Inclusion of whole flour from Latin-American crops into bread formulations as substitute of wheat delays glucose release and uptake

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Abstract

Bakery formulations limiting glucose availability for uptake without compromising product quality are required. Herein, bread formulations prepared by inclusion of whole flour from *Amaranthus hypochondriacus* (AB), *Chenopodium quinoa* (QB), *Salvia hispanica L* (ChB) or wheat (WWB) were compared to white bread (WB) in relation to glycaemic index (GI) in fasted animals. There was monitored the hepatic expression (mRNA) of PPAR-γ receptor as key regulator in substrate fractionation towards energy expenditure. GIs were associated to fluxes of glucose release (F_{Gluc}) and metabolic response (MTT assay) of HepG2 cells. ChB (19.7%) and AB (13.5%) decreased GI to a higher extent than QB (2.7%), but all increased expression of PPARγ in relation to WB. F_{Gluc} (AB>>ChB, WWB, WB>QB) showed a reciprocal relationship with $AUC_{in\ vivo}$, and decreased MTT conversion values (WB>WWB, ChB, AB, QB) by HepG2 cells. LAcs-containing bread formulations reducing GI, without compromising product quality, could constitute an advantageous strategy defeating metabolic diseases.

Keywords: Glycaemic index, amaranth, quinoa, chia, PPAR-γ, obesity, type 2 diabetes.

Introduction

Western diet commonly favors overnutrition with an altered food supply and a particular high intake, among other, of fat foods, sugary desserts and refined grains [1,2]. This usually takes place through bread consumption as staple food and part of the traditional diet. Thus, type and amount of dietary carbohydrates [3] are important determinants of postprandial glucose and insulin responses. The total rise in a person's blood glucose level following consumption of the food is nutritionally known as glycaemic index (GI) [4]. High-GI diets are associated with developing metabolic dysfunction and predispose to type 2 diabetes (T2D) [1] and overweight/obesity and associated risk factors in children and adolescents [5]. To tackle this worldwide spread pandemic it has been increased the fibre content by the inclusion of whole grains and/or external parts of the kernel [6] as well as the use of enzyme addition to bread. Currently, bakery formulations limiting glucose availability for uptake without compromising product quality are required.

Latin-American crops have also received increasing attention because of their advantageous immunutritional features [7]. Here, peroxisome proliferator-activated receptor (PPAR)-γ activation improves insulin and glucose parameters, resulting from an improvement of whole-body insulin sensitivity [8]. Previous research showed that inclusion of LAcs into bread formulations results effective in modulating the hepatic production of the inflammatory biomarkers [7] associated to the expression of proliferator-activated receptor-γ coactivator-1α (PGC1α) [9].

The objective of this study was to evaluate the impact of the inclusion of whole flour from different LAcs at different percentage levels, as a substitute to wheat flour in bread formulation, on glucose release from foods and glycaemic responses and changes in PPARγ expression in fasted animals.

Material and methods

Breadmaking. Commercial wheat and whole wheat flours, quinoa (*Chenopodium quinoa*) (Ecobasic – Bio, S.L., Spain), amaranth (*Amaranthus hypochondriacus*) (Corporación Proteína Americana, SCRL, Tehuacán Puebla, Mexico) and chia seeds (*Salvia hispanica*) (Primaria Raw Matrials, Valencia-Spain) were obtained from local supermarkets. Breadmaking processes were performed described elsewhere [10-12].

Flux of glucose release. The kinetics of glucose release was calculated using a bicameral chamber created with a 15,000-molecular weight cut-off dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). An aliquot (1.5 mL) of the intestinal digest [13] was pipetted into the upper chamber, and 1 mL of an isotonic solution [140 mM NaCl, 5 mM KCl] was added to the bottom compartment. Samples (300 µL) from the bottom compartment were collected every 5 minutes for 60 minutes using the isotonic solution to replace the volumes removed. Fluxes of glucose (F_{Gluc}, cm/s) were calculated from the linear slope of the glucose concentration in the bottom chamber [13].

Cell culture. HepG2 cell (ECACC 86010202, Salisbury, UK) cultures were placed (1 x10^4 cells/well) in the bottom chamber of the bicameral system and incubated with intestinal digests from the different bread formulations for 30 min. Then, metabolic responses were evaluated by monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) conversion on exposed cultures after 1.5 h [13].

Animals. Female Wistar albino rats, (3 weeks) were obtained from the University of Valencia Animal Service (SCSIE) and handled in strict accordance with the Guide for the Care and Use of Laboratory Animals (SCSIE, University of Valencia, Spain) (A1351244049254). Animals were randomly distributed into six different groups (n=5) and fasted for 5h prior to blood sampling: a control group and five groups that were

administered with the different experimental bread formulations. A 0.5 g aliquot of the different samples was administered and blood samples were taken. Sections (100 mg) of the liver were immersed in RNA later buffer (Qiagen, CA, USA) and snap-frozen in liquid nitrogen for gene expression analyses.

Glucose quantification. Blood glucose was determined using a commercial glucometer (Accu-Chek, Roche). Samples were taken at 0, 10, 20, 30, 45, 60 and 90 min. The data were used to plot time-course curves to calculate the area under the curve (AUC) for each treatment group (SigmaPlot v10.0, Systat Soft. Inc, UK). From the AUC values there were calculated apparent hydrolysis indexes (HI) in relation to a reference sample (white bread) as $HI=(AUC_{Bread\ formulation}/AUC_{White\ bread})x100$. Glycemic indexes were calculated as previously described (GI=39.71 + 0.549(HI)) [14].

mRNA expression analyses. rt-qPCR analyses were performed with primers designed for the following *Rattus norvegicus* genes: PPARγ (forward 5'- TGA TCC TAC GGC CAG ACA GA-3', reverse 5'-GGG AGG TTG TCC CTG GAA TG-3') and β-actin (forward 5'- CTC TTC CAG CCT TCC TTC CT-3'; reverse 5'- TAG AGC CAC CAA TCC ACA CA-3'), the latter used as a housekeeping gene [8].

Statistical analysis. SPSS v.15 software (SPSS Inc., Chicago, IL, USA) was used. One-way analysis of variance and the Tukey *post hoc* test were applied. Variance analysis by one-way method was used and the statistical significance was established at $P<0.05$ for all comparisons.

Results

Glycemic index. Animals fed with WB showed a rapid increase in glucose concentration reaching a maximum after 20 min (**Figure 1**). There was a slight decrease in the glycaemia up to 40 min from when it turned up again keeping the increasing trend up to 90 min. Only animals fed with QB exhibited a maximum concentration value at

60 min that resulted higher than values quantified for WB. When comparing bread LAcs-containing bread formulations with WWB there were quantified similar glucose concentration levels after 20 min, but slightly lower glucose values after 60 min for WWB.

Feeding WWB, AB or ChB significantly decreased (p<0.05) AUC values in relation to WB. Samples of QB rendered similar AUC values to the reference WB sample (**Table 1**). Notably, HI values calculated for WWB, AB and ChB samples decreased by 10.3%, 19.9% and 31.1%, respectively, when compared to WB. These values corresponded with significantly (p< 0.05) decreased GI values.

mRNA expression. There were quantified changes in the expression of PPARγ according to the following gradation (**Figure 2**): WB = WWB < ChB = AB < QB. Although there were calculated similar GI values for WWB and AB samples, feeding AB caused significantly higher changes in the hepatic transcripts of PPARγ. Otherwise, ChB with lower GI values than AB promoted similar changes in the expression levels of PPARγ.

Overall, taken together, these results reveal that GI values do not always associate with hepatic metabolic responses of key mediators of intrahepatic glucose accumulation or specific members of the PPAR gene family. As such, PPARγ overexpression can positively increase energy homeostasis and, thereby insulin-induced glucose metabolism influencing insulin resistance [15,16].

Fluxes of glucose (F_{Gluc}) and metabolic response. The analyses revealed significant effects of the inclusion of whole wheat flour from LAcs into bread formulations in the F_{Gluc} calculated from the different foods tested (**Figure 3**). F_{Gluc} were not reflected in increasing metabolic responses in HepG2 cells. All bread formulations with LAcs caused lower MTT conversion values than WB, in good accordance with the decreased

GI (Table 1). These data reveal significant differences in glucose uptake from the different foods. Statistical analyses showed a significant (P=0.03) reciprocal relationship (r=0.910) between $AUC_{in\ vivo}$ and F_{Gluc} at the 95% confidence level. The r^2 statistic indicates that the model as fitted explains 82.9% of the variability in $AUC_{in\ vivo}$.

Discussion

The present study provides new information concerning influence of LAcs to modulate the glucose release from bread formulations. This is reflected in decreased GI to fasted animals. These effects were accompanied of an upregulated PPARγ expression - key regulator of whole body glucose homeostasis and substrate distribution for energy expenditure. Notably, the effects of the inclusion of LAcs into bread formulations on GI can have important health consequences in metabolic disorders such as T2D, overweight/obesity and other risk factors of the associated metabolic syndrome. Whole grains have been used as an effective strategy to better control the glycaemic response because of their advantageous content in, among other, bran and complex polysaccharides [6]. Because of the negative impact of these practices in bread quality and the dietary contribution with immunonutritional gluten proteins it has been motivated an increasing interest on the use of ancient LAcs in bread formulations allowing to avoid several different technological problems and undesirable sensory characteristics of the final product [10-12].

This study shows that $AUC_{in\ vivo}$ is associated to the increased transcripts of PPARγ. Otherwise, contrasting discrepancies appear in animals fed with the different LAcs-containing bread formulation: QB *vs.* ChB and AB. Feeding QB increased PPARγ expression without significant changes in GI values in relation to WB. These discrepancies could be attributed to the remarkable differences that have been reported in the starch digestibility (Fig. 3) for pseudocereals [17]. These data can reflect the

potential importance of the breadmaking methods on starch digestibility and predicted GI for amaranth [22] and reveals the higher influence on *Chenopodium quinoa* grains used in bread formulations in relation to *Amaranthus hypochondriacus*. The particular nutritional composition in relation to fat and protein content could also help to explain, at least in part, the discrepancies in the starch digestibility of these pseudocereals predicting a relatively poor GI for AB and QB [17]. It should be not ruled out that the higher $AUC_{in\ vivo}$ values calculated for QB are majorly due to the delayed release of carbohydrates (Fig. 1). Therefore, the results indicate that GI represents the total rise in a person's blood glucose level following consumption of the food, but it could or not reflect the rapidity of the transfer of carbohydrates to bloodstream. Thus, GI could fail reflecting the physiological response(s) motivated by grain's composition or its biologically active food components.

Previous studies demonstrated that the glycaemic effect of foods depends on several different factors such as food texture and particle size [19], types of starch [20], the physical entrapment of starch molecules within food and food processing as well as other ingredients [21-22]. Thus, significant differences in GI values between samples containing different flour proportions (ChB, 5%; QB, 25%; AB, 25%; WWB, 100%) demonstrate the influence of bread formulation in glycaemic response(s). Moreover, it cannot be ruled out how household/industrial processing can affect GI of bread formulations containing ancient LAcs [18,23] that were out of the scope of this study.

The irrespective quantified changes in the transcripts of PPARγ (Fig. 2) in relation to calculated GI values (Table 1) for the different bread formulations provide insights about the influence of different flour's composition in glucose homeostasis. As such, PPARγ overexpression can be associated to increased energy expenditure that together with the decreased GI in relation to WB allow hypothesizing an improved insulin

sensitivity and down-regulated lipogenesis, but improved fat partitioning and metabolism. Consequently, these changes could lead to a more preserved mitochondrial function as well as less severity of inflammation processes that can be derived from increased oxidative stress because of high glycemic concentrations. The metabolic changes promoted by ChB and AB may be advantageous to those derived from feeding WWB, and also clinically relevant in metabolic diseases prevention such as T2D, obesity or the metabolic syndrome. Additionally, these beneficial health effects occur together with the fiber-mediated production of gut hormones (glucagon-like peptide-1 and peptide YY) or modulating inflammation by their interaction with specific G-protein coupled receptors (GPR43 and/or GPR41) [24] that can also significantly contribute to modulate insulin sensitivity.

Conclusions

From the study conducted there are supported positive effects decreasing GI that are derived from the inclusion of whole flour from *Amaranthus cruentus* at 25% (AB) or *Salvia hispanica* L. Besides, the effects derived from the inclusion of whole *Chenopodium quinoa* flour at 25% (QB) in bread formulations did not result too straightforward to understand from this experimental design. Starch hydrolysis of bread samples was significantly affected ($p<0.05$) by the type and proportion of flour amount added in the bread formulation. GI for AB and ChB was similar or even lower, respectively, to that calculated for WWB. When considering resistant starch, inversely related to the hydrolysis index, the lowest value is calculated for QB that rendered a similar GI to the reference WB. There could be attributed additional beneficial effects to AB- and ChB-induced upregulated expression (mRNA) of the PPARγ that plays a key preventive role in the development of insulin resistance, T2D, elevated triglycerides and low HDL levels and, a number of components of the metabolic syndrome. Further

214 human trials are necessary to confirm to what extent severity of insulin resistance can be
215 controlled with innovative bread formulations including ancient LAcs.
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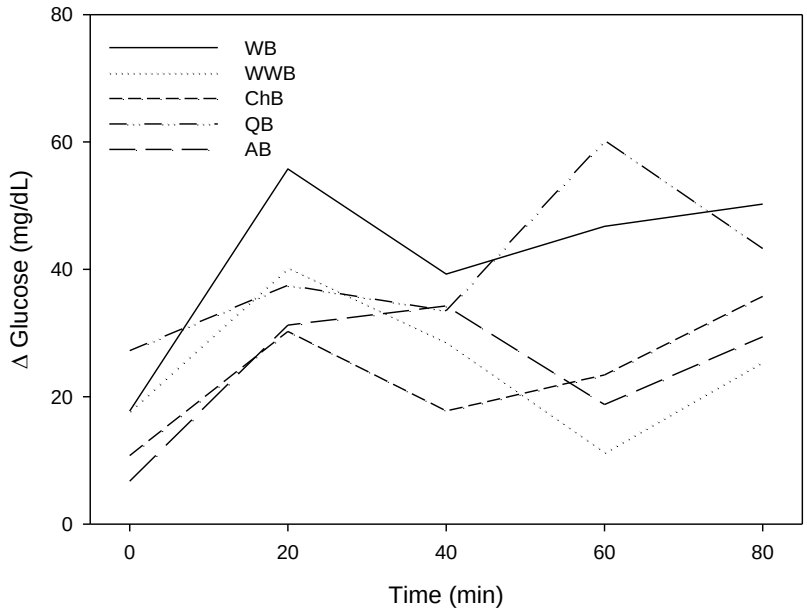
Table 1. Area under the curve (AUC), hydrolysis index (HI) and estimated glycemic index (GI) [18] of different bread formulations containing ancient Latin-American crops. Results are expressed as mean ± standard deviation (n=5). Different superscript letters indicate statistical ($p<0.05$) differences for each parameter.

Bread formulation	AUC	HI (%)	GI (%)
WB	7269.4 ± 516.3 [a]	100.0 ± 7.4 [a]	97.2 ± 4.1 [a]
WWB	5994.5 ± 405.2 [b]	89.7 ± 5.5 [b]	88.9 ± 4.0 [b]
ChB	4560.8 ± 604.4 [c]	68.9 ± 7.1 [c]	77.5 ± 5.0 [c]
QB	6949.4 ± 319.4 [a]	104.9 ± 5.3 [a]	97.3 ± 2.6 [a]
AB	5306.1 ± 451.4 [b]	80.1 ± 7.0 [b]	83.7 ± 4.9 [b]

Figure 1. Typical time-course glycaemic responses in animals fed with the different bread formulations.



298 **Figure 2.** Fold change in the hepatic expression (mRNA) of PPARγ receptor. Results
299 are expressed as mean ± standard deviation (n=5). Different superscript letters indicate
300 statistical ($p<0.05$) differences.

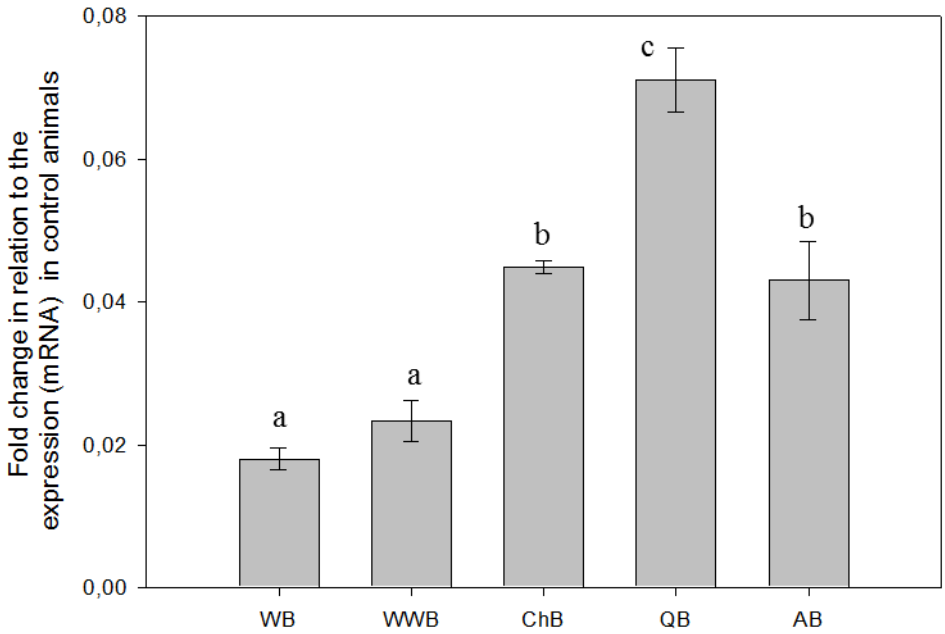


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Figure 3. Fluxes of glucose (F_{Gluc}) calculated from in vitro kinetics assays and metabolic responses associated to mitochondrial function (MTT) in HepG2 cells. Results are expressed as mean ± standard deviation (n=5). Different superscript letters indicate statistical (p<0.05) differences for each parameter.