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

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23 **ABSTRACT**

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

35

36 **Keywords:** Starch, glucose, bioaccessibility, quinoa, maize.

37

38 **Introduction**

39 Glucose homeostasis (GH) is one of the hallmarks of liver related diseases playing a critical role
40 in the pathogenesis of comorbidities associated to metabolic disorders such as type 2 diabetes (T2D)
41 and obesity. The latter constitute, moreover, due to an excessive glycaemia important risk factors,
42 together with sedentary lifestyle and altered food supply and preferences determining the
43 progression of liver dysfunction [1]. Recently, alterations in pancreatic β -cells metabolism in
44 response to hyperglycaemia has led to open a debate concerning T2D as a glycogen storage diseases
45 (GSD) of pancreatic β -cells [2]. Thus, both hyperglycaemia as well as hypoglycaemia in childhood
46 are associated with increased risk of physiopathological consequences [3] at a younger age and later
47 in life.

48 Several attempts have been made to develop digestion models to evaluate nutrient release from
49 food matrices, including the determination of their bioaccessibility (BA, %) [4-5]. Despite
50 differences in the variety of enzymes and other parameters, according to evidence-based
51 gastrointestinal physiology, there are scarce studies evaluating the fluxes of bioaccessible nutrients
52 [6] affecting physiological response(s). Simulated gastrointestinal digestion models, based on
53 solubility parameters, allow to evaluate food availability to the enzymatic action and hydrolysis
54 approaching glucose release [5]. However, these models did not reveal possible interactions
55 between the nutrients and food ingredients, potentially affecting their influence in response(s) to
56 signals for endocrine control.

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

64 digestion of carbohydrates, generally considered to be beneficial for the dietary management of
65 T2D, obesity and GSD [3,12].

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

68 **Materials and methods**

69 **Materials and reagents.** Commercial maize starch was purchased from ACH Food Companies
70 (Argo, USA), red quinoa starch was obtained in the laboratory by wet-milling [13] and black CF
71 was provided by Inca's treasure (Ecuador). Reagents were purchased from Sigma-Aldrich: α -
72 amylase (EC 3.2.1.1, A3176-1MU, USA, 16 U/mg), pepsin (EC 3.4.23.1, P7000, UK, 480 U/mg),
73 pancreatin from porcine (EC 232-468-9, P1750, USA, 4XUSP), bile extract porcine (EC 232-369-0,
74 B8631, USA) amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, 10115, Switzerland, 60.1
75 U/mg) and insulin from bovine pancreas (EC 234-291-2, I6634, USA, ≥ 25 USP units per mg).

76 **Samples.** Aliquots (100 mg) of maize and quinoa starch were weighted into microcentrifuge
77 tubes. Maize (MS_CF) and quinoa starch (QS_CF) were prepared with 100 mg of starch and mixed
78 with 20 mg defatted CF. Black CF was defatted with n-hexane (6 h) under reflux conditions in a
79 Soxhlet equipment (SoxtecTM 2050, Sweden) extracting all the lipid content from black CF (33%
80 g/100g dry matter). The proportion of black CF was chosen as the maximum percentage allowing
81 adequate solubility conditions. Three different set of samples were prepared with and without CF:
82 raw starches were kept in unheated water for 5 min; the others were subjected to different HTs to
83 obtain a partial (pretreated) (maize, 70°C/2min; quinoa, 60°C/1min) and total gelatinisation (maize,
84 100°C/5min; quinoa, 100°C/5min) [5]. Each set of samples was prepared in triplicate (n=3) for
85 simulated gastrointestinal digestion.

86 **Simulated gastrointestinal digestion.** Three independent set of samples for MS, QS and those
87 mixed with CF were subjected to a simulated gastrointestinal digestion procedure as described
88 elsewhere [6] with slight modifications. An oral phase was included previously to gastric digestion
89 where all samples were incubated in a solution of α -amylase (75 U/mL) for 30 seconds. The

90 digestion started with a peptic (gastric digestion) step (1h/37°C). Then, the intestinal phase of
91 digestion was initiated with a pancreatin-bile extract solution. 2 mL of the gastrointestinal digest
92 was carried out in the upper (donor compartment) chamber and 2 mL of an isotonic saline solution
93 in the bottom (acceptor compartment). The bicameral system consisted in a molecular weight cut-
94 off dialysis membrane (14 kDa) attached to a plastic insert ring to separate the “gastrointestinal
95 digest” from the acceptor compartment allowing to determine glucose dializability from the bottom
96 chamber considered the bioaccessible fraction for absorption.

97 Aliquots (250 µL) from the bottom compartment were collected at different intervals (15, 30, 45,
98 60 min), and isotonic solution was used to replace the volumes removed during sampling. Apparent
99 diffusion coefficients (D_{app}) were calculated from the linear slope of the glucose concentration in
100 the bottom chamber according to Laparra [6]. Glucose was quantified by enzymatic kit (D-Glucose
101 Assay Procedure, K-GLUC 07/11, Megazyme).

102 **Cell cultures.** The human hepatoblastoma (HB-8065[®]) cell line was obtained from the American
103 Type Culture Collection (Rockville, MD, USA) at passage 1 and used in experiments between
104 passages 9-11. Cell cultures were grown in Eagle’s Minimum Essential Medium (EMEM
105 Glutamax, Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were maintained
106 at 37°C in 5% CO₂, 95% air and the culture medium were changed every 2 days.

107 **Mitochondrial Enzymes activity (MTT assay).** Metabolic responses were evaluated by
108 monitoring MTT (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide) conversion
109 (TOX1, Sigma-Aldrich) according to a commercial kit [10]. For experimental studies cells were
110 seeded at a density of 3×10^5 cells/well onto 12 well plates (Costar, Cambridge, MA, USA) and
111 EMEM was replaced by 1 mL of minimum essential media (MEM) without fetal bovine serum.
112 Two different sets of cultures were prepared: i) untreated cell cultures, and ii) treated with insulin
113 (10 ng/mL) [14]. In order to detect early metabolic response(s) intestinal digestion, the bicameral
114 system fitted with the dialysis membrane was applied onto cells. Cell seeded plates were returned to
115 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 XVI software, and the significance level was established at $P < 0.05$.

Results and discussion

Starch digestibility and glucose bioaccessibility. Total glucose concentration at endpoint of the simulated gastrointestinal period was determined as an estimator of total starch hydrolysed (**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 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 MS₁₀₀ 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

142 glucose concentrations and Dapp. These data suggest different interactions between starch structure
143 and composition on glucose dialyzability probably due to differences in the length between
144 amylose/amylopectin chains in quinoa starch and/or their accessibility to digestive enzymes. The
145 inclusion of CF slightly decreased glucose concentrations in the acceptor compartment as supported
146 by reduced Dapp values. The decreased Dapp after addition of CF is concordant with several *in vivo*
147 studies, which have reported a better control on carbohydrate metabolism [17].

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

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

161 Different parameters to estimate glucose dializability from MS and QS starch (i.e., amount of
162 glucose extracted with the aliquot, cumulative glucose in basal media and Dapp) were statistically
163 associated. Cumulative glucose concentrations were determined as responsible for the major weight
164 of PC#1 (Eigenvalue, 3.93), meanwhile, the lower weight of PC#2 (Eigenvalue, 0.061) included the
165 other groups of parameters calculated. The analysis clearly discriminated between the digestibility
166 of MS and QS and those in presence of CF, which demonstrates the influence of the bioactive
167 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 [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] (**Figure 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 $0.92 \pm 0.02 \mu\text{g}$ 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 ($\text{PPAR}\gamma$) [7] inducing the 6-phosphofructo-2-kinase activity [21].

194 However, insulin is a physiologic homeostatic regulator of hepatocytes to the innate immune TLR4,
195 which dominance promotes a deficient insulin signaling [22]. From a molecular perspective,
196 immunonutritional agonists delay TLR4 retrieval to the plasmatic membrane, thus interfering its
197 functional activity [10]. Here, STPIs from CF could be thought as responsible for the inhibitory
198 effects reducing MTT conversion values by activation of the IRS1/PI3K/AKT pathway [20].
199 Overall, chia's influence on glucose metabolism highlights its potential promoting a more adequate
200 preservation insulin-induced glycogen storage or gluconeogenesis in order to prevent, or at least to
201 restrain, cell stress during disturbed glucose metabolism pathologies.

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

208 **Abbreviations:** Bioaccessibility, BA; Chia flour, CF; Dapp, Apparent diffusion coefficient; EC,
209 Enzyme Commission number; GH, Glucose homeostasis; GSD, Glycogen storage disease;
210 Hydrothermal treatment, HT; MS, Maize starch; MS_70, Treated maize starch at 70°C; MS_100,
211 Treated maize starch at 100°C; MS_CF, Maize starch with chia flour; MS_70_CF, Treated maize
212 starch with chia flour at 70°C; MS_100_CF, Treated maize starch with chia flour at 100°C; MTT,
213 (3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide; PC, Principal component; PCA,
214 Principal component analysis; PPAR γ , peroxisome proliferator-activated receptor gamma; QS,
215 Quinoa starch; QS_60, Treated quinoa starch at 60°C; Treated quinoa starch at 100°C; QS_CF,
216 Quinoa starch with chia flour; QS_60_CF, Treated quinoa starch with chia flour at 60°C;
217 QS_100_CF, Treated quinoa starch with chia flour at 100°C; STPI, serine-type protease inhibitor;
218 T2D, type 2 diabetes; TLR, Toll-like receptor.

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224 **Compliance with Ethical Standards**

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

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299

Figures

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 $\pm$ standard deviation ($n = 3$). Hydrothermal treatment: i) Maize (MS_70) and quinoa (QS_60) pretreated samples (maize 70°C/2min and quinoa 60°C/1min), ii) Maize (MS_100) and quinoa (QS_100) gelatinized samples (maize/quinoa 100°C/5min), mixed or not with chia. $t_{1/2}$, time that takes for reaching half the maximum total basal glucose concentration.

312 **Fig. 2:** (A) Principal component analysis of the kinetic parameters of glucose dializability from raw
 313 maize (MS) and quinoa (QS) starch mixed or not with chia flour (CF). (B-D) Lineweaver-Bürke's
 314 transformation from glucose concentration in the acceptor compartment. The average of three
 315 replications was represented and maximum velocity (Vmax) (inverse of y-intercept value) was
 316 calculated from the different raw starch formulations.
 317

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

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

Sample	Treatment	Soluble glucose (mg)		Bioaccessibility (%)	Dapp (cm ² /seg) (x10 ⁻²)
		before digestion	after digestion		
Raw	MS	0.89 $\pm$ 0.16 ^a	2.90 $\pm$ 0.21 ^a	7.6 $\pm$ 1.9 ^d	1.46 $\pm$ 0.29 ^b
	QS	2.94 $\pm$ 0.20 ^c	8.38 $\pm$ 0.18 ^f	4.5 $\pm$ 0.5 ^{ab}	2.84 $\pm$ 0.37 ^g
Pretreated	MS_70	1.95 $\pm$ 0.33 ^b	4.61 $\pm$ 0.20 ^b	6.1 $\pm$ 0.6 ^c	1.67 $\pm$ 0.17 ^{bcd}
	QS_60	5.74 $\pm$ 0.64 ^{de}	8.41 $\pm$ 0.23 ^f	4.4 $\pm$ 0.1 ^{ab}	2.27 $\pm$ 0.23 ^f
Gelatinised	MS_100	5.76 $\pm$ 0.80 ^{de}	7.72 $\pm$ 0.55 ^{de}	3.7 $\pm$ 0.2 ^a	1.59 $\pm$ 0.19 ^{bc}
	QS_100	5.62 $\pm$ 0.21 ^d	7.22 $\pm$ 0.23 ^d	4.0 $\pm$ 0.5 ^{ab}	1.53 $\pm$ 0.18 ^{bc}
Raw	MS_CF	0.91 $\pm$ 0.15 ^a	3.43 $\pm$ 0.60 ^a	5.3 $\pm$ 2.0 ^{bc}	1.09 $\pm$ 0.20 ^a
	QS_CF	3.07 $\pm$ 0.33 ^c	8.33 $\pm$ 0.18 ^f	3.7 $\pm$ 0.4 ^a	1.99 $\pm$ 0.19 ^{def}
Pretreated	MS_70_CF	1.97 $\pm$ 0.45 ^b	5.41 $\pm$ 0.06 ^c	4.6 $\pm$ 0.3 ^{abc}	1.61 $\pm$ 0.08 ^{bc}
	QS_60_CF	5.47 $\pm$ 0.86 ^d	8.37 $\pm$ 0.07 ^f	3.6 $\pm$ 0.0 ^{ab}	2.20 $\pm$ 0.10 ^{ef}
Gelatinised	MS_100_CF	6.67 $\pm$ 1.20 ^{ef}	8.15 $\pm$ 0.71 ^{ef}	2.4 $\pm$ 1.6 ^a	1.81 $\pm$ 0.35 ^{cde}
	QS_100_CF	7.28 $\pm$ 0.51 ^f	7.70 $\pm$ 0.15 ^{de}	3.7 $\pm$ 0.9 ^a	1.56 $\pm$ 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.