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

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ABSTRACT

High energy intake promotes imbalances in nutrients homeostasis contributing to the high prevalence of metabolic chronic diseases. The extent to what metabolic imbalances can be ameliorated by the inclusion of immunonutritional ingredients from flours favouring nutrients and calorie management remains poorly understood. Here, it is showed that partial replacement of wheat flour (WB) by these 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 derived from a high-fat diet consumption in hyperglycaemic animals. Feeding animals with bread formulations replacing wheat flour effectively reduced insulin resistance (by 2-fold, HOMA_{IR}). The reduction of starch content did not appear as determinant at controlling HOMA_{IR}. Only animals fed with RQ and Ch displayed increased plasmatic levels of triglycerides, which significantly contributed to mitigate HFD-induced hepatic lipid peroxidation, while elimination of PUFA negatively increased it. Feeding WQ and Ch samples caused an upward trend in hepatic TNF- α and IL-6 levels. Despite similarities between immunonutritional agonists from RQ and WQ, it was quantified higher IL-17 levels for WQ. All bread formulations except Ch_D significantly increased the hepatic granulocyte-monocyte colony stimulation factor levels. These results show that replacement of wheat flour, influencing the proportion of immunonutritional ingredients and protease inhibitory activity, in bread formulations improved metabolic imbalances to hyperglycaemic animals.

Keywords: Hyperglycaemia; High fat diet; Metabolic imbalances; Quinoa; Chia

1. Introduction

Western lifestyle (i.e., food choice and lack of physical activity) has led to an intense and continuous increase of the prevalence of metabolic diseases such as obesity, type 2 diabetes (T2D)¹ and of other major features of the metabolic syndrome. In early stages of metabolic disorders, there is an increased risk of developing T2D associated with alterations in insulin resistance². Here, hyperglycaemia also contributes to disrupt physiological lipid handling and management favouring dysfunctional alterations in hepatocytes³. Of note, recent data suggest that non-alcoholic fatty liver disease (NAFLD) incurs a high risk for the development of T2D, placing the liver in the centre of nutritional studies⁴. In this context, a significant bulk of evidence indicates the close relationship between innate immune signalling and hepatic metabolism⁵, worsening or improving the consequences derived from excessive nutrients availability.

Diet has acquired great importance due to its physiological involvement in the metabolic modulation where nutritional strategies can help either preventing or ameliorating metabolic alterations⁶. Prospective nutritional evaluations clearly established a direct relationship between consumption of white bread and the risk of becoming overweight/obese⁷. Besides, research efforts have evidenced the potential of replacing wheat flour by 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 increasing the expression of hepatic immunometabolic transcription factors such as PPAR- γ , helping to control glycaemic index and nutrients 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 population¹⁰, which exerts a key regulatory role on hepatic cholesteryl ester transfer protein raising high-density lipoprotein (HDL)-cholesterol¹¹. Additionally, *S. hispanica* is recognised as a rich source of polyunsaturated fatty acids that may play an important role in the management of insulin resistance¹². The promotion of a favourable immunometabolic environment may significantly contribute in 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 by that from Latin-American crops with metabolic and inflammation perturbations caused by 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 T2D¹⁵. Nevertheless, overfeeding a high-fat diet to animals made them develop insulin resistance where hyperglycaemia is associated with hypertriglyceridemia¹⁶. 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 resistance¹³. Longer periods of treatment cause the decline in insulin production (phase 2, 18-42 weeks) and decrease 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 wheat flour replacement by the inclusion of that from different varieties of *C. quinoa* (i.e., white and red) and processed *S. hispanica* seeds (i.e., whole and semi-defatted) in bread formulations on the impact of high fat diet consumption in hyperglycaemic animals at early stages of T2D development.

2. Materials and methods

2.1. Bread samples

Red and white quinoa seeds (Organic quinoa Real[®]) from ANAPQUI, La Paz, Boliva, were purchased from Ekologikoak (Bizkaia, Spain). Whole and semi-defatted chia flour were provided from 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 starch and lipid contents (Table 1)^{14,15}: red quinoa (RQ) at 25%, white quinoa (WQ) at 25%, chia (Ch) at 20 %, semi-defatted chia (Ch_D) at 20% and were compared to wheat bread (WB)¹⁶.

2.2 Immunonutritional agonists in flours: Bioaccessibility

Aliquots (0.5 g) of flours were processed and extracted and sequentially filtered through a 50-30KDa membrane (Amicon[®])¹⁷. Then, protease inhibitory activity was quantified in the <30 KDa fraction¹⁷.

Flours used for bread formulation were subjected to a simulated gastrointestinal digestion⁹ and bioaccessible immunonutritional agonists were monitored by HPLC-RP-DAD¹⁷. Briefly, flours were subjected to a two-step gastric (pepsin, 1h, 37°C) and intestinal (pancreatin/bile extract, 1h, 37°C)

116 digestion⁹. After addition of the pancreatin-bile extract solution, the volume brought to 10 mL with
117 isotonic saline solution and 2 mL of the gastrointestinal digestion was carried out in the upper (donor
118 compartment) chamber of a bicameral system. The bottom (acceptor compartment) of the system was
119 filled up with 2 mL of an isotonic saline solution.

120 The bioaccessible protein profile was defined on a 1260 Agilent HPLC system equipped with a
121 quaternary pump and a photodiode array detector set at 280 nm (Agilent Technologies, Waldbronn,
122 Germany). The separation (0.8 mL/min) was performed on a Poroshell 120 (7.5 cm x 4.6 mm) C18
123 column (Agilent)¹⁷.

124

125 *2.3. Animals*

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

131

132 *2.4. Experimental design*

133 All animals were injected intraperitoneally with streptozotocin (STZ) (two doses of 25 mg/kg in two
134 successive days)¹⁸ and hyperglycaemic animals were put under a high-fat diet (HFD)¹⁹ (EF AIN93G
135 modified, Ssniff Spezialdiäten GmbH, Soest, Germany) for 3 weeks. Mice were maintained under
136 controlled environment of temperature (21-23°C), humidity (55%) and a 12h:12h (light:dark) cycle
137 with food and water ad libitum. Animals were distributed in 5 groups (n=6/group) depending on the
138 bread administered. Bread formulations were administered 3 times per week (14 mg/day/animal)
139 during the period of study. The amount of administered bread was established according to daily
140 nutritional recommendation for bread consumption of 150g/day/70Kg body weight. The food intake
141 and body weight of each group were monitored every 2 days. After treatment, mice were sacrificed by
142 cervical dislocation.

143

144 *2.5. Biochemical parameters*

145 Blood samples were centrifuged (6,000×g/10 min) to get clear supernatants. Insulin and glucose
146 concentrations were determined in plasma samples. Insulin (RAB0817-1KT, Sigma-Aldrich,
147 Darmstadt, GER) and glucose (MAK263-1KT, Sigma-Aldrich, Darmstadt, GER) and triglycerides

148 (Cayman Chemical, n° 10010303, Michigan, USA) were measured using commercial kits. Insulin
149 resistance (IR) was defined as the homeostatic model assessment of insulin resistance (HOMAIR) value
150 and its behaviour was studied according to the treatment received. HOMAIR was calculated according
151 to the following formula¹⁹: [insulin (μU/ml)*glycaemia (mg/dL)]/405.

152

153 2.6. Determination of hepatic triglycerides and malondialdehyde levels

154 Liver sections were kept in Krebs's buffer (1 ml), containing the Complete Protease Inhibitor
155 Cocktail (Sigma), until analysis. Samples were thaw up and homogenised using a TissueRuptor
156 (Qiagen). Then, homogenates were centrifuged (10,000×g/15 min) to get clear supernatants. Then,
157 hepatic triglycerides determination was performed with a commercial kit (Cayman Chemical, n°
158 10010303, Michigan, USA) according to manufacturer's instructions. Malondialdehyde (MDA) was
159 determined in hepatic homogenates by using a thiobarbituric acid–reactive substances (TBARS) HPLC
160 method as described elsewhere²⁰. The HPLC analysis was performed on an Agilent 1260 system
161 (Germany) with a diode array detector by using a Poroshell C18 (5 μm 2.7 × 50 mm) (Agilent) with
162 detection at 532 nm. Total protein content in plasma and hepatic homogenates was determined with the
163 Pierce®-BCA method (ThermoFisher®, Rockford, USA) to normalise the results between the different
164 samples.

165

166 2.7 Hepatic inflammatory markers

167 Innate immune 'Toll-like' receptor (TLR)-4 was determined by quantitative reverse transcription
168 real-time polymerase chain reaction (qRT-PCR). Validated Gene Expression Assays for murine TLR4
169 (forward 5'-TGG GAA CAC ACG GTT GGA AA -3', reverse 5'-ACA GCA AGT TGT AGC ACT
170 ACT GA-3') was purchased from Applied Biosystems (Foster City, CA, USA). qRT-PCR was
171 performed with 500 ng of cDNA from liver sections, using the Universal PCR Master Mix (Applied
172 Biosystems, ThermoFisher®). Quantitative values were calculated by using the $2^{-\Delta Ct}$ method¹⁷.

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

2.8. Statistical analyses

Statistical analyses were performed with the software Statgraphics Centurion XVI. 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 diet.

Inclusion of flours from *C. quinoa*, white and red varieties, as well as *S. hispanica*, whole and defatted, was based considering different scenarios; i) to evaluate the effects derived from replacement of wheat immunonutritional agonists by those provided by *C. quinoa* and *S. hispanica*, and ii) to evaluate the interaction of immunonutritional agonists with different starch levels in presence of polyunsaturated fatty acids (PUFA) provided by *S. hispanica*, influencing insulin resistance development. According to their origin, white or red *C. quinoa*, reverse phase-HPLC-DAD analyses did not reveal significant differences between bioaccessible immunonutritional agonists. Notwithstanding, it was quantified significant variations in the proportion of immunonutritional compounds quantified in a low molecular (<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).

Levels of both carbohydrates and fat were taken into consideration to put animals under a High-fat diet (Table 3). Carbohydrate level of the diet used were elevated up to the highest level commonly used in the literature according to the experimental model (STZ-treated animals). Fat content was established at the highest percentage to be representative of a HFD as previously reported (30-50%)²¹. According to the literature, both HFD and 'very' HFD (58-60%) develop insulin resistant 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) previously reported¹³.

3.2. Changes in body weight

Animals fed with RQ, WQ and Ch_D exhibited a negative trend in body weight (BW) gain (Fig. 1) relative to WB, but only administration of RQ had a statistically significant effect (Fig. 1A). Notably,

the observed differences in HFD-induced BW gain was 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) (215.6 kcal/d) over the study period. Although not significant ($p>0.05$), due to the data dispersion, it could be found an opposite trend in mean values for body weight gain between animals administered with Ch or Ch_D formulations. Morphometric comparison of the hepatosomatic ratios revealed a positive trend in all groups of treatment in relation to WB. Only animals receiving RQ 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 does not appear as a major contributor controlling hyperglycaemia in comparison to RQ and WQ. Animals administered with RQ and Ch displayed a relative 2-fold lower glycaemia to animals administered with WB. Only animals fed with Ch_D did not display significant 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. Notably, all groups of treatment displayed reduced HOMA_{IR} values (Fig. 2C) evidencing the improved insulin resistance. HOMA_{IR} values were close to #3, even lower for RQ and Ch fed animals, which is considered the limit to establish an insulin resistance.

3.4. Lipid parameters

Plasmatic triglycerides concentrations (Fig. 3A) were increased ($p<0.05$) only in animals administered with Ch_D, but an increasing trend was also observed in those receiving RQ and Ch. Interestingly, highest mean triglycerides concentrations associate to those animals exhibiting the lowest glycaemia (Fig. 2A). Otherwise, it was not quantified significant differences in hepatic triglycerides concentrations between the different groups of treatment (Fig. 3B). However, all groups exceed normal hepatic triglyceride contents genetically determined in mice²².

Hepatic levels of malonaldehyde (MDA), a reactive metabolite derived from lipid peroxidation, was quantified to evaluate the influence of bread formulations in the control of HFD-induced excessive oxidative stress (Fig. 3C). Feeding RQ, WQ and Ch samples significantly reduced the degree of lipid peroxidation in relation to WB. Between the quinoa groups, it was quantified a significant difference ($P<0.05$), where RQ exhibited a more positive effect reducing hepatic MDA levels. Notably, animals

fed with Ch_D showed 1.5-fold higher MDA levels than its whole counterpart, from where it can be hypothesised a protector role 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 which obtained similar values (Fig. 4A). WQ and Ch 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 , and it was quantified a significant difference ($P<0.05$) between the concentrations in animals fed RQ and WQ. This behaviour was similar in IL-17 levels (Fig. 4D) suggesting that WQ could exert a greater influence in the hepatic inflammatory processes in comparison to RQ. In relation to variations in GM-CSF (Fig. 4E), only animals fed with Ch_D showed significantly ($P<0.05$) reduced levels of the cytokine in relation to WB. Besides, feeding WQ promoted upward trends in GM-CSF values suggestive of a more prominent anti-inflammatory innate immune response.

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* into bread formulations provides beneficial effects ameliorating the immunometabolic conditions derived from high energy intake. These effects appeared modifiable in hyperglycaemic animals fed a HFD during 3 weeks likely reproducing early features of T2D/metabolic syndrome²³.

According to the proximate composition of flours, the similar starch content in WB and those from *C. quinoa*, WQ and RQ, samples makes unlikely that better controlled variations in the plasmatic glucose concentrations could be attributed to starch (Table 1). Moreover, the statistical significance between insulin levels when animals receive RQ vs WQ 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 plasmatic glucose concentrations were inversely associated to 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.*

276 quinoa¹⁷. Pilot studies revealed slightly, but significant differences in the coefficients of diffusion for
277 the immunonutritional agonists from RQ and WQ (*data not shown*), where the slowest kinetic might be
278 associated to a better control in insulin production (Fig. 1B). Immunonutritional agonists found in the
279 flours have proved a significant potential to down-regulate the expression of key enzymes (i.e.,
280 GAPDH) involved in the glycolysis processes according to the following gradation²⁴: wheat, 34.9%;
281 quinoa, 44.2% and chia, 51.5%. These effects occur via their immunonutritional role interacting with
282 the innate immune ‘Toll-like’ receptor (TLR)-4^{24,25} (Fig. 4A). Accordingly, the lower
283 immunonutritional potential of RQ is supported by downward trends in the hepatic inflammatory
284 milieu, TNF α (Fig. 4B) and IL-6 (Fig. 4C), that is dependent on TLR4 engagement of adaptor
285 molecules and downstream molecular signalling.

286 To approach the comprehensive effect of n-3PUFAs, chia flour was defatted attaining a reduction of
287 the lipid fraction up to 70% (Table 1). It was observed an increase in the protein fraction that can be
288 attributed, at least in part, to the conversion factor used to estimate crude protein from total
289 nitrogen^{14,26}. Otherwise, methods targeting peptides containing three or more amino acid residues²⁷ are
290 affected to a lesser extent by protein compositional differences to provide greater concentration
291 accuracy. Accordingly, significant lower protein contents in defatted chia flours have been reported,
292 22.7 ± 0.7 g/100g²⁷, in comparison to those using the conversion of nitrogen^{14,26}, 29.4-31.7 g/100g.
293 Defatting causes a significant reduction (by 39.7%) of the α -amylase inhibitory activity of chia²⁸
294 attributed to the immunonutritional compounds. These effects can explain the upward trend of glucose
295 concentrations in animals fed Ch_D in relation to their counterparts receiving Ch (Fig. 2A).
296 Collectively, it can be hypothesized significant impairments in the tertiary structure of the compounds
297 responsible for the immunonutritional activity due to the loss of capability to interact with TLR4 (Fig.
298 4A). Previous data suggest that n-3PUFA may improve postprandial hyperglycaemia as well as insulin
299 secretion ability and hypertriglyceridemia, with impaired glucose metabolism^{29,30}. Neither duration nor
300 dosage appear to explain the observed heterogeneity in response to n-3PUFAs³⁰. Here, the significant
301 reduction of the lipid fraction could be concordant with the variation observed in plasmatic glucose,
302 however, it cannot explain the improved control on insulin levels (Fig. 2B-C). This observation
303 transpires in the sense of supporting the role of immunonutritional compounds.

304 Immunonutritional agonists from quinoa and chia have been shown to significantly down-regulate
305 the expression of the fatty acid translocase (i.e., CD36)¹⁰, thereby modulating the breadth of signalling
306 for TLR4²⁴. In the last few years, different studies pointed out the significant effect of regulation of the
307 TLR4/NF- κ B-pathway on obesity-associated imbalances in biochemical parameters (i.e.,

triglycerides)^{18,31,32}. Commonly, the reduction of plasmatic 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 a mechanism to prevent the liver from developing insulin resistance³³. These observations agree with findings relative to the important regulatory and sequential role of innate and adaptive immunity to establish tissue lipid homeostasis³⁴. When considering the influence of n3-PUFA data did not support potential positive effects of PUFA regulating the plasmatic triglycerides 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. Here, TLR4 has been identified as a mediator able to induce epoxy-oxylipin products promoting the protective effects of soluble epoxide hydrolase³⁵. This protective effect appears quite well reflected in the reduced MDA levels quantified in animals fed with Ch in comparison to Ch_D. 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 TLR4³⁶.

Here, the extent to what RQ and WQ affect MDA levels (Fig. 3C) appears inversely associated to the level of TLR4 transcripts (Fig. 4A), which commonly associates with protein content. These differences are revealed by the upward trends in TNF α and IL-6 production (Fig. 4B-C). Collectively, lower mRNA levels of TLR4 with increased cytokine production may interpret the experimental data showing faster kinetics if 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 and negative effects, on metabolic control and insulin sensitivity is still under debate³⁷⁻³⁹. The lack of significant differences in the phenolic content between RQ and WQ samples⁴⁰ transpires in the sense of a protein-mediated effect as unique promoter of different TLR4 transcript levels. Also, neither the isolated polyphenols profile nor total antioxidant capacity (DPPH method) estimated for *S. hispanica* flours, semi-defatted or not, could be associated to the extractable or bioaccessible total antioxidant capacity estimated for these fractions⁴¹.

TLR4 mRNA levels together with the trend for both cytokines quantified in animals receiving Ch is tempting to suggest similarities between immunonutritional agonists in WQ and Ch. But 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 action⁴². Accordingly, variations in MDA levels between WQ

and Ch are explained (Fig. 3A-C), at least in part, by lower PKC expression levels favoured by Ch helping to ameliorate the effects of fat oversupply. The decreased hepatic IL-17 levels (Fig. 4D) also supports positive effects on the high-fat diet-induced metabolic stress by inhibiting fatty acid β -oxidation⁴³. With this on mind, the negative balance in GM-CSF levels (Fig. 4E) attained by feeding Ch allows suggesting a better controlled HFD-induced impaired insulin sensitisation (Fig. 2A-B) as reflected in lower HOMA_{IR} (Fig. 2C). Overall, the lack of these inflammatory response(s) helps supporting the partial loss of bioactivity by immunonutritional agonists during defatting as previously suggested.

5. Conclusions

Replacement of wheat flour by those 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 derived from high fat diet consumption to hyperglycaemic animals. A relative short STZ/HFD model (3 weeks) allows monitoring both hyperglycaemia and hypertriglyceridemia as well as insulin sensitization and resistance development. Here, immunonutritional agonists rather than other nutrients such as PUFA significantly contributed to a higher extent to the beneficial outcomes. These results support food composition rather than calorie intake as a major determinant of the nutritional consequences. Hence more intense research into food and health based on the advances made in life sciences is needed. These data help opening new avenues for the potential use of these bread formulations helping to tailor personalised nutritional intervention strategies to ameliorate the severity of chronic metabolic diseases. Further research with human trials is needed to determine the magnitude of these beneficial effects.

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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).

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

References

- [1] S. N. Bhupathiraju and F.B. Hu, Epidemiology of obesity and diabetes and their cardiovascular complications, *Circ Res*, 2016, **118**(11), 1723–1735.
- [2] K.C. Sung, W.S. Jeong, S.H. Wild and C.D. Byrne, Combined influence of insulin resistance, overweight/obesity, and fatty liver as risk factors for type 2 diabetes, *Diabetes Care*, 2012, **35**(4), 717–722.
- [3] K.H. Williams, N.A. Shackel, M.D. Gorrell, S.V. McLennan and S.M. Twigg, Diabetes and nonalcoholic fatty liver disease: A pathogenic duo, *Endocr Rev*, 2013, **34**(1), 84–129.
- [4] K. Linder, F. Springer, J. Machann, F. Schick, A. Fritsche, H. U. Häring, G. Blumenstock, M. B. Ranke, N. Stefan, G. Binder and S. Eehalt, Relationships of body composition and liver fat content with insulin resistance in obesity-matched adolescents and adults, *Obesity*, 2014, **22**(5), 1325–1331.
- [5] L. Bai and H. Li, Innate immune regulatory networks in hepatic lipid metabolism, *J Mol Med (Berl)*, 2019, **97**(5), 593–604.
- [6] H. Kolb and S. Martin, Environmental/lifestyle factors in the pathogenesis and prevention of type 2 diabetes, *BMC Med*, 2017, **15**(1), 131.
- [7] C. de la Fuente-Arrillaga, M. A. Martinez-Gonzalez, I. Zazpe, Z. Vazquez-Ruiz, S. Benito-Corchon and M. Bes-Rastrollo, Glycemic load, glycemic index, bread and incidence of overweight/obesity in a Mediterranean cohort: the SUN project, *BMC Public Health*, 2014, **14**, 1091.
- [8] J. M. Laparra and M. Haros, Inclusion of ancient Latin-American crops in bread formulation improves intestinal iron absorption and modulates inflammatory markers, *Food Funct*, 2016, **7**(2), 1096–1102.
- [9] J.M Laparra and M. Haros, Inclusion of whole flour from Latin-American crops into bread formulations as substitute of wheat delays glucose release and uptake, *Plant Foods Hum Nutr*, 2018, **73**(1), 13–17.
- [10] J. Laparra, B. Fotschki and C. Haros, Immunonutritional consequences of different serine-type protease inhibitors in a C57BL/6 hepatocarcinoma model, *Oncotarget*, 2019a, **10**(7), 760–772.
- [11] S. J. L. van der Tuin, Z. Li, J. F. P. Berbée, I. Verkouter, L. E. Ringnalda, A. E. Neele, J. B. van Klinken, S. S. Rensen, J. Fu, M. P. J. de Winther, A. K. Groen, P. C. N. Rensen, K. W. van Dijk and Y.

Wang, Lipopolysaccharide lowers cholesteryl ester transfer protein by activating F4/80⁺Clec4f⁺Vsig4⁺Ly6C⁺ kupffer cell subsets, *J Am Heart Assoc*, 2018, **7**(6). pii: e008105.

[12] M. Lepretti, S. Martucciello, M. A. Burgos Aceves, R. Putti and L. Lionetti, Omega-3 Fatty Acids and Insulin Resistance: Focus on the Regulation of Mitochondria and Endoplasmic Reticulum Stress. *Nutrients*, 2018, **10**(3), 350.

[13] D. A. Barrière, C. Noll, G. Roussy, F. Lizotte, A. Kessai, K. Kirby, K. Belleville, N. Beaudet N, J. M. Longpré, A. C. Carpentier, P. Geraldès and P. Sarret, Combination of high-fat/high-fructose diet and low-dose streptozotocin to model long-term type-2 diabetes complications. *Sci Rep*, 2018, **8**(1),424.

[14] M. T. Fernández-Espinar, J. V. Gil, M. Segura-Campos and M. Haros, in *CHÍA (Salvia hispanica L.) The old food of the future*, J. J. P. Jiménez and F. M. Rodríguez, Editorial Universidad de Sevilla, Seville, Spain, 2016, Charter 3, pp. 127–135.

[15] J. Ballester-Sánchez, M. C. Millán-Linares, M. T. Fernández-Espinar and C. M. Haros, Development of Healthy, Nutritious Bakery Products by Incorporation of Quinoa, *Foods*, 2019, **8**(9), 379.

[16] E. Iglesias-Puig and M. Haros, Evaluation of performance of dough and bread incorporating chia (*Salvia hispanica* L.), *Eur Food Res Technol*, 2013, **237**(6), 865–874.

[17] J. M. Laparra and C. M. Haros, Plant seed protease inhibitors differentially affect innate immunity in a tumor microenvironment to control hepatocarcinoma, *Food Funct*, 2019b, **10**(7), 4210–4219.

[18] J. M. Laparra, M. Díez-Municio, F. J. Moreno and M. Herrero, Kojibiose ameliorates arachidic acid-induced metabolic alterations in hyperglycaemic rats, *Br J Nutr*, 2015, **114**(9), 1395–1402.

[19] D. A. De Magalhães, W. T. Kume, F. S. Correia, T. S. Queiroz, E. W. A. Neto, M. P. Dos Santos, N. H. Kawashita and S. A. De França, High-fat diet and streptozotocin in the induction of type 2 diabetes mellitus: a new proposal. *An Acad Bras Cienc*, 2019, **91**(1), 14.

[20] J. F. Young, S. E. Nielsen, J. Haraldsdóttir, B. Daneshvar, S. T. Lauridsen, P. Knuthsen, A. Crozier, B. Sandström, and L. O. Dragsted, Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status, *Am J Clin Nutr*, 1999, **69**(1), 87–94.

[21] S. Gheibi, K. Kashfi and A. Ghasemi, A practical guide for induction of type-2 diabetes in rat: Incorporating a high-fat diet and streptozotocin. *Biomed Pharmacother*, 2017, **95**, 605–613.

[22] X. Lin, P. Yue, Z. Chen and G. Schonfeld, Hepatic triglyceride contents are genetically determined in mice: results of a strain survey, *Am J Physiol Gastrointest Liver Physiol*, 2005, **288**(6), G1179–G1189.

435 [23] K. Srinivasan, B. Viswanad, L. Asrat, C. L. Kaul and P. Ramarao, Combination of high-fat diet-
 436 fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological
 437 screening, *Pharmacol Res*, 2005, 52(4), 313–320.

438 [24] M. Srdić, I. Ovčina, B. Fotschki, C. M. Haros and J. M. Laparra, *C. quinoa* and *S. hispanica* L.
 439 Seeds Provide Immunonutritional Agonists to Selectively Polarize Macrophages, *Cells*, 2020, 9(3),
 440 593.

441 [25] Y. Junker, S. Zeissig, S. J. Kim, D. Barisani, H. Wieser, D. A. Leffler, V. Zevallos, T. A.
 442 Libermann, S. Dillon, T. L. Freitag, C. P. Kelly and D. Schuppan, Wheat amylase trypsin inhibitors
 443 drive intestinal inflammation via activation of toll-like receptor 4. *J Exp Med*, 2012, 209(13), 2395–
 444 2408.

445 [26] R. González-Luna, R. González-Luna, S. Moreno, D. Quistián, M. M. Yust, M.C. Millán-Linares,
 446 F. Millán and J. Pedroche in *CHÍA (Salvia hispanica L.) The old food of the future*, J. J. P. Jiménez and
 447 F. M. Rodríguez, Editorial Universidad de Sevilla, Seville, Spain, 2016, Charter 2, pp. 87–93.

448 [27] M. E. Valverde, D. Orona-Tamayo, T. Hernández-Pérez and O. Paredes-López, in *CHÍA (Salvia*
 449 *hispanica L.) The old food of the future*, J. J. P. Jiménez and F. M. Rodríguez, Editorial Universidad de
 450 Sevilla, Seville, Spain, 2016, Charter 1, pp. 17–26.

451 [28] M. Segura-Campos, E. Martínez-Leo, M. T. Fernández-Espinar, J. V. Gil and C. M. Haros, in
 452 *CHÍA (Salvia hispanica L.) The old food of the future*, J. J. P. Jiménez and F. M. Rodríguez, Editorial
 453 Universidad de Sevilla, Seville, Spain, 2016, Charter 3, pp. 137–146.

454 [29] T. Sawada, H. Tsubata, N. Hashimoto, M. Takabe, T. Miyata, K. Aoki, S. Yamashita, S. Oishi, T.
 455 Osue, K. Yokoi, Y. Tsukishiro, T. Onishi, A. Shimane, Y. Taniguchi, Y. Yasaka, T. Ohara, H. Kawai
 456 and M. Yokoyama, Effects of 6-month eicosapentaenoic acid treatment on postprandial hyperglycemia,
 457 hyperlipidemia, insulin secretion ability, and concomitant endothelial dysfunction among newly-
 458 diagnosed impaired glucose metabolism patients with coronary artery disease. An open label, single
 459 blinded, prospective randomized controlled trial. *Cardiovasc Diabetol*, 2016, 15:121.

460 [30] L. L. O'Mahoney, J. Matu, O. J. Price, K. M. Birch, R. A. Ajjan, D. Farrar, R. Tapp, D. J. West, K.
 461 Deighton, M. D. Campbell, Omega-3 polyunsaturated fatty acids favourably modulate cardiometabolic
 462 biomarkers in type 2 diabetes: A meta-analysis and meta-regression of randomized controlled trials.
 463 *Cardiovasc Diabetol*, 2018, 17:98.

464 [31] X. Chen, S. Wu, C. Chen, B. Xie, Z. Fang, W. Hu, J. Chen, H. Fu and H. He, Omega-3
 465 polyunsaturated fatty acid supplementation attenuates microglial-induced inflammation by inhibiting

the HMGB1/TLR4/NF- κ B pathway following experimental traumatic brain injury. *J Neuroinflammation*, 2017, **14**(1):143.

[32] S. J. L. van der Tuin, Z. Li, J. F. P. Berbée, I. Verkouter, L. E. Ringnalda, A. E. Neele, J. B. van Klinken, S. S. Rensen, J. Fu, M. P. J. de Winther, A. K. Groen, P. C. N. Rensen, K. W van Dijk and Y. Wang, Lipopolysaccharide Lowers Cholesteryl Ester Transfer Protein by Activating F4/80+Clec4f+Vsig4+Ly6C- Kupffer Cell Subsets. *J Am Heart Assoc*, 2018, **7**(6). pii: e008105.

[33] K. Uchimura, M. Hayata, T. Mizumoto, Y. Miyasato, Y. Kakizoe, J. Morinaga, T. Onoue, R. Yamazoe, M. Ueda, M. Adachi, T. Miyoshi, N. Shiraishi, W. Ogawa, K. Fukuda, T. Kondo, T. Matsumura, E. Araki, K. Tomita and K. Kitamura, The serine protease prostaticin regulates hepatic insulin sensitivity by modulating TLR4 signalling. *Nat Commun*, 2014, **5**, 3428.

[34] K. Mao, A. P. Baptista, S. Tamoutounour, L. Zhuang, N. Bouladoux, A. J. Martins, Y. Huang, M. Y. Gerner, Y. Belkaid and R. N. Germain, Innate and adaptive lymphocytes sequentially shape the gut microbiota and lipid metabolism, *Nature*, 2018, **554**(7691), 255–259.

[35] S. Thomson, M. L. Edin, F. B. Lih, M. Davies, M. M. Yaqoob, B. D. Hammock, D. Gilroy, D. C. Zeldin and D. Bishop-Bailey, Intimal smooth muscle cells are a source but not a sensor of anti-inflammatory CYP450 derived oxylipins. *Biochem Biophys Res Commun*, 2015, **463**(4), 774–780.

[36] C. R. Stewart, L. M. Stuart, K. Wilkinson, J. M. van Gils, J. Deng, A. Halle, K. J. Rayner, L. Boyer, R. Zhong, W. A. Frazier, A. Lacy-Hulbert, J. E. Khoury, D. T. Golenbock and K. J. Moore, CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol*, 2010, **11**(2), 155–161.

[37] M. Lechleitner, T. Koch, M. Herold, A. Dzien and F. Hoppichler, Tumour necrosis factor- α plasma level in patients with type 1 diabetes mellitus and its association with glycaemic control and cardiovascular risk factors, *J Intern Med*, 2000, **248**(1), 67–76.

[38] A. L. Carey and M. A. Febbraio, Interleukin-6 and insulin sensitivity: friend or foe? *Diabetologia*, 2004, **47**(7), 1135–1142.

[39] M. Sadagurski, L. Norquay, J. Farhang, K. D'Aquino, K. Copps and M. F. White, Human IL6 enhances leptin action in mice, *Diabetologia*, 2010, **53**(3), 525–535.

[40] J. Ballester-Sánchez, J. V. Gil, C. M. Haros and M. T. Fernández-Espinar, Effect of incorporating white, red or black quinoa flours on free and bound polyphenol content, antioxidant activity and colour of bread, *Plant Foods Hum Nutr*, 2019, **74**(2), 185–191.

496 [41] M. Pellegrini, R. Lucas-Gonzalez, E. Sayas-Barberá, J. Fernández-Lopez, J. A. Pérez-Álvarez and
497 M. Viuda-Martos, Bioaccessibility of phenolic compounds and antioxidant capacity of chia (*Salvia*
498 *hispanica* L.) seeds, *Plant Foods Hum Nutr*, 2017, **73**(1), 47–53.

499 [42] C. Schmitz-Peiffer and T. J. Biden, Protein kinase C function in muscle, liver, and β -cells and its
500 therapeutic implications for type 2 diabetes. *Diabetes*, 2008, **57**(7), 1774–83.

501 [43] T. Shen, X. Chen, Y. Li, X. Tang, X. Jiang, C. Yu, Y. Zheng, H. Guo and W. Ling, Interleukin-
502 17A exacerbates high-fat diet-induced hepatic steatosis by inhibiting fatty acid β -oxidation, *Biochim*
503 *Biophys Acta Mol Basis Dis*, 2017, **1863**(6), 1510–1518.

504

505 **Figure caption**

506 **Fig. 1** Variations of body weight gain (**A**) and liver to body weight ratio (**B**) in animals fed with high
507 fat diet and administered with the different bread formulations: WB, white bread; RQ, red quinoa flour
508 (25%)-containing bread; WQ, white quinoa flour (25%)-containing bread; Ch, chia flour (20%)-
509 containing bread; Ch_D, semi-defatted chia flour (20%)-containing bread. Untreated controls are
510 represented by the dotted line. *Indicates statistically significant ($p<0.05$) differences in relation to
511 controls.

512

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

519

520 **Fig. 3** Plasmatic triglycerides (**A**), hepatic triglycerides (**B**) and peroxidised lipids (**C**) in animals fed
521 with high fat diet and administered with the different bread formulations: WB, white bread; RQ, red
522 quinoa flour (25%)-containing bread; WQ, white quinoa flour (25%)-containing bread; Ch, chia flour
523 (20%)-containing bread; Ch_D, semi-defatted chia flour (20%)-containing bread. Untreated controls
524 are represented by the dotted line. *Indicates statistically significant ($p<0.05$) differences in relation to
525 WB.

526

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

534

Components g/100g d.m.	Flours				
	Wheat ¹	Red quinoa ¹	White quinoa ¹	Chia ²	defatted chia ²
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

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

537 The data are reported on dry basis. Values are expressed as mean ± standard deviation (n = 3). d.m. dry
538 matter. ¹ Ballester-Sánchez et al. ⁴⁵; ² Fernández-Espinar et al. ²⁷

540 **Table 2.** Content of immunonutritional agonists quantified in flours from different botanical origin and their estimated bioaccessibility and

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

541 protease inhibitory activity. Results are expressed as mean ± SD (n=4).

542 ¹ Analysis of the different varieties, white vs red, did not reveal significant differences for the extractability of the immunonutritional
543 agonists. a-c Different superscript letters indicate statistically significant differences within the same column.

544

545 **Table 3.** Comparison of the high fat diet used in this study with some reported in the literature.

Carbohydrate (%)	High fat diet		Ref.
	Fat (%)	Protein (%)	
43	42	15	This study
42 - 59.8	20 - 38	13 - 20.1	
35 - 42	40 - 45	18 - 20	¹ Gheibi et al., 2017
5 - 27.5	58 - 60	15.5 - 35	

546 ¹ Values revised by Gheibi et al. ¹⁵

547

548

549
550

Fig. 1

Fig. 2

Fig. 3

Fig. 4