdown parameter (PV–HPV) can give information about stability under heating conditions. Potato starch showed a very high value, displaying a structural fragility that could lead to easier destruction of the structure when it is cooked (Haros et al., 2006). Notably, the rice and quinoa starches exhibited a lower breakdown value than maize starch, which suggests a better preserved structure, favouring a lower peak glucose concentration and a slower rate of fall than with conventional maize starch.

During cooling, an important parameter to consider is retrogradation, which is the tendency to restructuration and can be measured through the setback parameter (CPV–HPV). Wheat and potato showed the highest setback viscosities, indicating a low resistance to retrogradation and, as a result, a higher rearrangement. The formation of double helices in this rapid process of restructuration is mainly attributed to amylose, which possesses a larger flexible structure than amylopectin (Van Soest, de Wit, Tournois, & Vliegenthart, 1994). However, the lack of differences in the setback values of the maize and quinoa starches, despite the amylose content determined for maize (amylose 22%) and quinoa (amylose 7%) (data not shown), suggests that other starch characteristics are involved in the retrogradation process.

The gelatinisation parameters were determined by DSC analysis (Table 2). Onset temperature (T_o) showed the same trend as P_{temp}: rice > maize > potato > wheat > quinoa, as was expected. This relationship between gelatinisation and pasting temperatures was also confirmed previously by other researchers (Li, Wang, & Zhu, 2016). Low values in starch gelatinisation and pasting processes might suggest a less crystalline structure, which could result in higher enzymatic susceptibility (Lin, Zhang, Zhang, & Wei, 2017; Srichuwong et al., 2017). The gelatinisation enthalpy (ΔH_G) varied from 10 to 12 J/g, except in the case of potato, which had a value of 16 J/g, demonstrating that higher energy was required to disrupt the crystalline structure. The resistance

Table 1
Pasting properties of starch from various sources.

Parameter	Units	Starch				
		Maize	Quinoa	Wheat	Potato	Rice
P_{temp}	°C	75.5 ± 0.5^{c}	66.5 ± 2.1^{a}	69.3 ± 0.0^{b}	69.4 ± 0.0^{b}	82.0 ± 0.6^{d}
P_{time}	min	5.1 ± 0.0^{b}	7.0 ± 0.0^{e}	6.1 ± 0.0^{c}	3.2 ± 0.1^{a}	6.6 ± 0.1^{d}
PV	cP	2906 ± 23^{b}	3380 ± 384^{c}	4607 ± 12^{d}	9751 ± 40^{e}	2385 ± 30^{a}
HPV	cP	1852 ± 23^{ab}	2653 ± 447^{d}	3321 ± 105^{e}	1552 ± 71^{a}	2111 ± 30^{c}
CPV	cP	3089 ± 18^{ab}	3867 ± 474^{c}	5371 ± 87^{d}	3475 ± 49^{bc}	2830 ± 30^{a}
Breakdown	cP	1054 ± 45^{c}	728 ± 63^{b}	1287 ± 93^{d}	8200 ± 111^{e}	274 ± 1^{a}
Setback	cP	1237 ± 41^{b}	1215 ± 28^{b}	2051 ± 18^{c}	1924 ± 121^{c}	720 ± 1^{a}

P_{temp}: Pasting temperature, P_{time}: Peak time, PV: Peak viscosity, HPV: Hot paste viscosity, CPV: Cool paste viscosity, Breakdown: PV-HPV, Setback: CPV-HPV, cP: centipoises.
Mean ± standard deviation, $n = 3$. Values in the same row followed by the same letter are not significantly different ($P < 0.05$).

Table 2
Thermal properties of starch from various sources.

Parameter	Units	Starch				
		Maize	Quinoa	Wheat	Potato	Rice
Gelatinisation						
Onset temperature, T_o	°C	64.9 ± 0.0^{d}	50.7 ± 2.3^{a}	55.1 ± 0.2^{b}	60.8 ± 0.2^{c}	69.7 ± 0.7^{e}
Peak temperature, T_p	°C	69.6 ± 0.1^{c}	58.7 ± 1.6^{a}	60.1 ± 0.1^{a}	65.3 ± 1.1^{b}	76.4 ± 0.5^{d}
Conclusion temperature, T_c	°C	76.0 ± 0.1^{d}	69.3 ± 1.4^{b}	66.5 ± 1.1^{a}	73.4 ± 0.5^{c}	83.1 ± 0.8^{e}
Enthalpy of gelatinisation, ΔH_G	J/g	12.5 ± 0.1^{b}	10.4 ± 0.6^{a}	10.2 ± 0.6^{a}	16.6 ± 1.3^{c}	12.5 ± 0.2^{b}
Retrogradation						
Onset temperature, T_o	°C	44.3 ± 0.1^{c}	36.9 ± 1.0^{a}	43.7 ± 1.0^{c}	44.6 ± 1.2^{c}	41.2 ± 0.1^{b}
Peak temperature, T_p	°C	54.1 ± 1.5^{b}	46.5 ± 0.2^{a}	52.4 ± 1.5^{b}	60.8 ± 0.1^{c}	52.2 ± 2.1^{b}
Conclusion temperature, T_c	°C	63.2 ± 0.8^{bc}	56.3 ± 0.5^{a}	61.3 ± 0.8^{b}	71.5 ± 0.3^{d}	64.4 ± 2.0^{c}
Enthalpy of retrogradation, ΔH_R	J/g	3.5 ± 0.7^{ab}	1.6 ± 0.3^{a}	2.1 ± 0.5^{a}	5.8 ± 0.0^{b}	6.0 ± 2.2^{b}

Mean ± standard deviation, $n = 3$. Values in the same row followed by the same letter are not significantly different ($P < 0.05$).

produced by potato may be interpreted as high crystallinity, which could interfere with the accessibility of the enzyme (Shi, Gao, & Liu, 2018). Retrogradation parameters were measured after 7 days at 4 °C, and quinoa starch presented the highest resistance to retrogradation of amylopectin (Table 2). In long-term retrogradation, amylopectin is mainly responsible for reorganisation of structure (Van Soest et al., 1994). The presence of short chains in quinoa might contribute to a less compacted starch structure, leading to a starch with low retrogradation, which could be displayed as better digestibility (Lin et al., 2017; Srichuwong et al., 2017). On the other hand, the consumption of retrograded starches may be beneficial for health, owing to the lower depletion of total digestible starch than gelatinised starch (Chung, Lim, & Lim, 2006). Moreover, rearrangement of the crystalline structure could hinder α-amylase action and trigger a slow rate of intestinal digestion, which could be reflected in a lower glucose concentration peak *in vivo*.

3.2. In vitro starch digestion and GI estimation

In this study, the hydrolysis percentages of various common starches were compared with that of raw maize starch (Table 3). The digestion method used has been proved suitable for establishing variations in susceptibility to enzyme interaction depending on structural differences between the samples considered (Rosin, Lajolo, & Menezes, 2002; Sanz-Penella et al., 2014).

Significant differences ($p < 0.05$) were found in the total (%) starch hydrolysed (TSH_{120}) as a function of the sample considered. Native potato presented the lowest value compared to any other raw starch studied. This is supported by previous studies, which indicate that the lack of peripheral channels in potato starch granules inhibits the penetration of α-amylase, whereas the presence of superficial pores, such as in maize starch, could enable enzymatic action (Dhital, Shrestha, & Gidley, 2010; Lehmann & Robin, 2007). Moreover, the smaller specific surface area of large granules in potato starch compared to the others cereals may difficult the access and attachment of enzyme (Lehmann and Robin, 2007; Srichuwong et al., 2005). As indicated at Fig. 1, rice and maize starches had similar hydrolysis, wheat achieved greater hydrolysis and quinoa presented the highest hydrolysis value, reaching around 70% hydrolysis, which is curious, considering that it was uncooked starch. These results are in good agreement with Srichuwong et al. (2017), who investigated starches obtained by various isolation processes and reported a similar trend in hydrolysis relating to short amylopectin chains, but without giving other digestion parameters. It is highlighted that the amylose content in cereals is generally about 15–30% (Waterschoot et al., 2015) which is corroborated by our cereals starches (maize, 22%; rice, 21%; wheat, 25%). Nevertheless, quinoa presented only about 7% of amylose which could influence in the high digestibility displayed. The lower presence of amylose reported could favour the digestibility due to a higher amylose content has been associated with reduced susceptibility to enzymatic hydrolysis (Chung, Liu, Lee, & Wei, 2011).

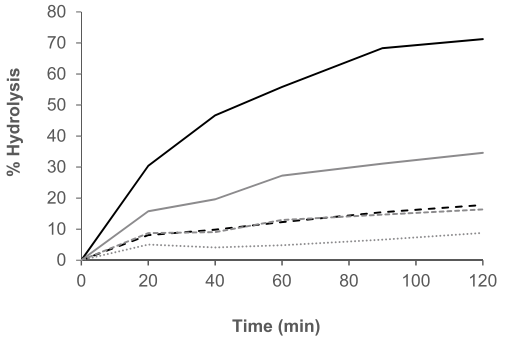

Fig. 1. Hydrolysis of raw starches. Symbols: ▪ ▪ ▪, maize starch; ——, quinoa starch; ——, wheat starch; ▪ ▪ ▪, rice starch; ·····, potato starch.

In order to determine whether the various sources of starch released the same amount of glucose during digestion, the area under the curve (AUC) and hydrolysis index (HI) were calculated (Table 3). The analyses revealed that the raw starches obtained from quinoa and wheat had significantly higher AUC values than the raw starch obtained from maize. It is important to remember here that major differences would be determined by the structural fragility and short amylopectin proportion, as indicated above. When the various raw starches were tested after thermal processing at 100 °C (GS), there were no significant differences in GI values except for potato GS, which continued to have the lowest GI, owing to the lack of digestibility, as was also observed previously (Shi et al., 2018).

After analysing the raw starches (as negative controls) and the gelatinised starches (as positive controls), the effect of the hydrothermal

Table 3
Effect of treatment on *in vitro* digestibility of starch.

Sample	Treatment (°C-min)	TSH_{120} (%)	AUC	HI (%)	GI	Slope (SH/min)
Maize	Raw	34.0 ± 3.4^{bc}	3340 ± 362^{ab}	43.1 ± 4.7^{ab}	63.4 ± 2.6^{ab}	0.4 ± 0.1^{a}
	70 °C-2 min	65.4 ± 1.4^{ef}	6403 ± 54^{d}	82.6 ± 0.7^{d}	85.1 ± 0.4^{d}	3.9 ± 1.2^{abc}
	GS	81.9 ± 5.2^{gh}	8315 ± 642^{e}	107.3 ± 8.3^{e}	98.6 ± 4.6^{e}	8.9 ± 1.0^{de}
Quinoa	Raw	73.8 ± 2.6^{fg}	6352 ± 568^{d}	82.0 ± 7.3^{d}	84.7 ± 4.0^{d}	2.2 ± 0.5^{abc}
	60 °C-1 min	83.7 ± 2.4^{gh}	8766 ± 432^{e}	113.1 ± 5.6^{e}	101.8 ± 3.1^{e}	14.4 ± 3.5^{f}
	GS	91.9 ± 2.7^{h}	8014 ± 200^{e}	103.4 ± 2.6^{e}	96.5 ± 1.4^{e}	3.9 ± 0.4^{abc}
Wheat	Raw	51.6 ± 6.8^{d}	4886 ± 510^{c}	63.0 ± 6.6^{c}	74.3 ± 3.6^{c}	1.1 ± 0.3^{ab}
	60 °C-1 min	82.2 ± 3.7^{gh}	8139 ± 445^{e}	105.0 ± 5.7^{e}	97.4 ± 3.2^{e}	4.5 ± 1.0^{bc}
	GS	81.6 ± 0.2^{gh}	7866 ± 334^{e}	101.5 ± 4.3^{e}	95.4 ± 2.4^{e}	5.1 ± 1.6^{cd}
Potato	Raw	20.7 ± 1.8^{a}	2069 ± 246^{a}	26.7 ± 3.2^{a}	54.4 ± 1.7^{a}	0.4 ± 0.3^{a}
	70°C-1 min	21.8 ± 4.3^{ab}	2464 ± 538^{ab}	31.8 ± 7.5^{ab}	57.2 ± 4.1^{ab}	2.2 ± 0.8^{abc}
	GS	56.1 ± 7.1^{de}	4841 ± 490^{c}	62.5 ± 6.3^{c}	74.0 ± 3.5^{c}	2.4 ± 0.1^{abc}
Rice	Raw	36.6 ± 1.2^{c}	3794 ± 253^{bc}	49.0 ± 3.3^{bc}	66.6 ± 1.8^{bc}	0.6 ± 0.2^{a}
	75 °C-2 min	59.9 ± 6.0^{de}	6213 ± 259^{d}	80.2 ± 3.3^{d}	83.7 ± 1.8^{d}	4.4 ± 1.0^{bc}
	GS	82.7 ± 6.1^{gh}	8411 ± 570^{e}	108.5 ± 7.4^{e}	99.3 ± 4.0^{e}	9.1 ± 1.9^{e}

The data are reported on dry basis. Mean ± standard deviation, $n = 3$. Values in the same column followed by the same letter are not significantly different ($P < 0.05$). TSH_{120}: Total starch hydrolysed at 120 min, AUC: Area under the curve of starch digestion from 0 to 120 min, HI: Hydrolysis index, GI: Glycaemic index, SH: Starch hydrolysed, GS: Gelatinised starch.
The slope was calculated using the Lineweaver-Burk's transformation from the cumulative curves.

treatment was investigated, taking into account the effect of the pasting and thermal properties on the enzymatic hydrolysis of starch, in order to develop food with specific characteristics. The degree of gelatinisation has been reported as one of the main rate-limiting factors in the binding of enzymes to starch for digestion of starches (Wang et al., 2019). The treatment was applied to attain partial gelatinisation of starch in order to evaluate to what extent alterations in starch structure caused by heat-moisture processing affect its digestibility. The pretreatment temperature was selected on the basis of the parameters shown in Tables 1 and 2 The thermal pretreatment of maize and rice starches led to a higher hydrolysis rate than in the case of their raw counterparts, as was observed by Chung et al. (2006) in waxy rice starch subjected to various thermal treatments. Pretreatment of the maize and rice starches, consisting of the application of 70 °C (maize) and 75 °C (rice) for 2 min, led to an HI that was approximately 75% of the HI of the corresponding gelatinised starches. However, the pretreated maize did not hydrolyse totally and did not exceed the hydrolysis values of the raw quinoa. A similar tendency was observed by Ahmadi-Abhari et al. (2013), who reported that wheat starch began to lose crystallinity, and consequently starch digestibility improved, but total hydrolysis was not achieved. In the current investigation, pretreated wheat starch began to lose crystallinity and thus improved its digestibility and reached HI values similar to those obtained for pretreated quinoa starch. The higher hydrolysis observed in the pretreatment of wheat and quinoa in comparison with maize and rice could be due to their low T_p and high PV values, which suggest greater susceptibility to disintegration of their structure (Li et al., 2016).

Data from the hydrolysis parameters were transformed according to Lineweaver-Burk's model in order to obtain approximate values of the kinetic parameters of starch digestion, helping to gain insight into the potential physiological effects (Sanz-Penella et al., 2014). Although the raw quinoa and wheat starches had higher slopes (Table 3), they were accompanied by high hydrolysis, which means that a lower dose would be required. This would help to reduce the digestive inconveniences resulting from the consumption of high amounts of raw maize starch. Furthermore, although the slope values calculated for both raw and gelatinised quinoa starch were similar, the values for gelatinised maize were significantly higher than those of the raw counterpart.

Collectively, these structural changes in quinoa starch may help to maintain glucose concentrations for a longer time and lead to a less rapid rate of fall than in the case of maize starch. Notably, although there are many studies on differences in the techno-functional characteristics of starches and their digestibility, it is not clear how these differences would relate to the rate or efficiency of hydrolysis by pancreatic amylase.

4. Conclusions

To sum up, from this study it can be concluded that it may be possible to modify digestibility by controlling starch properties through variations in temperature or cooking time, which could be useful when designing GI-specific formulations for impaired glucose metabolism. Maize and rice starches showed similar technological characteristics, which were concordant with the lack of differences in digestion. Potato starch showed high resistance to digestibility, whereas quinoa and wheat were more susceptible to enzymatic attack. Furthermore, pasting and thermal parameters for quinoa starch indicated structural changes at granule and molecular level that were reflected in its digestibility. Raw maize starch has been used for years by patients with glycogen storage disease despite the short duration of its effect and the gastrointestinal problems associated with it. Raw quinoa starch could offer a promising potential for extending normoglycaemia in these patients. The results indicate the starches and their pretreatment, taking into account their physico-chemical characteristics, could be a potential useful dietary source for patients who have an altered glucose metabolism. Knowing these parameters and how enzymatic susceptibility is affected is essential to a better understanding of the changes in starch structure which could be applied to develop specific formulations. This proposal gives information in order to develop simple formulations with cereals/pseudcereals/tubers flours to control the starch digestibility. Taking into account their behaviour according the source, composition, grade of crystallinity and/or structure in the food matrices to control the glucose homeostasis. However, this is a preliminary study which could open the door to future investigations designed to attain a better understanding of the physiological effects *in vivo*.

Declaration of competing interest

The authors state that there are no conflicts of interest regarding the publication of this article. This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Raquel Selma-Gracia: Validation, Visualization, Investigation, Formal analysis, Writing - review & editing, Writing - original draft. **José Moisés Laparra:** Supervision, Writing - original draft, Funding acquisition. **Claudia Monika Haros:** Conceptualization, Methodology, Supervision, Writing - review & editing, Resources, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2020.105687.

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ORIGINAL PAPER

Kinetic Approach to the Influence of Chia Flour on Glucose Bioaccessibility from Hydrothermally Treated Maize and Quinoa Starch

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Abstract
Starch structure and bioactive ingredients play an implicit role in the control of glucose release at intestinal level reducing the risk of inadequate metabolic response(s). This study performs a comparative kinetic approach to glucose release from hydrothermally treated (HT) maize (MS) and quinoa (QS) starch. Besides, chia flour (CF) (20%, *w*/w) was added to evaluate its influence of on the apparent diffusion coefficients (Dapp) when subjected to simulated gastrointestinal digestion. Hepatocyte cultures were used to monitor mitochondrial enzymes activity (test MTT) to bioaccessible glucose concentrations. With an increasing temperature, Dapp for both QS and its mixtures with CF were kept unaltered, while those for MS were disrupted progressively affecting glucose bioaccessibility. Principal component analysis revealed differences between maize and quinoa starches, but common features in the corresponding mixtures with CF. Data indicated that quinoa starch helps controlling glucose release and that addition of CF decreased mitochondrial activity in presence of insulin.

Keywords Starch · Glucose · Bioaccessibility · Quinoa · Maize

Abbreviations

BA	Bioaccessibility
CF	Chia flour
Dapp	Apparent diffusion coefficient
EC	Enzyme Commission number
GH	Glucose homeostasis
GSD	Glycogen storage disease
HT	Hydrothermal treatment
MS	Maize starch
MS_70	Treated maize starch at 70 °C
MS_100	Treated maize starch at 100 °C
MS_CF	Maize starch with chia flour
MS_70_CF	Treated maize starch with chia flour at 70 °C
MS_100_CF	Treated maize starch with chia flour at 100 °C
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide
PC	Principal component
PCA	Principal component analysis
PPARγ	Peroxisome proliferator-activated receptor gamma
QS	Quinoa starch
QS_60	Treated quinoa starch at 60 °C, treated quinoa starch at 100 °C
QS_CF	Quinoa starch with chia flour
QS_60_CF	Treated quinoa starch with chia flour at 60 °C
QS_100_CF	Treated quinoa starch with chia flour at 100 °C
STPI	Serine-type protease inhibitor
T2D	Type 2 diabetes
TLR	Toll-like receptor

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Introduction

Glucose homeostasis (GH) is one of the hallmarks of liver related diseases playing a critical role in the pathogenesis of comorbidities associated to metabolic disorders such as type 2 diabetes (T2D) and obesity. The latter constitute, moreover,

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due to an excessive glycaemia important risk factors, together with sedentary lifestyle and altered food supply and preferences determining the progression of liver dysfunction [1]. Recently, alterations in pancreatic β-cells metabolism in response to hyperglycaemia has led to open a debate concerning T2D as a glycogen storage diseases (GSD) of pancreatic β-cells [2]. Thus, both hyperglycaemia as well as hypoglycaemia in childhood are associated with increased risk of physiopathological consequences [3] at a younger age and later in life.

Several attempts have been made to develop digestion models to evaluate nutrient release from food matrices, including the determination of their bioaccessibility (BA, %) [4, 5]. Despite differences in the variety of enzymes and other parameters, according to evidence-based gastrointestinal physiology, there are scarce studies evaluating the fluxes of bioaccessible nutrients [6] affecting physiological response(s). Simulated gastrointestinal digestion models, based on solubility parameters, allow to evaluate food availability to the enzymatic action and hydrolysis approaching glucose release [5]. However, these models did not reveal possible interactions between the nutrients and food ingredients, potentially affecting their influence in response(s) to signals for endocrine control.

Recent studies have demonstrated the benefits of inclusion of chia (*Salvia hispanica* L) flour delaying glucose release and uptake, increasing the expression of biomarkers influencing nutrient fate and energy metabolism [7]. Chia's high fiber content (*i.e.*, soluble mucilage) is able to influence glucose availability [8]. In addition, chia flour is a good source of immunonutritional agonists; protease inhibitors (STPIs) able to interact with the innate immune 'Toll-like receptor' (TLR)-4 [9, 10]. Immunonutritional agonists have been examined as therapeutic agents, in the control of appetite, energy intake, and carbohydrate metabolism [11], helping to slow down the digestion of carbohydrates, generally considered to be beneficial for the dietary management of T2D, obesity and GSD [3, 12].

The objective of this study is to evaluate kinetic features for glucose release from hydrothermally treated maize and quinoa starch, and the influence of bioactive ingredients provided by CF.

Materials and Methods

Materials and Reagents Commercial maize starch was purchased from ACH Food Companies (Argo, USA), red quinoa starch was obtained in the laboratory by wet-milling [13] and black CF was provided by Inca's treasure (Ecuador). Reagents were purchased from Sigma-Aldrich: α-amylase (EC 3.2.1.1, A3176-1MU, USA, 16 U/mg), pepsin (EC 3.4.23.1, P7000, UK, 480 U/mg), pancreatin from porcine (EC 232–468-9, P1750, USA, 4XUSP), bile extract porcine (EC 232–369-0, B8631, USA) amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, 10,115, Switzerland, 60.1 U/mg) and insulin from bovine pancreas (EC 234–291-2, I6634, USA, ≥25 USP units per mg).

Samples Aliquots (100 mg) of maize and quinoa starch were weighted into microcentrifuge tubes. Maize (MS_CF) and quinoa starch (QS_CF) were prepared with 100 mg of starch and mixed with 20 mg defatted CF. Black CF was defatted with *n*-hexane (6 h) under reflux conditions in a Soxhlet equipment (Soxtec™ 2050, Sweden) extracting all the lipid content from black CF (33% g/100 g dry matter). The proportion of black CF was chosen as the maximum percentage allowing adequate solubility conditions. Three different set of samples were prepared with and without CF: raw starches were kept in unheated water for 5 min; the others were subjected to different hydrothermal treatments (HT) to obtain a partial (pretreated) (maize, 70 °C/2 min; quinoa, 60 °C/1 min) and total gelatinisation (maize, 100 °C/5 min; quinoa, 100 °C/5 min) [5]. Each set of samples was prepared in triplicate (n = 3) for simulated gastrointestinal digestion.

Simulated gastrointestinal digestion Three independent set of samples for MS, QS and those mixed with CF were subjected to a simulated gastrointestinal digestion procedure as described elsewhere [6] with slight modifications. An oral phase was included previously to gastric digestion where all samples were incubated in a solution of α-amylase (75 U/mL) for 30 s. The digestion started with a peptic (gastric digestion) step (1 h/37 °C). Then, the intestinal phase of digestion was initiated with a pancreatin-bile extract solution. 2 mL of the gastrointestinal digest was carried out in the upper (donor compartment) chamber and 2 mL of an isotonic saline solution in the bottom (acceptor compartment). The bicameral system consisted in a molecular weight cut-off dialysis membrane (14 kDa) attached to a plastic insert ring to separate the "gastrointestinal digest" from the acceptor compartment allowing to determine glucose dializability from the bottom chamber considered the bioaccessible fraction for absorption.

Aliquots (250 μL) from the bottom compartment were collected at different intervals (15, 30, 45, 60 min), and isotonic solution was used to replace the volumes removed during sampling. Apparent diffusion coefficients (Dapp) were calculated from the linear slope of the glucose concentration in the bottom chamber according to Laparra [6]. Glucose was quantified by enzymatic kit (D-Glucose Assay Procedure, K-GLUC 07/11, Megazyme).

Cell cultures The human hepatoblastoma (HB-8065©) cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 1 and used in experiments between passages 9–11. Cell cultures were

grown in Eagle's Minimum Essential Medium (EMEM Glutamax, Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were maintained at 37 °C in 5% CO_2, 95% air and the culture medium were changed every two days.

Mitochondrial Enzymes Activity (MTT Assay) Metabolic responses were evaluated by monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) conversion (TOX1, Sigma-Aldrich) according to a commercial kit [10]. For experimental studies cells were seeded at a density of 3×10^5 cells/well onto 12 well plates (Costar, Cambridge, MA, USA) and EMEM was replaced by 1 mL of minimum essential media (MEM) without fetal bovine serum. Two different sets of cultures were prepared: i) untreated cell cultures, and ii) treated with insulin (10 ng/mL) [14]. In order to detect early metabolic response(s) intestinal digestion, the bicameral system fitted with the dialysis membrane was applied onto cells. Cell seeded plates were returned to the incubator for additional for 30 min.

Principal Component and Statistical Analyses The principal component analysis (PCA) was performed using Statgraphics© Plus (version 5.1). Multiple ANOVA and Fisher's least significant differences (LSD) were applied to establish statistically significant differences. The statistical analyses were analyzed with Statgraphics Centurion XVl software, and the significance level was established at $P < 0.05$.

Results and Discussion

Starch Digestibility Starch hydrolysis was estimated from soluble glucose concentrations at endpoint of the digestion period (Table 1). After simulated gastrointestinal digestion, raw MS displayed significantly lower digestibility than raw QS, which could be attributed to the lower amylose/amylopectin ratios in QS [5, 15]. Besides, the digestibility percentage for QS is estimated around 60%, which results higher than that calculated (39.8%) from other solubility methods [7]. Digestibility values were increased according to the HTs applied only for MS, however, this increase was reflected in significant decreases in bioaccessible glucose (BA, %). Meanwhile, BA (%) remained constant in all hydrothermally treated QS samples suggesting the highest digestibility of raw QS. This result is supported by reduced pasting and onset temperature as well as peak viscosity values indicative of the great susceptibility to enzymatic activity of QS [5]. Despite not observing statistically significant ($P > 0.05$) differences in starch digestibility in MS and QS mixed with CF, all HTs displayed a downward trend in BA (%). In this vein, consumption of wheat bread-containing chia seeds promoted a lower postprandial glycaemia in comparison with bread without seeds [16].

Evaluation of Fluxes of Glucose Total basal glucose concentrations for MS and QS and the effect of HT on those are shown in Fig. 1. To show clearly the fit of the data points to a 2-parameter exponential distribution, data were plotted against the theoretical curves.

HT of MS significantly increased total dialyzable glucose concentration. In fact, glucose for MS_100 was 6-fold higher than MS ($P > 0.05$); however, Dapp values resulted not statistically different. From data for QS, it was calculated a clear inverse relationship between total basal glucose concentrations and Dapp. These data suggest different interactions between starch structure and composition on glucose dialyzability probably due to differences in the length between amylose/amylopectin chains in quinoa starch and/or their accessibility to digestive enzymes. The inclusion of CF slightly decreased glucose concentrations in the acceptor compartment as supported by reduced Dapp values. The decreased Dapp after addition of CF is concordant with several *in vivo* studies, which have reported a better control on carbohydrate metabolism [17].

The contribution of chia seeds to dietary soluble fiber through its mucilage content has been suggested to contribute slowing down food glucose release [18]. However, the lack of significant differences in the amounts of glucose extracted with the aliquots at different time points between samples mixed or not with CF does not support a potential role for the mucilage reducing glucose dializability (Fig. 1 E-F). It could be expected that this effect must be revealed at a higher magnitude during earlier digestion times when the free glucose to mucilage ratio is low, and thereby it is unlikely that this interaction could manifest in a linear correlation (Fig. 1E-F). Notably, the advanced $t_{1/2}$ for both MS_CF (11.5%) and QS_CF (6.6%) in presence of CF could be due not only to the mucilage content. Menga et al. [17] showed that chia (whole) seeds displayed 2-fold-higher slowly digestible starch fraction of rice flour in comparison to the isolated mucilage.

Principal Component Analysis (PCA) To synthesize the information for identifying patterns in such a way to highlight similarities as well as differences between the analyzed samples data obtained from the kinetic analysis were subjected to PCA (Fig. 2A).

Different parameters to estimate glucose dializability from MS and QS starch (*i.e.*, amount of glucose extracted with the aliquot, cumulative glucose in basal media and Dapp) were statistically associated. Cumulative glucose concentrations were determined as responsible for the major weight of PC#1 (Eigenvalue, 3.93), meanwhile, the lower weight of PC#2 (Eigenvalue, 0.061) included the other groups of parameters calculated. The analysis clearly discriminated between the digestibility of MS and QS and those in presence of CF, which demonstrates the influence of the bioactive components found in CF on

Table 1 Effect of inclusion of chia flour in glucose release. Values are expressed as mean ± standard deviation ($n = 3$)

Sample	Treatment	Soluble glucose (mg)		Bioaccessibility (%)	Dapp (cm^2/seg) ($\times 10^{-2}$)
		before digestion	after digestion		
Raw	MS	0.89 ± 0.16^{a}	2.90 ± 0.21^{a}	7.6 ± 1.9^{d}	1.46 ± 0.29^{b}
	QS	2.94 ± 0.20^{c}	8.38 ± 0.18^{f}	4.5 ± 0.5^{ab}	2.84 ± 0.37^{g}
Pretreated	MS_70	1.95 ± 0.33^{b}	4.61 ± 0.20^{b}	6.1 ± 0.6^{c}	1.67 ± 0.17^{bcd}
	QS_60	5.74 ± 0.64^{de}	8.41 ± 0.23^{f}	4.4 ± 0.1^{ab}	2.27 ± 0.23^{f}
Gelatinised	MS_100	5.76 ± 0.80^{de}	7.72 ± 0.55^{de}	3.7 ± 0.2^{a}	1.59 ± 0.19^{bc}
	QS_100	5.62 ± 0.21^{d}	7.22 ± 0.23^{d}	4.0 ± 0.5^{ab}	1.53 ± 0.18^{bc}
Raw	MS_CF	0.91 ± 0.15^{a}	3.43 ± 0.60^{a}	5.3 ± 2.0^{bc}	1.09 ± 0.20^{a}
	QS_CF	3.07 ± 0.33^{c}	8.33 ± 0.18^{f}	3.7 ± 0.4^{a}	1.99 ± 0.19^{def}
Pretreated	MS_70_CF	1.97 ± 0.45^{b}	5.41 ± 0.06^{c}	4.6 ± 0.3^{abc}	1.61 ± 0.08^{bc}
	QS_60_CF	5.47 ± 0.86^{d}	8.37 ± 0.07^{f}	3.6 ± 0.0^{ab}	2.20 ± 0.10^{ef}
Gelatinised	MS_100_CF	6.67 ± 1.20^{ef}	8.15 ± 0.71^{ef}	2.4 ± 1.6^{a}	1.81 ± 0.35^{cde}
	QS_100_CF	7.28 ± 0.51^{f}	7.70 ± 0.15^{de}	3.7 ± 0.9^{a}	1.56 ± 0.05^{bc}

Superscript letters indicate statistical differences between samples within the same column ($P < 0.05$). MS: maize starch; MS_70: maize starch at 70 °C; MS_100: maize starch at 100 °C; MS_CF: maize starch with chia flour; MS_70_CF: maize starch with chia flour at 70 °C; MS_100_CF: maize starch with chia flour at 100 °C; QS: quinoa starch; QS_60: quinoa starch at 60 °C; QS_100: quinoa starch at 100 °C; QS_CF: quinoa starch with chia flour; QS_60_CF: quinoa starch with chia flour at 60 °C; QS_100_CF: quinoa starch with chia flour at 100 °C; Bioaccessibility = (total glucose in the acceptor compartment / total glucose after digestion at 60 min) x·100; Dapp: apparent diffusion coefficient

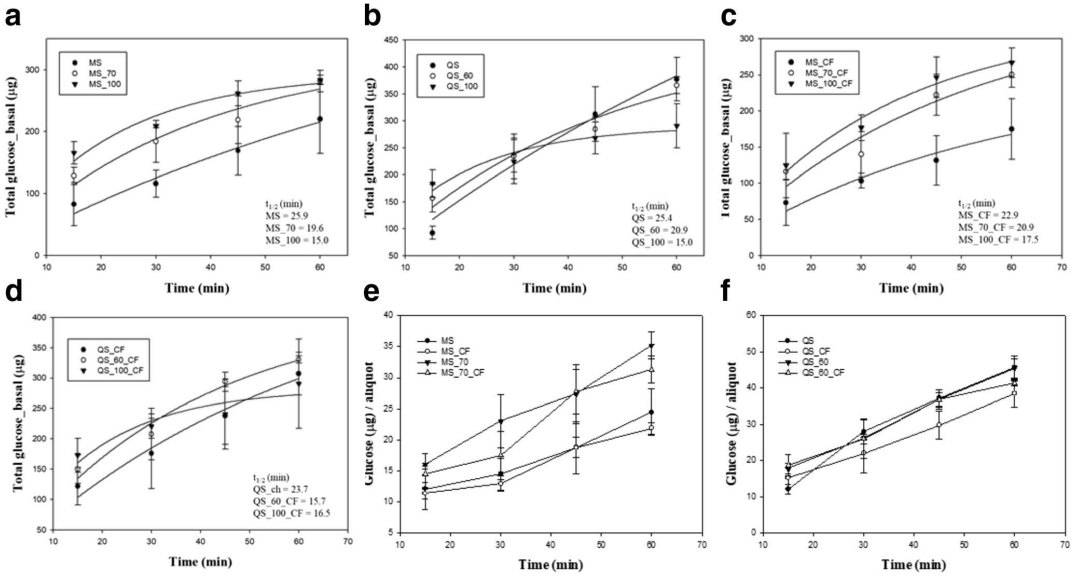

Fig. 1 (A-D) Kinetics of basal glucose accumulations in the acceptor compartment from maize (MS) and quinoa (QS) starch digestion and the influence of chia flour (CF). Raw starches: maize (MS), quinoa (QS), maize with chia flour (MS_CF) and quinoa with chia flour (QS_CF). (E-F) Amount of glucose extracted with the aliquots in acceptor compartment (bioaccessible) during simulated gastrointestinal digestion. Results are expressed as mean ± standard deviation ($n = 3$). Hydrothermal treatment: i) Maize (MS_70) and quinoa (QS_60) pretreated samples (maize 70 °C/2 min and quinoa 60 °C/1 min), ii) Maize (MS_100) and quinoa (QS_100) gelatinized samples (maize/quinoa 100 °C/5 min), mixed or not with chia. $t_{1/2}$, time that takes for reaching half the maximum total basal glucose concentration

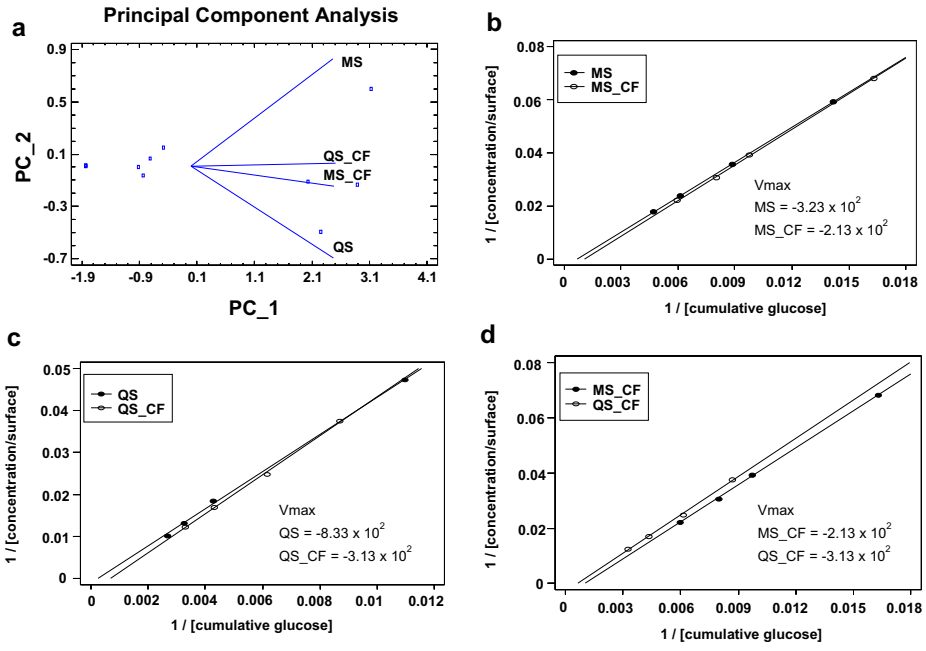

Fig. 2 (A) Principal component analysis of the kinetic parameters of glucose dializability from raw maize (MS) and quinoa (QS) starch mixed or not with chia flour (CF). (B-D) Lineweaver-Bürke's transformation from glucose concentration in the acceptor compartment. The average of three replications was represented and maximum velocity (Vmax) (inverse of y-intercept value) was calculated from the different raw starch formulations

glucose BA changes. Moderate differences in PCA were detected in MS and QS in presence of CF, compared to their non-added counterparts. This is in accordance with previous studies, where immunonutritional agonists found in CF [19] have proved their metabolic modulatory affecting glucose utilization and mitochondrial function [10].

As an approach to estimate the influence of STPIs from CF, we performed a Lineweaver-Bürke's transformation of the bioaccessible glucose concentrations (Fig. 2B-C). Notably, addition of CF differentially increased V_{max} values from MS (by 34%) and QS (by 62%) (Fig. 2D). The latter observations also transpire in the way of a direct effect on enzyme activity rather than the mucilage-mediated physico-chemical modulation of glucose dialyzability.

Hepatic Metabolic Response to Bioaccessible Glucose Glucose undergoes poor biotransformation at intestinal level, while hepatocytes perform regulatory response(s) on glucose uptake [14, 20], as well as liver function is affected by immunonutritional agonists [19] (Fig. 3). Raw MS and QS samples were used for metabolic assays because of the marked differences caused by HT.

Significant differences were quantified in total bioaccessible glucose from MS and QS (Fig. 3A). However,

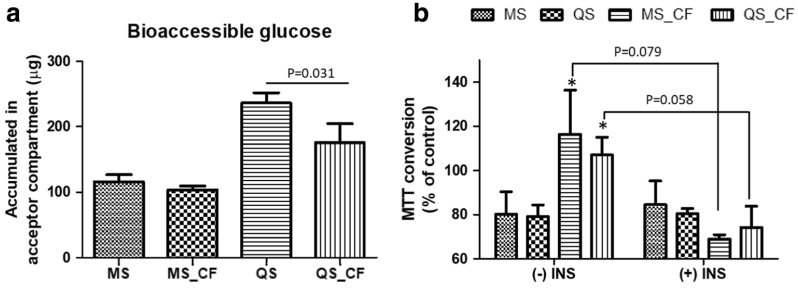

Fig. 3 (A) Total bioaccessible glucose from raw maize (MS) and quinoa (QS) starch mixed or not with chia flour (CF). (B) MTT conversion changes by HepG2 cells exposed ((+) INS) or not ((−) INS) to insulin (INS). Results are expressed as mean ± standard deviation ($n = 2$). *Indicates statistically significant ($P < 0.05$) differences in relation to treatment

mitochondrial metabolic responses did not mirror these differences in glucose concentration when insulin was added (Fig. 3B). Addition of CF provided 0.92 ± 0.02 μg bioaccessible immunonutritional agonists [19]. When HepG2 cells were exposed to insulin (10 ng/mL) a significant inhibitory response was observed in those samples mixed with CF in comparison with MS and QS. Collectively, these data could suggest the participation of cellular molecular mechanisms influencing glucose utilization and insulin signaling. Interaction of immunonutritional agonists provided by chia with the innate immune 'Toll-like' receptor (TLR)-4 with an inhibitory effect on key enzymes in the glycolytic process can explain the observed effects [10].

The increased mitochondrial activities of cell cultures in presence of CF, without insulin exposure, is concordant with its stimulatory capacity on the expression of peroxisome proliferator-activated receptor gamma (PPARγ) [7] inducing the 6-phosphofructo-2-kinase activity [21]. However, insulin is a physiologic homeostatic regulator of hepatocytes to the innate immune TLR4, which dominance promotes a deficient insulin signaling [22]. From a molecular perspective, immunonutritional agonists delay TLR4 retrieval to the plasmatic membrane, thus interfering its functional activity [10]. Here, STPIs from CF could be thought as responsible for the inhibitory effects reducing MTT conversion values by activation of the IRS1/PI3K/AKT pathway [20]. Overall, chia's influence on glucose metabolism highlights its potential promoting a more adequate preservation insulin-induced glycogen storage or gluconeogenesis in order to prevent, or at least to restrain, cell stress during disturbed glucose metabolism pathologies.

Conclusions

Determination of glucose Dapp allows defining both qualitative and quantitative differences between MS and QS starch. Notably, hydrothermal pretreatment of QS had negative effects on Dapp for glucose. Despite differences in glucose kinetics of MS and QS, not significantly different MTT values were quantified in presence or not of insulin. However, inclusion of CF displayed an opposite trend, indicating that, apart from mucilage, chia's compounds can interact in cellular response.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Inclusion of *Salvia hispanica* L. and *Chenopodium quinoa* into bread formulations improves metabolic imbalances derived from a high-fat intake in hyperglycaemic mice

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High-energy intake causes imbalances in nutrient homeostasis contributing to a high prevalence of metabolic chronic diseases. The extent to what metabolic imbalances can be ameliorated by the inclusion of immunonutritional ingredients obtained from flours favouring nutrient and calorie management remains poorly understood. Herein, it is demonstrated that partial replacement of wheat flour (WB) with that from *Chenopodium quinoa* varieties [red (RQ, 25% w/w) and white (WQ, 25% w/w)] as well as from *Salvia hispanica* L., [whole (Ch, 20% w/w) and semi-defatted (Ch_D, 20% w/w)] in bread formulations ameliorates the metabolic and inflammation consequences of high-fat diet consumption in hyperglycaemic animals. Feeding animals with bread formulations replacing wheat flour effectively reduced insulin resistance (by 2-fold, HOMAir). The reduction in starch content did not appear as a determinant of controlling HOMAir. Only animals fed with RQ and Ch diet displayed increased plasma levels of triglycerides, which significantly contributed to mitigate HFD-induced hepatic lipid peroxidation. The latter was increased in animals receiving Ch_D diet, where PUFAs were eliminated from chia's flour. Feeding with WQ and Ch samples caused an upward trend in hepatic TNF-α and IL-6 levels. Despite similarities between immunonutritional agonists in animals fed with RQ and WQ, IL-17 levels were quantified higher for animals fed with WQ. All bread formulations except Ch_D samples significantly increased the hepatic granulocyte–monocyte colony stimulation factor levels. These results indicated that replacement of wheat flour with that from quinoa and chia improved the metabolic imbalances in hyperglycaemic animals.

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1. Introduction

Western lifestyle (*i.e.*, food choice and lack of physical activity) has led to an intense and continuous increase in the prevalence of metabolic diseases such as obesity, type 2 diabetes (T2D)[1] and other major features of metabolic syndrome. In early stages of metabolic disorders, there is an increased risk of developing T2D associated with alterations in insulin resistance.[2] Hyperglycaemia also disrupts physiological lipid handling and management causing dysfunctional alterations in hepatocytes.[3] Of note, recent data have suggested that non-alcoholic fatty liver disease (NAFLD) poses a high risk for the development of T2D, placing the liver in the centre of nutritional studies.[4] In this context, a significant bulk of evidence indicates the close relationship between innate immune signalling and hepatic metabolism,[5] worsening or improving the consequences of excessive nutrient availability.

Diet has acquired great importance due to its physiological involvement in the metabolic modulation, where nutritional strategies can help either prevent or ameliorate metabolic alterations.[6] Prospective nutritional evaluations clearly established a direct relationship between consumption of white bread and the risk of becoming overweight/obese.[7] Besides, research efforts have evidenced the potential of replacing wheat flour with that obtained from Latin-American crops (*Chenopodium quinoa* and *Salvia hispanica*) in bread formulations reducing glycaemic index as well as the hepatic inflammatory milieu.[8,9] These studies evidenced the positive effects of increasing the expression of hepatic immunometabolic transcription factors such as PPAR-γ, helping to control glycaemic index and nutrient fate in energy metabolism. Recent research has also evidenced the positive effects of immunonutritional protease inhibitors found in *C. quinoa* and *S. hispanica* to increase the hepatic F4/80$^+$ cell population,[10] which exerts a key regulatory function on hepatic cholesteryl ester transfer

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protein raising high-density lipoprotein (HDL)-cholesterol.[11] Additionally, *S. hispanica* is recognised as a rich source of polyunsaturated fatty acids that may play an important role in the management of insulin resistance.[12] The promotion of a favourable immunometabolic environment may significantly contribute to the prevention and treatment of T2D. These are novel perspectives connecting the altered 'gut-liver' milieu to risk factors for immunometabolic imbalances influencing T2D severity and progression. However, current evidence for an association of bread formulations replacing wheat flour with that from Latin-American crops with metabolic and inflammation perturbations caused by high-fat diet (HFD) in glucose homeostasis has largely been inferential.

Research efforts have been made to develop animal models that reproduce the transition from pre-diabetes to diabetes, which occurs mainly associated with the apparition of hyperglycaemia to emulate the long-term complications of T2D.[13,14] The repeated administration of low doses of streptozotocin to mimic an early prediabetic (hyperinsulinemic) phase with modest dysglycaemia failed to reproduce the insulin resistance axis typically seen in T2D.[15] Nevertheless, overfeeding a high-fat diet to animals made them develop insulin resistance, where hyperglycaemia is associated with hypertriglyceridemia.[16] Lessons from preclinical long-term (56 weeks) models of T2D reveal three distinct phases: the earliest effects starting from 2 weeks characterized, among other, by hyperglycaemia and insulin resistance (phase 1).[13] Longer periods of treatment cause a decline in insulin production (phase 2, 18–42 weeks) and decrease the fasting and post-IPGTT glucose levels (phase 3, 42–56 weeks). Taken together, these studies seem to suggest that translational value of animal models can be further enhanced when attaining a better understanding of the role of environmental factors that influence the onset, severity and progression of the early stages of the disease.

The objective of this study was to evaluate the influence of wheat flour replacement with that from *C. quinoa* (*i.e.*, white and red) and *S. hispanica* seeds (*i.e.*, whole and semi-defatted) in bread formulations on metabolic consequences of HFD consumption by hyperglycaemic animals. Inclusion of flours from *C. quinoa*, white and red varieties, as well as *S. hispanica*, whole and defatted, was based on different scenarios; (i) to evaluate the effects derived from replacement of wheat immunonutritional agonists with those provided by *C. quinoa* and *S. hispanica*, and (ii) to evaluate the interaction of immunonutritional agonists with different starch levels in the presence of polyunsaturated fatty acids (PUFAs) provided by *S. hispanica*, influencing insulin resistance development.

2. Materials and methods

2.1. Bread samples

Red and white quinoa seeds (Organic quinoa Real©) from ANAPQUI, La Paz, Bolivia, were purchased from Ekologikoak (Bizkaia, Spain). Whole and semi-defatted chia flours were provided by Primaria Premium Raw Materials Company (Valencia, Spain). Wheat flour was purchased from a local market. Five bread formulations were prepared containing different proportions of flours to adjust the starch and lipid contents (Table 1):[14,15] red quinoa (RQ) at 25%, white quinoa (WQ) at 25%, chia (Ch) at 20%, and semi-defatted chia (Ch_D) at 20%, and they were compared to wheat bread (WB) formulations.[16] These formulations were chosen to provide similar contents of the immunonutritional agonists[17] within the bread.

2.2. Immunonutritional agonists in flours: bioaccessibility

Aliquots (0.5 g) of flours were processed, extracted and sequentially filtered through a 50–30 kDa membrane (Amicon®).[17] Then, protease inhibitory activity was quantified in the <30 kDa fraction.[17]

Flours used for bread formulation were subjected to simulated gastrointestinal digestion[9] and bioaccessible immunonutritional agonists were monitored by HPLC-RP-DAD.[17] Briefly, flours were subjected to a two-step gastric (pepsin, 1 h, 37 °C) and intestinal (pancreatin/bile extract, 1 h, 37 °C) digestion.[9] After addition of the pancreatin–bile extract solution, the volume was made up to 10 mL with an isotonic saline solution and 2 mL of the gastrointestinal digestion was carried out in the upper (donor compartment) chamber of a bicameral system. The bottom (acceptor compartment) of the system was filled up with 2 mL of an isotonic saline solution.

The bioaccessible protein profile[17] was obtained using a 1260 Agilent HPLC system equipped with a quaternary pump and a photodiode array detector set at 280 nm (Agilent Technologies, Waldbronn, Germany). The separation (0.8 mL min^{-1}) was performed using a Poroshell 120 (7.5 cm × 4.6 mm) C18 column (Agilent).

Table 1 Proximal compositions of raw materials used to obtain flours

	Flours				
Components (g per 100 g d.m.)	Wheat[a]	Red quinoa[a]	White quinoa[a]	Chia[b]	Defatted chia[b]
Starch	66.2 ± 1.3	62.6 ± 1.1	61.8 ± 1.7	1.7 ± 0.4	1.8 ± 0.2
Proteins	12.4 ± 0.1	12.8 ± 0.7	13.0 ± 0.7	21.4 ± 1.7	29.4 ± 0.2
Lipids	1.1 ± 0.1	5.3 ± 0.2	5.4 ± 0.6	34.4 ± 0.4	7.6 ± 0.3
Ash	0.48 ± 0.08	2.32 ± 0.04	2.37 ± 0.02	4.77 ± 0.04	6.28 ± 0.03

The data are reported on dry basis. Values are expressed as mean ± standard deviation (n = 3). d.m. dry matter. [a] Ballester-Sánchez *et al.*[15] [b] Fernández-Espinar *et al.*[14]

2.3. Animals

In this study, 5-week-old male C57BL/6 mice with an average weight of 15–18 g were obtained from the Centro de Investigaciones Biológicas (CIB-CSIC) in Madrid, Spain. Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of CSIC (Consejo Superior de Investigaciones Científicas) and the protocol was approved by its Ethics Committee (Ethic code, Proex 080/19).

2.4. Experimental design

All animals were injected intraperitoneally with streptozotocin (STZ) (two doses of 25 mg kg^{-1} in two successive days) to induce hyperglycaemia.[18] Then animals were put under a high-fat diet (HFD)[19] (EF AIN93G modified, Ssniff Spezialdiäten GmbH, Soest, Germany) for 3 weeks. Mice were maintained in a controlled environment of temperature (21–23 °C) and humidity (55%) under a 12 h : 12 h (light : darkness) cycle with free access to food and water. Animals were distributed in individual groups (n = 6 per group) according to the formulation of the bread administered. Bread formulations were administered 3 times per week (14 mg per day per animal) during the period of study. The amount of bread administered was established according to the daily nutritional recommendation for bread consumption of 150 g er day per 70 kg body weight. The food intake and body weight of each group were monitored every 2 days. After treatment, mice were killed by cervical dislocation. Animals treated with STZ and put under a HFD, but not receiving bread formulations were used as controls.

2.5. Biochemical parameters

Blood samples were centrifuged (6000g per 10 min) to get clear supernatants. Insulin and glucose concentrations were determined in plasma samples. Insulin (RAB0817-1KT, Sigma-Aldrich, Darmstadt, GER), glucose (MAK263-1KT, Sigma-Aldrich, Darmstadt, GER) and triglycerides (Cayman Chemical, n° 10010303, Michigan, USA) were measured using commercial kits. Insulin resistance (IR) was defined as the homeostatic model assessment of insulin resistance (HOMAir) value, and its behaviour was studied according to the treatment received. HOMAir was calculated according to the following formula:[19] [insulin (μU mL^{-1}) × glycaemia (mg dL^{-1})]/405.

2.6. Determination of hepatic triglycerides and malondialdehyde levels

Liver sections were kept in Krebs's buffer (1 mL), containing the Complete Protease Inhibitor Cocktail (Sigma), until analysis. Samples were thawed and homogenised using a TissueRuptor (Qiagen). Then, the homogenates were centrifuged (10 000g per 15 min) to get clear supernatants. Hepatic triglyceride determination was performed using a commercial kit (Cayman Chemical, n° 10010303, Michigan, USA) according to the manufacturer's instructions. Malondialdehyde (MDA) was determined in hepatic homogenates using a thiobarbituric acid–reactive substance (TBARS) HPLC method as described elsewhere.[20] The samples (50 μL aliquots) were chromatographed with a linear gradient of water and acetonitrile with 0.1% triflouroacetic acid (0–60%, 17 min). Four malondialdehyde standards were included (0.025–0.25 μM) in each TBARS procedure and HPLC analysis, and the concentration of MDA in the samples was calculated from a standard curve. The HPLC analysis was performed using an Agilent 1260 system (Germany) equipped with a diode array detector with a Poroshell C18 (5 μm 2.7 × 50 mm) (Agilent) at 532 nm. The total protein content in plasma and hepatic homogenates was determined by the Pierce®-BCA method (ThermoFisher®, Rockford, USA) to normalise the results between the different samples.

2.7. Hepatic inflammatory markers

Innate immune 'Toll-like' receptor (TLR)-4 was determined by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). Validated Gene Expression Assays for murine TLR4 (forward 5′-TGG GAA CAC ACG GTT GGA AA-3′, reverse 5′-ACA GCA AGT TGT AGC ACT ACT GA-3′) was purchased from Applied Biosystems (Foqter City, CA, USA). qRT-PCR was performed with 500 ng of cDNA from liver sections, using the Universal PCR Master Mix (Applied Biosystems, ThermoFisher®). Quantitative values were calculated by the $2^{-\Delta Ct}$ method.[17]

The cytokine profile was quantified in liver samples homogenised in an isotonic solution using a TissueRuptor (Qiagen) and sonicated. Tumor necrosis factor (TNF)-α (cat. no. 32673019, Friesoythe; GER), granulocyte macrophage colony-stimulating factor (GM-CSF) (cat. no. 32673129, Friesoythe; GER), interleukin-6 (IL-6) (cat. no. 32670069, Friesoythe; Germany) and interleukin-17 (IL-17) (cat. no. 32670179, Friesoythe; GER) (ImmunoTools) were determined by ELISAs according to the manufacturer's instructions. The results of the ELISA assays were normalised according to the total protein content (Pierce®-BCA).

2.8. Statistical analyses

Statistical analyses were performed using the Statgraphics Centurion XVI software. For normally distributed data, ANOVA and the Student t test were applied. Statistical significance was established at $P < 0.05$ for all comparisons.

3. Results

3.1. Immunonutritional compounds in bread formulations and high-fat diets

According to their origin, white or red *C. quinoa*, reverse phase-HPLC-DAD analyses did not reveal significant differences between bioaccessible protease inhibitors displaying immunonutritional agonist activity.[10,17] Previous research showed that immunonutritionally active fraction from *C. quinoa* and *S. hispanica* provides serine-type protease inhibitors within complexes with a certain homology between

different crops.[17] Immunonutritional agonists were identified as glycoproteins with an N-terminal glucuronamide linkage in *S. hispanica*, while they were glucosides in *C. quinoa*. The most protease inhibitory activity was identified in complexes of low molecular weight (<30 kDa). In this study, there were quantified significant variations in the proportion of immunonutritional compounds quantified in a low-molecular-weight (<30 kDa) protein fraction from *C. quinoa* and *S. hispanica* (Table 2). Despite variations in the total amount of immunonutritional agonists, *C. quinoa* provided a similar proportion of those within the <30 kDa fraction than that of *T. durum*. However, that fraction from *S. hispanica* was up to 4.2-fold higher. Immunonutritional agonists displayed significant differences in relation to their bioaccessibility (Table 2).

The levels of both carbohydrates and fat were taken into consideration to put animals under a high-fat diet. The carbohydrate level of the diet used (43%) was elevated up to the highest level commonly used in the literature according to the experimental model (STZ-treated animals).[13,18] The fat content was established at the highest percentage to be representative of a HFD (42%) as reported previously (30–50%).[21] According to the literature, both HFD and 'very' HFD (58–60%) develop insulin resistance in animals. Moreover, to target early events responsible for the onset and progression of immunometabolic changes, the study period was fitted to the earlier phase (2–18 weeks) reported previously.[13]

3.2. Changes in body weight

Animals fed with RQ, WQ and Ch_D samples exhibited a negative trend in body weight (BW) gain (Fig. 1) relative to WB, but only administration of RQ samples had a statistically significant effect (Fig. 1A). Notably, the observed differences in HFD-induced BW gain were not due to reduced food intake. There were no significant differences in daily food (energy) intake between the different groups of treatment (90.2 kJ d^{-1}) (215.6 kcal d^{-1}) over the study period. Although not significant ($p > 0.05$), due to data dispersion, an opposite trend could be found in the mean values for body weight gain between animals administered with Ch or Ch_D formulations. Morphometric comparison of the hepatosomatic ratios (liver/BW) revealed a positive trend in all groups of treatment in relation to WB. Only animals receiving RQ samples exhibited a significant ($P = 0.03$) increase relative to WB (Fig. 1B).

3.3. Glycaemic parameters

Feeding animals with RQ, WQ and Ch samples significantly decreased serum glucose concentrations (Fig. 2A) in relation to those receiving WB. The significant reduction in starch provided by Ch and Ch_D samples does not appear as a major contributor controlling hyperglycaemia in comparison to RQ and WQ samples. Animals administered with RQ and Ch samples displayed a 2-fold lower glycaemia than animals admi-

Table 2 Content of immunonutritional agonists quantified in flours from different botanical origins and their estimated bioaccessibility and protease inhibitory activity of fraction <30 kDa. Results are expressed as mean ± SD ($n = 4$)

	Molecular size (mg protein per g flour)				
Botanical origin	>50 kDa	>30 kDa	<30 kDa	Bioaccessibility[a] (μg min^{-1})	Prot. inh activity (%)
Triticum durum	0.099 ± 0.002[a]	0.022 ± 0.007[a]	0.011 ± 0.003[a]	0.41 ± 0.03	−2.20 ± 0.08[a]
Chenopodium quinoa[b]	0.029 ± 0.001[b]	0.017 ± 0.002[a]	0.013 ± 0.005[a]	0.99 ± 0.08	−0.60 ± 0.08[b]
Salvia hispanica	0.147 ± 0.006[c]	0.082 ± 0.021[b]	0.046 ± 0.001[b]	0.62 ± 0.04	−0.83 ± 0.05[b]

[a] Bioaccessibility of immunonutritional agonists is expressed as the rate of appearance in the basal compartment representing the proportion of those available for absorption or to exert bioactive effects. [b] Analysis of the different varieties, white *vs.* red, did not reveal significant differences for the extractability of the immunonutritional agonists. a–c Different superscript letters indicate statistically significant differences within the same column.

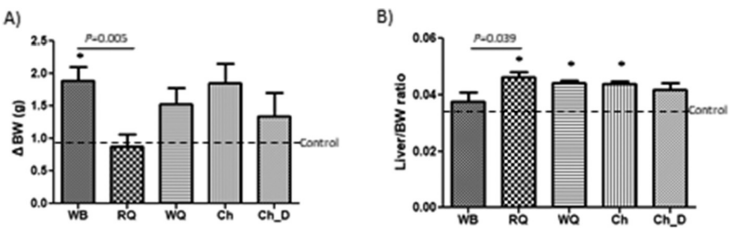

Fig. 1 Variations in body weight gain (A) and liver-to-body-weight ratio (B) in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to controls.

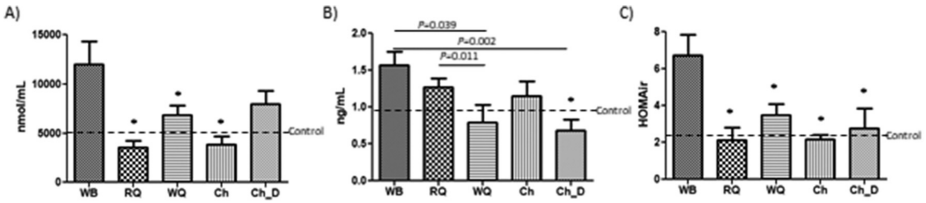

Fig. 2 Biochemical parameters (plasma glucose, A; insulin, B and HOMAir, C) in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to WB.

nistered with WB. Only animals fed with Ch_D samples did not display significantly decreased glucose concentrations. All groups of treatment displayed a positive trend decreasing insulin levels in comparison to WB (Fig. 2B). This effect on insulin regulation reached statistical significance in those animals administered with WQ and Ch_D samples. Notably, all groups of treatment displayed reduced HOMAir values (Fig. 2C), evidencing the improved insulin resistance. HOMAir values were close to #3, even lower for RQ and Ch sample-fed animals, which is considered the limit to establish an insulin resistance.

3.4. Lipid parameters

Plasma triglyceride concentrations (Fig. 3A) were increased ($p < 0.05$) only in animals administered with Ch_D samples, but an increasing trend was also observed in those receiving RQ and Ch samples. Interestingly, highest mean triglyceride concentrations are associated with those animals exhibiting the lowest glycaemia (Fig. 2A). Otherwise, significant differences were not quantified in hepatic triglyceride concentrations between different groups of treatments (Fig. 3B). However, all groups exceed normal hepatic triglyceride contents genetically determined in mice.[22]

Hepatic levels of malondialdehyde (MDA), a reactive metabolite derived from lipid peroxidation, were quantified to evaluate the influence of bread formulations in the control of HFD-induced excessive oxidative stress (Fig. 3C). Feeding with RQ, WQ and Ch samples significantly reduced the degree of lipid peroxidation in relation to WB. Between the quinoa groups, a significant difference ($P < 0.05$) was quantified, where RQ samples exhibited a more positive effect reducing the hepatic MDA levels. Notably, animals fed with Ch_D samples showed 1.5-fold higher MDA levels than its whole counterpart, from where a protector role can be hypothesised for chia's fat in the hepatic lipid peroxidation.

3.5. Hepatic Inflammatory markers

Hepatic expression (mRNA) levels of TLR4 increased compared to WB, except for Ch_D that obtained similar values (Fig. 4A). Animals fed with WQ and Ch samples displayed a slight upward trend in TNF-α levels compared to WB (Fig. 4B). Changes in IL-6 concentrations (Fig. 4C) mirrored those of TNF-α in animals fed with WQ and Ch, and a significant difference was quantified ($P < 0.05$) between the concentrations in animals fed with RQ and WQ samples. This behaviour was similar in IL-17 levels (Fig. 4D), suggesting that WQ samples could exert a greater influence in the hepatic inflammatory processes in comparison to RQ samples. In relation to variations in GM-CSF (Fig. 4E), only animals fed with Ch_D samples showed significantly ($P < 0.05$) reduced levels of the cytokine in relation to WB. Besides, feeding with WQ samples promoted upward trends in GM-CSF values suggestive of a more prominent anti-inflammatory innate immune response. This suggestion is based on the GM-CSF-mediated expansion of the myeloid population contributing to ameliorate the hepatic metabolic stress.

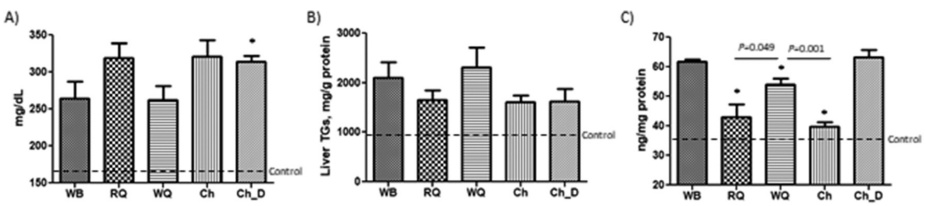

Fig. 3 Plasma triglycerides (A), hepatic triglycerides (B) and peroxidised lipids (C) in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to WB.

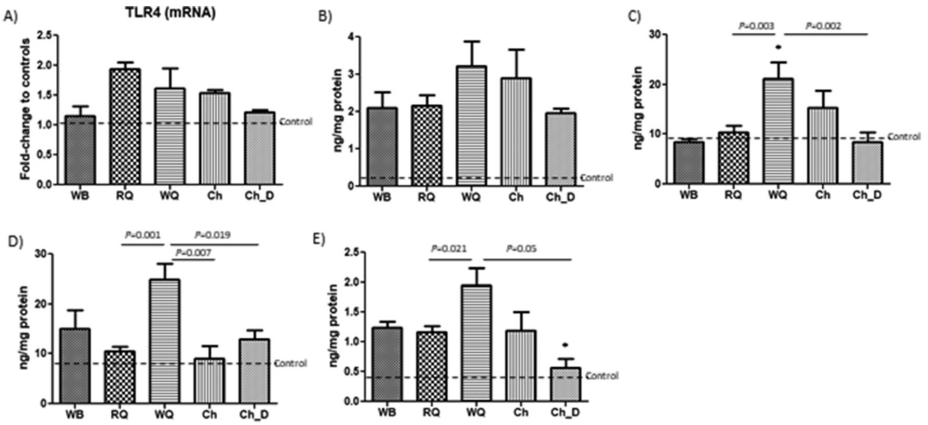

Fig. 4 Hepatic inflammatory markers: (A) Toll-like' receptor (TLR)-4; (B) tumour necrosis factor (TNF)-α; (C) interleukin (IL)-6; (D) IL-17; and (E) granulocyte–monocyte colony stimulating factor (GM-CSF), in animals fed with a high-fat diet and administered with different bread formulations: WB, white bread; RQ, red quinoa flour (25%)–containing bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread; Ch_D, semi-defatted chia flour (20%)–containing bread. Untreated controls are represented by the dotted line. * Indicates statistically significant ($p < 0.05$) differences in relation to WB.

4. Discussion

This study shows that replacement of wheat flour with those obtained from different varieties of *C. quinoa* (*i.e.*, white and red) and processed seeds (*i.e.*, whole and semi-defatted) of *S. hispanica* in bread formulations provides beneficial effects ameliorating the immunometabolic conditions derived from high-energy intake. These effects appeared modifiable in hyperglycaemic animals fed with a HFD during 3 weeks likely reproducing early features of T2D/metabolic syndrome.[23]

According to the proximate composition of flours, similar starch contents in WB and those from C. quinoa, WQ and RQ, samples makes unlikely that better controlled variations in the plasma glucose concentrations could be attributed to starch (Table 1). Moreover, the statistical significance between insulin levels when animals receive RQ *vs.* WQ samples allows hypothesizing that these changes are not completely derived from glycaemia and may imply the participation of immunonutritional agonists found in *C. quinoa.*[17] Variations in the plasma glucose concentrations were inversely associated with the protease inhibitory activity, but directly to the higher bioaccessibility estimated for immunonutritional agonists from wheat (*T. durum*) and quinoa (*C. quinoa*) flours (Table 2). In wheat flours, these compounds have been found as part of homodimeric and heterotetrameric complexes, while monomeric units are majorly present in flours from quinoa and chia.[17,24] Major structural differences rely on the lack of glycoconjugate prosthetic groups in immunonutritional agonists from wheat, but carriage of an N-terminal glucuronamide linkage in *S. hispanica* and glucosides in *C. quinoa.*[17] Pilot studies revealed slight, but significant, differences in the coefficients of diffusion for the immunonutritional agonists from RQ and WQ samples (*data not shown*), where the slowest kinetic might be associated with a better control over insulin production (Fig. 1B). Immunonutritional agonists found in the flours have been proved to have a significant potential to down-regulate the expression of key enzymes (*i.e.*, GAPDH) involved in the glycolysis processes according to the following gradation:[24] wheat, 34.9%; quinoa, 44.2% and chia, 51.5%. These effects occur *via* their immunonutritional role interacting with the innate immune 'Toll-like' receptor (TLR)-4[24,25] (Fig. 4A). Accordingly, the lower immunonutritional potential of RQ samples is supported by downward trends in the hepatic inflammatory milieu, TNFα (Fig. 4B) and IL-6 (Fig. 4C), which is dependent on TLR4 engagement of adaptor molecules and downstream molecular signalling.

To approach the comprehensive effect of n-3 PUFAs, chia flour was defatted attaining a reduction in the lipid fraction up to 70% (Table 1). An increase in the protein fraction was observed, which could be attributed, at least in part, to the conversion factor used to estimate crude protein from total nitrogen.[14,26] Otherwise, methods targeting peptides containing three or more amino acid residues[27] are affected to a lesser extent by protein compositional differences to provide greater concentration accuracy. Accordingly, significant lower protein contents in defatted chia flours have been reported, 22.7 ± 0.7 g per 100 g,[27] in comparison to those using the conversion of nitrogen,[14,26] 29.4–31.7 g per 100 g. Defatting causes a significant reduction (by 39.7%) in the α-amylase inhibitory activity of chia[28] attributed to the immunonutritional compounds. These effects can explain the upward trend in glucose concentrations in animals fed with Ch_D samples in relation to their counterparts receiving Ch samples (Fig. 2A). Collectively, significant impairments can be hypothesized in the tertiary structure of the compounds responsible for the immunonutritional activity due to the loss of capability to

interact with TLR4 (Fig. 4A). Previous data suggest that n-3 PUFA may improve postprandial hyperglycaemia as well as insulin secretion ability and hypertriglyceridemia, with impaired glucose metabolism.[29,30] Neither duration nor dosage appears to explain the observed heterogeneity in response to n-3 PUFAs.[30] Herein, the significant reduction in the lipid fraction could be concordant with the variation observed in plasma glucose; however, it cannot explain the improved control on insulin levels (Fig. 2B and C). This observation transpires in the sense of supporting the role of immunonutritional compounds.

Immunonutritional agonists from quinoa and chia have been shown to significantly down-regulate the expression of the fatty acid translocase (*i.e.*, CD36),[10] thereby modulating the breadth of signalling for TLR4.[24] In the last few years, different studies pointed out the significant effect of regulation of the TLR4/NF-κB-pathway on obesity-associated imbalances in biochemical parameters (*i.e.*, triglycerides).[11,18,31] Commonly, the reduction in plasma triglyceride concentrations was derived either from inhibition of its downstream molecular signalling or delaying the intracellular retrieval of the receptor allowing signal transduction. In this scenario, proteolytic cleavage and shedding of TLR4 has been demonstrated as the mechanism to prevent the liver from developing insulin resistance.[32] These observations agree with the findings relative to the important regulatory and sequential role of innate and adaptive immunity to establish tissue lipid homeostasis.[33] When considering the influence of n3-PUFAs, data did not support potential positive effects of PUFAs regulating the plasma triglyceride concentrations, in accordance with the lack of correlation between the effects and level of n3-PUFA intake. Health effects of n-3 PUFAs are partly mediated by their oxidized metabolites, *i.e.*, eicosanoids and other oxylipins. Herein, TLR4 has been identified as a mediator able to induce epoxy-oxylipin products, promoting the protective effects of soluble epoxide hydrolase.[34] This protective effect appears quite well reflected in the reduced MDA levels quantified in animals fed with Ch samples in comparison to those receiving Ch_D samples. While important for the clearance of these molecules from the circulation, CD36-dependent signalling has also been implicated in the pro-inflammatory effects of modified endogenous ligands of TLR4.[35]

Herein, the extent to what RQ and WQ samples affect MDA levels (Fig. 3C) appears inversely associated with the level of TLR4 transcripts (Fig. 4A), which is commonly associated with the protein content. These differences are revealed by the upward trends in TNFα and IL-6 production (Fig. 4B and C). Collectively, lower mRNA levels of TLR4 with increased cytokine production may interpret the experimental data, showing faster kinetics of TLR4 mRNA translation and protein expression, which aggravate glycaemia-induced metabolic stress. However, the role of TNF-α and IL-6, which can elicit either positive or negative effects, on metabolic control and insulin sensitivity is still under debate.[36–38] The lack of significant differences in the phenolic content between RQ and WQ samples[39] transpires in the sense of a protein-mediated effect as a unique promoter of different TLR4 transcript levels. Moreover, neither the isolated polyphenols profile nor total antioxidant capacity (DPPH method) estimated for *S. hispanica* flours, semi-defatted or not, could be associated with the extractable or bioaccessible total antioxidant capacity estimated for these fractions.[40]

TLR4 mRNA levels together with the trend for both cytokines quantified in animals receiving Ch samples are tempting to suggest similarities between immunonutritional agonists in animals receiving WQ and Ch samples. However, it should not be ruled out that immunonutritional agonists also promote physiological variations in lipid-dependent kinases (*i.e.*, protein kinase C – PKC).[24] These kinases have shown wide-ranging roles in signal transduction and modulation of insulin action.[41] Accordingly, variations in MDA levels between animals fed with WQ and Ch samples are explained (Fig. 3A–C), at least in part, by lower PKC expression levels favoured by Ch samples helping to ameliorate the effects of fat oversupply. The decreased hepatic IL-17 levels (Fig. 4D) also support positive effects on the high-fat diet-induced metabolic stress by inhibiting fatty acid β-oxidation.[42] With this in mind, the negative balance in GM-CSF levels (Fig. 4E) attained by feeding with Ch samples allows suggesting a better controlled HFD-induced impaired insulin sensitisation (Fig. 2A and B) as reflected in lower HOMAir (Fig. 2C). Overall, the lack of these inflammatory response(s) helps supporting the partial loss of bioactivity by immunonutritional agonists during defatting as suggested previously.

5. Conclusions

The replacement of wheat flour with that from *C. quinoa* (*i.e.*, white and red) or *S. hispanica* (*i.e.*, whole and semi-defatted) in bread formulations ameliorates the severity of metabolic consequences of high-fat diet consumption in hyperglycaemic animals. A relative short STZ/HFD model (3 weeks) allows us to monitor both hyperglycaemia and hypertriglyceridemia as well as insulin sensitization and resistance development. Herein, protease inhibitors displaying immunonutritional activity provided by flours rather than other nutrients, such as PUFAs, significantly contributed to a higher extent to the beneficial outcomes. These results indicated that the immunonutritional potential of different bread formulations rather than calorie intake or starch content appears as a major determinant of the control of glucose homeostasis. This opens new avenues on food formulation, influencing innate immunity to shape nutrient homeostasis. Further research with human trials is needed to determine the magnitude of these beneficial effects.

Compliance with ethical standards

Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of

Laboratory Animals of CSIC (Consejo Superior de Investigaciones Científicas) and the protocol was approved by its Ethics Committee (Proex 080/19).

Conflicts of interest

The authors declare that they have no conflict of interest.

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Immunonutritional Bioactives from *Chenopodium quinoa* and *Salvia hispanica* L. Flour Positively Modulate Insulin Resistance and Preserve Alterations in Peripheral Myeloid Population

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Abstract: Innate immunity plays a determinant role in high fat diet (HFD)-induced insulin resistance. This study compares the effects of immunonutritional bioactives from *Chenopodium quinoa* (WQ) or *Salvia hispanica* L. (Ch) when used to partially replace wheat flour (WB) into bread formulations. These flours were chosen to condition starch and lipid content in the products as well as because their immunonutritional activity. To be administered with different bread formulations, HFD-fed C57BL/6J mice were distributed in different groups: (i) wild type, (ii) displaying inherited disturbances in glucose homeostasis, and (iii) displaying dietary iron-mediated impairment of the innate immune TLR4/TRAM/TRIF pathway. We analyze the effects of the products on glycaemia and insulin resistance (HOMA-IR), plasmatic triglycerides, intestinal and hepatic gene expression and variations of myeloid (MY), and lymphoid (LY) cells population in peripheral blood. Our results show that feeding animals with WQ and Ch formulations influenced the expression of lipogenic and coronary risk markers, thus attaining a better control of hepatic lipid accumulation. WQ and Ch products also improved glucose homeostasis compared to WB, normalizing the HOMA-IR in animals with an altered glucose and lipid metabolism. These positive effects were associated with positive variations in the peripheral myeloid cells population.

Keywords: insulin resistance; innate immunity; myeloid population; obesity

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1. Introduction

Nowadays, more than 1600 million people worldwide are overweight or obese. According to the World Health Organization (WHO), this global health problem affects all groups of the population and this number will continue increasing by >140% in 2050. Obesity, together with other chronic diseases (i.e., type 2 diabetes–T2D, hypertension, dyslipidemia, physical inactivity, chronic kidney and liver diseases, and smoking), contributes an additional risk factor to cardiometabolic health, while hyperglycaemia confers a substantial independent risk for the adverse physiological outcomes [1]. For example, gestational hyperglycaemia and obesity are independently associated with adverse outcomes during pregnancy [1,2]. Worldwide hyperglycaemia kills some 3.4 million people a year (WHO). Beyond alleviating glucose levels, the greatest benefits for disease prevention appear to be derived from improving the 'glycemic control', while the normalization of glycaemia results in a more modest reduction of the effects [3].

Non-alcoholic fatty liver disease (NAFLD) incurs a high risk for the development of T2D and other major features of the metabolic syndrome [4]. Metabolic imbalances,

including those affecting glucose homeostasis, arise from complex interactions between genetic and environmental factors [5]. While the host's endogenous factors are rather difficult to influence, the environmental (i.e., dietary) factors are predominant and addressable from a preventive or therapeutic approach. Besides, emerging evidence supports the metabolic (re)programming of defined components of the innate immune system (i.e., innate immune both myeloid and lymphoid cells), which act as key mediators to induce obesity [6,7]. Furthermore, significant changes in the subpopulations of lymphocytes have been reported in young adults with metabolic syndrome [8]. Notably, intestinal innate immune 'Toll-like' receptor (TLR)-4 is a well-known element, which has been identified as an essential regulator of insulin resistance and accumulation of macrophages within the adipose tissue [5]. These immune changes are critical mechanism(s) to normalize the distinctive stamp of obesity in the glucose homeostasis dysregulation. In this context, immunonutritional strategies can play an important role in controlling the severity and progression of the disease. However, whether the use of bread formulation prepared by replacing wheat flour [9] modulates myeloid and lymphoid populations and glycaemic control remains elusive.

The question of how endogenous (i.e., transgenerational inheritance) [10] and environmental factors (i.e., diet) [11] influence early stages and the onset of alterations in lipid and glucose homeostasis has promoted the development of different preclinical models. The exposure of non-pregnant female mice to the obesogenic tributyltin (TBT) enabled a transgenerational inheritance of disturbances in glucose homeostasis [10] and inhibition of the insulin receptor expression [12]. Furthermore, it caused a permanently metabolic (re)programming towards the hepatic fat accumulation and weight gain, particularly in conditions of caloric excess [10]. Besides, it was demonstrated that there was a development of hypoglycemia associated with hypertriglyceridemia and a transitory insulin resistance in animals fed a high fat diet (HFD) [11]. These long term (56 weeks) preclinical models of T2D revealed that an elevated hyperglycaemia associates with early insulin resistance after 2 weeks under HFD feeding. Metabolic alterations caused by HFD that result in and are associated with hepatic steatosis implies a dysmetabolic hepatocellular iron uptake [13]. In addition to this, HFD-induced activation of the TLR4/MyD88 pathway leads to an inhibited macrophage proliferation, which associates with macrophage infiltration into adipose tissue [14]. The TLR4/MyD88-dependent signaling likely contributes to promote hepatic inflammation [15], whereas the production of type I interferons via TLR4/TRIF-dependent signaling appears to exert protective roles in the metabolic dysfunction [16]. This raises interest in evaluating to what extent modulating the immunonutritional potential of food formulations can influence changes in the proportions of peripheral myeloid immune cells in individuals with alterations in the glucose homeostasis.

Bread formulations enriched with *Chenopodium quinoa* and *Salvia hispanica* L. flours provide an effective alternative to improving metabolic imbalances derived from a HFD intake in hyperglycaemic mice [9]. These effects were attributed to immunonutritional bioactives (protease inhibitors found in the low molecular weight albumins/globulin fractions) that interact with TLR4 [17–20]. Notwithstanding, this interaction appears different to that exerted by immunonutritional bioactives from wheat [21]. At the molecular level, compounds derived from *C. quinoa* and *Salvia hispanica* L. display glycoside and glucuronide groups bound to the amino acid backbone, while those from wheat (*Triticum durum*) lack glycosidic prosthetic groups [17]. Proteome analyses on human-like macrophages shed some light on the biological activity of protease inhibitors from *C. quinoa* and *S. hispanica* L, supporting their correlation with TLR4/TRIF signaling [19] that contrasts with TLR4/MyD88 of wheat [21].

In this study, we hypothesize that partial replacement of wheat flour by that of *C. quinoa* and *S. hispanica* L. will improve the immunonutritional potential of bread formulations. More specifically, such substitution will positively contribute to ameliorate alterations of glucose and lipid homeostasis through the modulation of the innate immune potential within the 'gut-liver' axis. To this end, different preclinical models that reproduce

major features of the obesogenic and hyperglycemic conditions mimicking the human disease are used. These results can provide novel points of view with significant implications for the ongoing debate about developing appropriate interventions to maximize the beneficial effects and immunometabolic control of obesity.

2. Materials and Methods

2.1. Bread Samples

Distinct bread formulations were prepared containing different proportions of flour from white quinoa (WQ) at 25% and chia (Ch) at 20% and were compared to wheat bread (WB) [22]. The different proportions of *C. quinoa* and *S. hispanica* flour were chosen to normalize the protein content in the products because as immunonutritional bioactives derive from it. The chemical composition of *C. quinoa*- and *S. hispanica* L-containing bread formulations is shown in Table 1.

Table 1. Proximal composition of *C. quinoa*- and *S. hispanica* L-containing bread formulations administered in the study.

Component g/100g d.m.	Bread		
	Wheat [23]	**White Quinoa [23]**	**Chia [24,25]**
Starch	66.2 ± 1.3	61.8 ± 1.7	53.3 ± 0.1
Proteins	12.4 ± 0.1	13.2 ± 2.5	14.1 ± 0.5
Lipids	1.1 ± 0.1	2.2 ± 0.1	7.8 ± 0.1
Ash	0.5 ± 0.1	1.5 ± 0.0	1.3 ± 0.0
Iron (μmol/g) [26]	0.4 ± 0.1	0.6 ± 0.0	0.7 ± 0.0
InsP6 (μmol/g) [26]	*n*.d.	2.0 ± 0.0	1.7 ± 0.0

Values are expressed as mean ± standard deviation (n = 3). d.m. dry matter; *n*.d. not detected; $InsP_6$, phytic acid.

2.2. Animals

C57BL/6 mice with 6 weeks of age were obtained from the Centro de Investigaciones Biológicas (CIB-CSIC) in Madrid, Spain. Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of CSIC (Consejo Superior de Investigaciones Científicas) and the protocol was approved by its Ethics Committee (Proex No.080/19). Mice were maintained under a controlled environment of temperature (21–23 °C) and humidity (55%) and a 12 h:12 h (light:dark) cycle with food and water ad libitum. After treatment, mice were sacrificed by cervical dislocation.

2.3. Experimental Design

Different models were used to reproduce the main metabolic and immunological alterations in the functionality of the "gut-liver" axis and were of great relevance in the influence of glucose homeostasis (Figure 1). All bread formulations were administered (14 mg/day/animal) to the different animal models; three times per week for 3 weeks to model 1 (Figure 1A) and for 2 consecutive days to model 2 (Figure 1B).

The amount of bread administered was established according to the daily nutritional recommendation for bread consumption (i.e., 150 g/day/70 kg body weight) that was previously proved to be effective controlling glucose homeostasis [9]. Food intake and changes in body weight of each group were monitored every 2 days. Livers were removed and weighed to calculate the whole body to liver weight (hepatosomatic index). After treatment, mice were killed by cervical dislocation. Animals put on a HFD but not receiving bread formulations were used as controls.

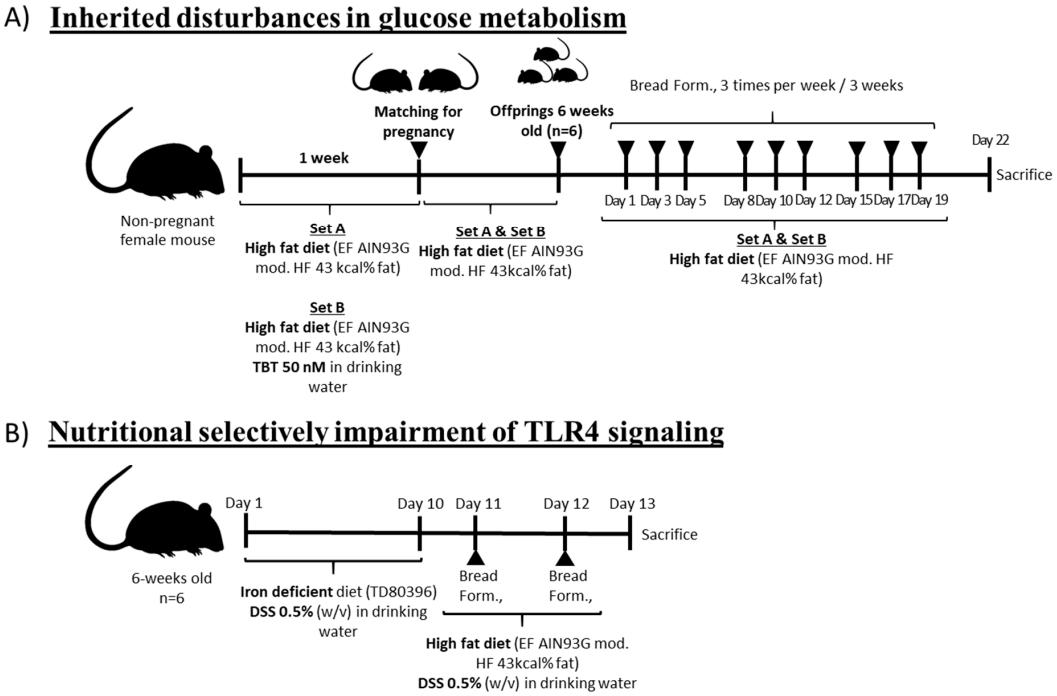

Figure 1. Schematic representation of the two experimental models used to mimic early disturbances in glucose homeostasis and the administration pattern for the bread formulations (bread form: wheat-, *Chenopodium quinoa*- and *Salvia hispanica*-based bread formulations. Model 1 (**A**): Male animals (n = 6/group) were split into two groups depending on their origin; (i) pregnant female mice under standard conditions (F0_A) or (ii) pregnant female mice exposed (F0_B) to the obesogenic tributyltin TBT 50 nM (*w*/*v*) via drinking water to develop a state of obesity [10]. After, both F1_A and F1_B generations were kept on a HFD until reaching 6 weeks of age. Model 2 (**B**): Male mice (n = 6/group, 6 weeks-old) from pregnant females under standard conditions were kept on an iron-deficient diet (AIN93G modified, Ssniff Spezialdiäten GmbH, Soest, Germany), exposed to 0.5%, and administered with dextran sulfate sodium (DSS) (*w*/*v*) via drinking water [27] for 10 days. After, animals were put on a HFD and continued to be exposed to 0.5% DSS for an additional 2 days.

2.4. Biochemical Parameters

Blood samples were centrifuged (6000× *g*/10 min) to get clear supernatants. Glucose, insulin, and triglycerides concentrations were determined in plasma samples. Glucose was determined by glucose kit (KA1648, Abnova, Taoyuan, Taiwan), insulin by ELISA kit (RAB0817-1KT, Sigma-Aldrich, Darmstadt, GER) and triglycerides with a commercial kit (Cayman) (n° 10010303). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) value was used to define insulin resistance according to the following formula: [(insulin (µIU/mL)* glycaemia (mg/dL)]/405 [28].

2.5. Hemogram

Complete blood count was performed on an automated hemocytometer (Abacus Junior Vet, ELECTROMEDINTER SL) to calculate total red blood cells (RBC) and leukocyte counts (WBC) as well as the lymphocytes (LY), myeloid (MY) (macrophages, monocytes), and granulocytes (GR) (granulocytes, eosinophils, neutrophils) population percentages.

2.6. Transcripts of Hepatic Lipogenic and Coronary Risk Markers and Macrophage Identifiers

Validated Gene Expression Assays for murine fatty acid synthase (FASN) (forward 5′-TTC CCA CCA AGT GTG GGT AT -3′, reverse 5′-TGG GAC CTT CAG CTT GCT TC -3′), sterol regulatory element-binding protein 1 (SREBP1a) (forward 5′- TCA AAA CCG CTG TGT CCA GT -3′, reverse 5′- GAC GTC TCA ACC CGC TAG G -3′), prostaglandin-endoperoxide synthase 2 (PTGS2) (forward 5′- AAA AGA GAA CGT GAG AGG GCA -3′, reverse 5′- TCA AAC TGG GAA CGG GTG AC -3′), arachidonate 15-lipoxygenase (ALOX15) (forward 5′- TCC CAT TCT AGG GGA GAG GG -3′, reverse 5′- CCT TGA CCA GCT CAG TAG GC -3′), CD68 (forward 5′- AGA AGT GCA ATG GTG GGT CT-3′, reverse 5′- TGG GGC TTA AAG AGG GCA AG -3′), CD206 (forward 5′- TGC AAG CTT GTA GGA AGG AGG -3′, reverse 5′- GAT TAG AGT GGT GAG CAG GC -3′) and β-actin (forward 5′-GGC TCC TAG CAC CAT GAA GAT CAA -3′, reverse 5′- AGC TCA GTA ACA GTC CGC CTA GAA -3′) was purchased from Applied Biosystems (Foqter City, CA, USA). Qrt-PCR was performed with 500 ng of cDNA from liver sections, using the Universal PCR Master Mix (Applied Biosystems, Foster, CA, USA) Quantitative values were calculated by using the $2^{-\Delta Ct}$ method [9].

2.7. Statistical Analyses

The results are presented as the mean and standard error of the mean (SEM). The statistical analysis between the different groups of treatment within a same experimental model was conducted using one-way analysis of variance (ANOVA) and the Kruskal–Wallis post hoc test by ranks. Because of the different conditions used to obtain the animals for the different models, the comparison between groups of animals receiving the same bread formulation was performed by comparison of the means. Statistical analyses were performed with the software Statgraphics Centurion XVI and significance was established at $p < 0.05$ for all comparisons.

3. Results and Discussion

3.1. Immunonutritional Bioactives

This study evaluates the modulatory role of the inclusion of *C. quinoa* and *S. hispanica* L. into *T. aestivum*-based bread formulations on the HFD-induced immunometabolic effects in conditions of caloric excess as well as hepatic steatosis, which is elicited by prenatal exposure to the obesogenic TBT [10]. Partial replacement of wheat flour by that from *C. quinoa* or *S. hispanica* L. into bread formulations was evaluated as an immunonutritional strategy to provide glycoside- and glucuronide-carrying proteins (2S seed storage protein), which have shown to display immunonutritional potential (Figure 2). Here, is shown the molecular weight of the molecular backbone as well as the glycoside (Figure 2A) and glucuronide (Figure 2B) linkage as deduced from the RP-HPLC-Ms/Ms [17] analyses on protein bands. These glycoside and glucuronide groups were not found to be associated with the wheat-derived immunonutritional bioactives [17]. Interestingly, the effects observed could not only be directly associated to these structural features but to their capacity to interact with TLR4 (discussed below). The inclusion of *C. quinoa* or *S. hispanica* L. flours modified the content of bioactive 2S globulins according to the following gradation: 2.21 ± 0.04 mg/g (WB) = 1.74 ± 0.32 mg/g (WQ) < 8.19 ± 0.11 mg/g (Ch). Focusing on the control of glucose homeostasis, the particular composition of the products help to provide additional information on how glycaemia results affected by the different carbohydrate and lipid changes (Table 1) [9].

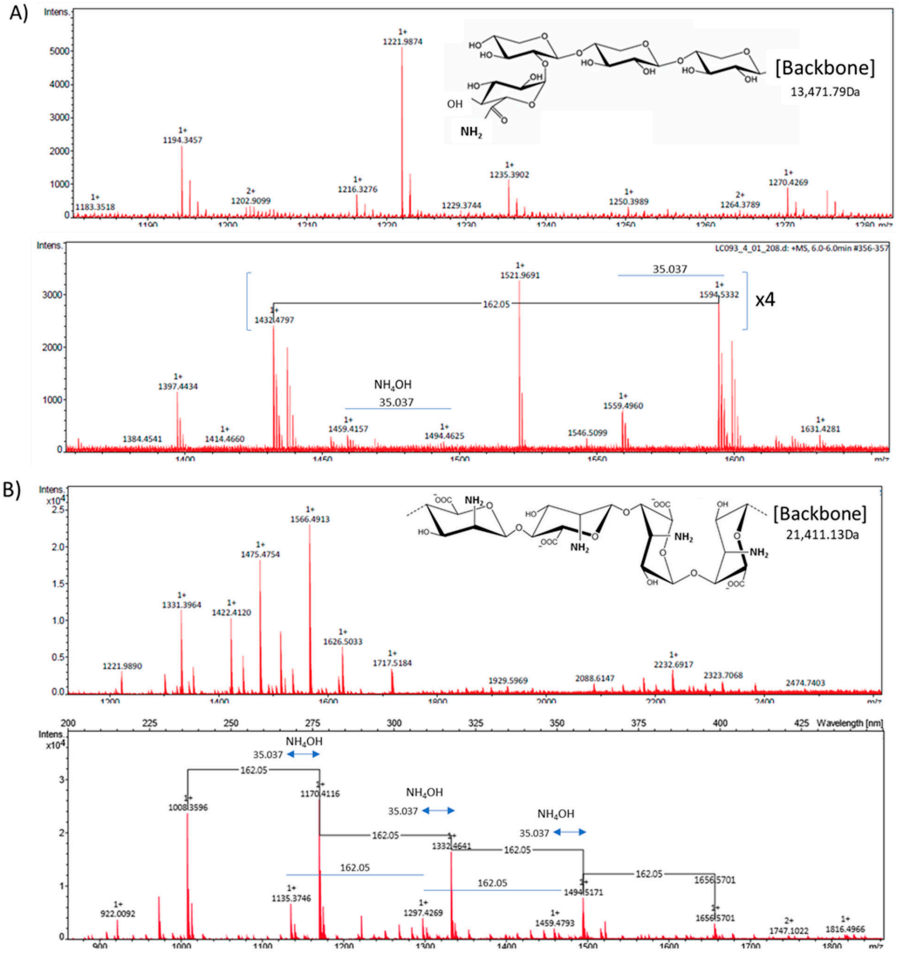

Figure 2. Mass spectrometry '*m*/*z*' signals from the bioactive fraction obtained from *C. quinoa* (**A**) and *S. hispanica* L (**B**) [17,19].

3.2. Immunnutritonal Influence of Obesogenic Effects

To determine whether the different products affect hepatic lipid homeostasis, C57BL/6J mice were put on a 43% HFD (Figure 3), as established in previous studies [9]. Significant differences in the daily food (energy) intake between wild type or TBT-exposed mice in the different groups of treatment (30.0 kcal/day) (143.7 kJ/day) were not quantified. Feeding WB, only TBT-treated animals increased the body weight (BW) gain. However, contrasting patterns were observed for WQ and Ch (Figure 3A). Despite the model, animals receiving WQ increased BW, whereas those fed with Ch maintained a BW similar to that observed in the control group. These effects are attributed to bioactive ingredients in bread formulations, since it was previously shown that F1 animals directly exposed in utero to TBT display low effects on BW gain in eight-week-old animals [10]. Wild type and TBT-exposed animals fed HFD and administered with WQ showed a similar hepatosomatic (liver to BW) index of the animals compared to controls (Figure 3B). Otherwise, mice fed with WB and Ch showed higher liver/BW ratios. It is important to point out here that these variations were associated with changes in the liver weight for WB (1.30 ± 0.24 g), whereas in those administered with Ch (1.09 ± 0.13 g) and WQ (1.06 ± 0.05 g) it remained unaltered. As

shown, iron deficiency favored higher values of the hepatosomatic index in all groups of treatment (Figure 3C), without causing differences in liver weight. In agreement with the TBT-derived obesogenic effects, animals coming from pregnancy females exposed to TBT displayed elevated triglycerides (TGs) levels (Figure 3D) that support alterations in hepatic lipid homeostasis. Plasmatic TGs levels in TBT-exposed animals ranged between 11.1 ± 6.6 and 45.8 ± 16.4%; higher than their respective counterparts. To further evaluate hepatic response(s), we quantified the relative variation of hepatic transcripts (mRNA) for lipogenic (i.e., FASN and SREBP1a) as well as coronary risk (i.e., ALOX15 and PTGS2) markers (Figure 3E–H). Rt-qPCR analyses of hepatic tissue revealed opposite patterns in the expression of FASN as a function of the treatment and feeding. In perinatal TBT-exposed mice, feeding WQ up-regulated FASN gene expression, whereas animals fed with WB and Ch exhibited down-regulation on this parameter. Data for SREBP1a showed similar trends for both models with significant higher expression levels in animals fed WQ. When considering the effects in the expression of ALOX15 and PTGS2, those were sharply increased in animals fed with WQ and Ch, but only slightly affected in those receiving WB. In this regard, it can be hypothesized that the administration of bread formulations containing *C. quinoa* or *S. hispanica* L. flour could help controlling the HFD/TBT-induced disturbances in lipid homeostasis. However, administration of WB seems to impair the physiological control of these biomarkers mostly in TBT-exposed animals.

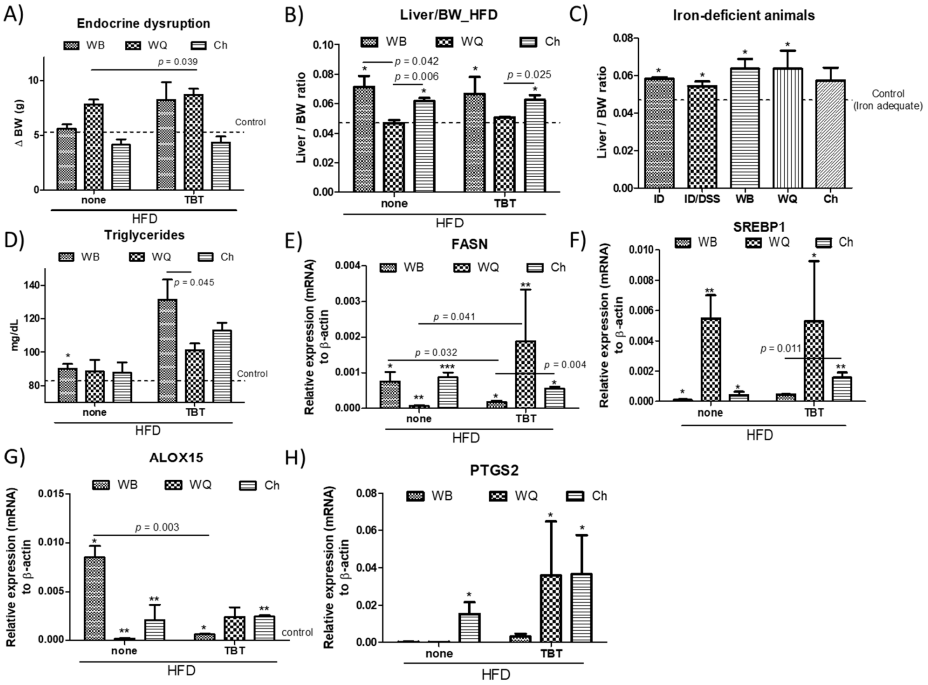

Figure 3. Effects of bread formulations-WB, wheat bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread-on body weight (BW) gain (**A**), changes in the hepatosomatic index (Liver/BW ratio) in wild type and tributyltin-exposed mice (**B**) as well as iron deficient mice (**C**), plasmatic triglyceride concentrations (**D**), and transcript levels (mRNA) of lipogenic markers-fatty acid synthase (FASN) (**E**) and sterol regulatory element-binding protein 1 (SREBP1a) (**F**), and coronary risk markers-arachidonate 15-lipoxygenase (ALOX15) (**G**) and prostaglandin-endoperoxide synthase (PTGS2) (**H**). Results are expressed as mean ± mean standard error (n = 3–6). Untreated controls are represented by the dotted line. *, **, *** indicates statistical differences between animals put under the same experimental model.

Carbohydrates and polyunsaturated fatty acids (PUFA) are identified as positive [29] and negative [30], respectively, regulators of FASN transcripts. This dietary regulation fails to explain the changes observed in the FASN mRNA levels, and the apparent decrease of lipogenic markers in animals fed with WQ. TBT exposure raises the possibility of TBT-mediated alterations in molecules regulating glucose levels as well as fatty acid breakdown, for example, decreasing adiponectin [12,31]. FASN activity is required to promote cholesterol synthesis to facilitate TLR4 signal transduction and proinflammatory macrophage activation [32]. One of the most well-established transcription factors affecting FASN is SREBP; SREBP1 promotes fatty acid synthesis, while SREBP2 is more specific to cholesterol synthesis [33]. SREBP1 amplifies autophagy [34] that can regulate the hepatocellular lipid accumulation by its selective degradation. Accordingly, increased ALOX15 expression levels in animals fed WQ and Ch may suggest that mediators such as lipoxin, resolvin, and protectin, among other metabolites, could contribute to ameliorate hepatic inflammation and insulin resistance [35]. Furthermore, PTGS2 expression levels support protective effects against diet-induced steatosis by increased transcripts of hepatic cyclooxygenase (COX)-2 expression [36]. Collectively, these data suggest a potential increase of phagocytic conditions in animals fed with the different bread formulations (i.e., WQ >> Ch > WB) that may interpret the results from animals fed with WQ and Ch as a better control of the lipid accumulation.

3.3. Control of Alterations in Glucose Homeostasis

Hepatic lipogenesis results from insulin stimulation via lipogenic gene expression. To ascertain the possible association between changes in lipid homeostasis and alterations in insulin resistance, we measured plasmatic glucose concentrations and insulin levels to determine the HOMA-IR (Figure 4). This approach allowed to observe significant HOMA-IR increases in the HFD-groups administered with WB and Ch in relation to controls (Figure 4A). Feeding WQ and Ch products significantly reduced HOMA-IR relative to WB in perinatal TBT-exposed animals on a HFD. The effect on HOMA-IR values was similar, while inducing different trends in hyperglycaemic animals (streptozotocin-induced) fed with WQ and Ch on a HFD [9]. Glucose concentrations in both preclinical models were equal when feeding all bread formulations, showing increased values in relation to the control mice (Figure 4B). However, only animals fed with WQ and Ch displayed increased insulin levels. After removing the TBT stress, the animals could recover glucose homeostasis control via insulin receptor signal and insulin levels [12]. Here, both WQ and Ch bread formulations enabled a better control on insulin production (WQ > Ch >> WB) in perinatal TBT-exposed animals (Figure 4C). When comparing the effects of *C. quinoa*- and *S. hispanica* L-containing bread formulations on the relative variations in insulin production to 'non-treated' animals on the HFD, a similar reduction of insulin production in both hyperglycemic (streptozotocin-induced) [9] and perinatal TBT-exposed animals was observed. Taken together, variations in HOMA-IR seem to occur in TBT-exposed animals as an apparently improved insulin sensitivity (Figure 4B,C), as downward insulin levels seem to suggest. This is also supported by the absence of differences in postprandial glycaemia despite the significantly reduced carbohydrate content in Ch formulation, as well as the negligible effect of dietary polyunsaturated fatty acids from Ch in comparison to animals fed with WQ. Thus, changes in the immunonutritional bioactive protein fraction [9] seems to significantly take control on glucose homeostasis independently to the disease stage.

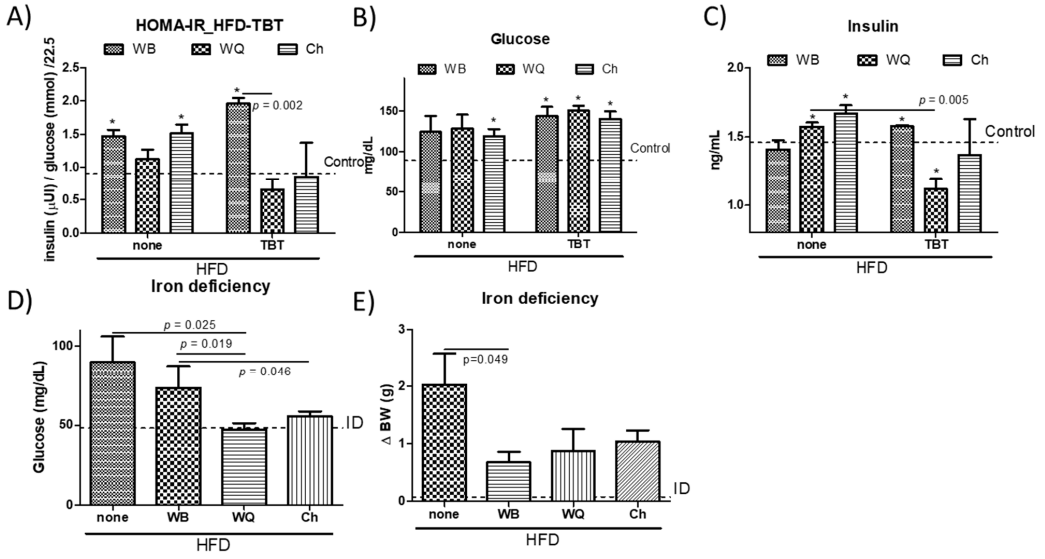

Figure 4. Biochemical parameters (HOMA-IR, **A**; glucose, **B** and insulin, **C**) in wild type and tributyltin (TBT)-exposed mice, glucose levels in iron-deficient (ID) (**D**) animals and body weight gain in ID animals (**E**), all fed with a high-fat diet and administered with different bread formulations: WB, wheat bread; WQ, white quinoa flour (25%)–containing bread and Ch, chia flour (20%)–containing bread. Results are expressed as mean ± mean standard error ($n = 6$). Untreated controls are represented by the dotted line. * indicates statistically significant ($p < 0.05$) differences in relation to controls.

Because insulin resistance relates to intestinal epithelial TLR4 expression and activity [5], we examined whether the different bread formulations influenced glucose homeostasis in animals displaying a nutritional impairment of TLR4 signaling. Low iron levels selectively impair TLR4 signaling in macrophages through the TLR4/TRAM/TRIF pathway [37]. Research efforts identified the TRIF-dependent TLR signaling as a preventive factor in hepatic inflammation and diet-induced lipid accumulation [15,38]. Feeding with the different products, glucose concentrations in iron-deficient mice (ID, Haemoglobin, 11.1 ± 0.8 g/dL) were significantly reduced (Figure 4D): WB, 46%; WQ, 63% and Ch, 50% in relation to ID animals fed only with HFD, and WB, 52%; WQ, 68% and Ch, 58% to perinatal TBT-exposed mice. Animals receiving bread formulations exhibited significant lower BW values relative to those not receiving an additional feeding to the HFD, although this difference lacked statistical significance between the different products (Figure 4F). These observations transpire in the sense that dietary starch amount contributes but does not determine glycaemia, which seemed to depend, to a significant extent, on innate immune signals that stem at the intestinal level. Experimental data may allow to interpret a differential engagement of the TLR4/TRIF signaling by WQ and Ch (Figure 2), in line with a previous report [19]. Notably, the structural differences between bioactives provided by *C. quinoa* and *S. hispanica* L flours in comparison to wheat support their differential capacity to induce innate immunity via TLR4/MyD88-dependent signaling. This suggestion also aligns with the different FASN expression levels in TBT-exposed animals, as only TLR4/MyD88-dependent signaling impairs adiponectin signaling, contributing to insulin resistance [39].

3.4. Variations on Peripheral Immune Populations

The effects on immune cells and hepatocytes can modulate metabolism in T2D and obesity conditions and, reciprocally, the nutritional status influences immune homeostasis. The alterations in peripheral leukocyte populations in different animal groups are shown

in Tables 2 and 3. Wild type and TBT-exposed mice that were fed a HFD did not show differences in the RBC or WBC of the animals. WB administration caused downward trends in peripheral MY cells of wild type animals but significant increases in GR percentages in TBT-exposed animals. These alterations were not observed in those mice who were feed with WQ or Ch formulations. In contrast, upward trends in the MY population were observed through feeding with these samples. The variations relative to controls calculated in peripheral MY and LY populations in mice that were fed with the different products are shown in Figure 5. In animals without perinatal TBT exposure (Figure 5A), opposite significant variations in the MY population after feeding WB or WQ and Ch formulations were observed. These variations only reached statistical significance between animals fed WB and WQ. Meanwhile, variations in the LY population were no longer observed. By contrast, in perinatal TBT-exposed animals, MY variations were narrowed, losing statistical significance between the different groups of treatment (Figure 5B). Otherwise, feeding with WQ and Ch caused significantly increased LY in relation to WB. We do not have direct evidence to explain the increases in the LY population. In this sense, a possible explanation could be that the expansion of lymphoid cells and disturbances of hepatic glucose homeostasis may occur as sequential events derived from losses in metabolic control by myeloid cells. When considering iron-deficient mice, feeding both WQ and Ch products promoted clear increases in the MY population, which points out the significant influence of TLR4/TRIF signaling in these changes. However, innate immune signature in MY reached statistical significance only in animals fed with Ch, while changes in the LY population were not observed (Figure 5C).

Table 2. Total red blood cells (RBC) and leukocyte (WBC) counts, and lymphoid (LY), myeloid (MY), and granulocytes (GR) percentages (%) in peripheral blood of wild type animals and those perinatally-exposed to tributyltin. Wheat bread (WB), white quinoa bread (WQ) and chia bread (Ch). Results are expressed as mean ± standard deviation (n = 5–6). Different superscript letters (a–c) indicate statistical differences ($p < 0.05$).

Treatment		**WB**		**WQ**		**Ch**	
Parameter	**Control**	**None**	**TBT**	**None**	**TBT**	**None**	**TBT**
RBC ($\times 10^9$/L)	8.8 ± 0.5^{a}	8.5 ± 0.1^{a}	8.6 ± 0.4^{a}	8.7 ± 0.3^{a}	8.6 ± 1.3^{a}	7.6 ± 1.5^{a}	7.4 ± 1.6^{a}
WBC ($\times 10^9$/L)	4.1 ± 0.9^{a}	3.4 ± 1.1^{a}	7.0 ± 1.2^{b}	3.7 ± 1.1^{a}	5.3 ± 1.2^{a}	3.8 ± 1.1^{a}	4.9 ± 2.8^{a}
LY (%)	91.7 ± 5.3^{a}	92.5 ± 3.5^{a}	84.9 ± 5.1^{a}	89.8 ± 4.6^{a}	93.2 ± 2.9^{a}	93.2 ± 3.5^{a}	94.6 ± 1.6^{a}
MY (%)	3.3 ± 1.5^{a}	2.9 ± 1.1^{a}	3.1 ± 0.3^{a}	4.0 ± 0.8^{a}	3.4 ± 1.4^{a}	3.7 ± 1.3^{a}	4.5 ± 1.7^{a}
GR (%)	4.9 ± 1.8^{a}	2.9 ± 1.1^{a}	12.0 ± 4.8^{b}	2.4 ± 1.9^{a}	3.4 ± 1.6^{a}	3.3 ± 1.6^{a}	1.1 ± 0.7^{c}

Table 3. Total red blood cells (RBC) and leukocyte (WBC) counts, and lymphoid (LY), myeloid (MY), and granulocytes (GR) percentages (%) in peripheral blood of iron-deficient mice. Wheat bread (WB), white quinoa bread (WQ) and chia bread (Ch). Results are expressed as mean ± standard deviation (n = 5–6). Different superscript letters (a–c) indicate statistical differences ($p < 0.05$).

Treatment		**WB**	**WQ**	**Ch**
Parameter	**Control**			
RBC ($\times 10^9$/L)	6.6 ± 0.8^{a}	7.3 ± 0.8^{a}	6.5 ± 0.9^{a}	6.5 ± 0.7^{a}
WBC ($\times 10^9$/L)	2.3 ± 0.6^{a}	3.1 ± 1.1^{a}	3.4 ± 1.3^{a}	3.6 ± 1.8^{a}
LY (%)	95.9 ± 1.4^{b}	93.5 ± 1.4^{ab}	93.7 ± 3.8^{ab}	90.8 ± 7.2^{a}
MY (%)	3.8 ± 0.1^{a}	3.3 ± 0.7^{a}	4.0 ± 0.9^{a}	4.0 ± 0.2^{a}
GR (%)	2.9 ± 0.6^{ab}	2.8 ± 1.5^{ab}	1.5 ± 0.8^{a}	3.8 ± 2.2^{b}

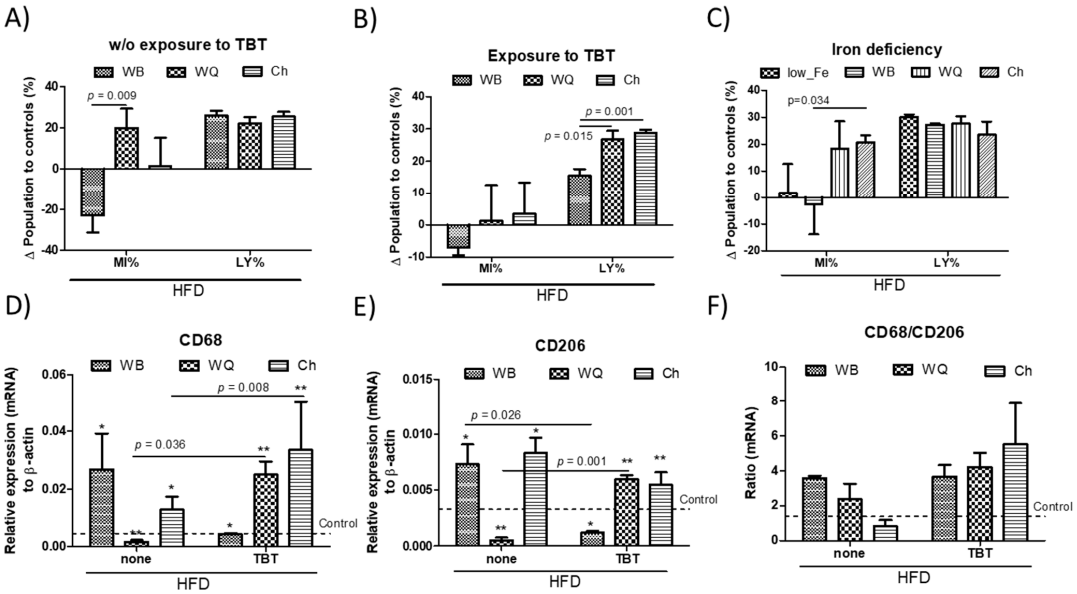

Figure 5. Peripheral relative variations of myeloid (MY) and lymphoid (LY) populations in wild type (**A**) and tributyltin (TBT)-exposed mice (**B**) as well as in iron-deficient (**C**) animals. Hepatic rt-qPCR analysis (mRNA) of selected macrophage marker genes; CD68 (**D**), CD206 (**E**) and their relative variation (**F**). Animals were fed with different bread formulations-WB, wheat bread; WQ, white quinoa flour (25%)–containing bread; Ch, chia flour (20%)–containing bread. Results are expressed as mean ± mean standard error (n = 6). Untreated controls are represented by the dotted line. *, ** indicates statistical differences between animals put under the same experimental model.

Differences in immune stimulation can promote the modulation of cell-mediated immunity within the gut-liver axis, modulating the proportion of immune cells infiltrating into liver, and thereby HFD-induced insulin resistance. Hence, rt-qPCR analysis allowed to evaluate the relative mRNA levels of selected macrophage marker genes (Figure 5D–F). Since HFD-induced insulin resistance is commonly accompanied with chronic inflammation, the mRNA levels of CD68 (M1 phenotype) and CD206 (M2 phenotype) were measured (Figure 5D,E). In HFD-animals, the results show that feeding with WQ and Ch significantly down-regulated the CD68 gene expression in relation to WB. However, animals with perinatal TBT exposure displayed rather different results, showing a significant up-regulation of the CD68 transcripts in animals fed with WQ and Ch. In contrast, WB significantly decreased those transcripts in relation to their counterparts without TBT exposure. By comparison, CD206 transcripts exhibited similar trends but at a lower level than CD68. Experimental data show that both WB and Ch products up-regulate hepatic CD68 and CD206 mRNA levels, respectively, indicating that feeding does not block macrophage infiltration. These results allowed to calculate relative variations between macrophage's M1/M2 phenotypes higher than those in controls (Figure 5F), which were not observed in those animals fed with Ch without perinatal TBT exposure.

Myeloid cells (i.e., monocytes and macrophages) dominantly express the TLR4 receptor in relation to lymphoid (i.e., ILCs and Th cells) populations. Taken together, data transpire in the sense of a peripheral myeloid trafficking/polarization as a key cell-mediated coordination process between hepatic metabolic lipid alterations and long-term, adaptive immune responses. This is in line with the generally accepted alterations in the macrophage metabolism contributing to impair liver dysfunction [40]. Increased myelopoiesis and M1 polarization may represent an integrated mechanism to control circulating nutritional fatty acids induced TLR4-mediated activation of macrophages [41] ending on and inhibited pro-

liferation [14] and malfunction in the lympho-myeloid populations [42]. Furthermore, proliferation/survival and cytokine production of ILC2s, active contributors to diet-induced obesity [6], is suppressed by IFN-γ and, to a lesser extent, by IL-27, which expression in human macrophages occurs in an IFNα mediated fashion [43]. This could be an example of the important role of diet influencing reciprocal interaction between innate immunity and the metabolic system.

These data are consistent with previous studies demonstrating a role for innate lymphoid cells determining insulin secretion and glycaemia [6]. For example, alterations in the myeloid cell populations have been identified as potentially relevant effectors in the metabolic control of glucose homeostasis. However, the underlying innate immune signaling allowing myeloid control of liver metabolism is unclear. Because WQ and Ch provide immunonutritional agonists are able to interact with the innate immune TLR4, intestinal TLR4 may act as a key trigger of the immunometabolic processes. Notably, the immunonutritionally-induced resistance to obesity in animals developing a transgenerational increased liver fat accumulation, in which insulin resistance also occurs, was rescued by feeding with WQ and Ch but not with WB. These findings support that intestinal TLR4 signaling was likely important in the control of glycaemia and insulin resistance. Collectively, data evidenced a role for immunonutritional agonists from *C. quinoa* and *S. hispanica* in intestinal innate immunity in the control of glucose homeostasis.

4. Conclusions

In summary, the reciprocal contributions of lymphoid cells populations and lipid metabolism to immunonutritional outcomes of the products cannot be excluded. Indeed, lipid metabolism is important for the expansion and function of the lymphoid cells population, enhancing TLR4-mediated proinflammatory signaling [44]. Fatty acid synthase-dependent MyD88 palmitoylation is necessary for TLR4-induced inflammation [32]; however, both WQ and Ch may interact with TLR4 triggering MyD88-independent signaling in myeloid cells [19]. Thereby, WQ and Ch products improved glucose homeostasis in comparison to WB, this is consistent with previous studies demonstrating that it associates with early increased peripheral myeloid cells population [45,46]. Collectively, it is likely that immunonutritional agonists may contribute to maintaining the function of hepatic myeloid populations [i.e., monocytes and macrophages], which act as critical effectors determining the function of innate lymphoid cells (i.e., Tregs and ILCs) in the regulation of glucose homeostasis in mice on a HFD.

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Institutional Review Board Statement: Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of CSIC (Consejo Superior de Investigaciones Científicas) and the protocol was approved by its Ethics Committee (Proex 080/19).

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