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Effects of dietary melanoidins on digestive physiology, nutrient digestibility and plasmatic antioxidant capacity of the rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

Melanoidins are complex molecules usually found within dietary matrixes suffering heating steps during the production process. These compounds are known to exert a number of negative effects on the digestibility of nutrients and digestive enzymes in mammals, but also determines an increase in the antioxidant capacity of the diet with potential effects for animal health. In the present work, digestive and nutritional consequences of the ingestion of glucose-glycine melanoidins were tested in juvenile rainbow trout (*Oncorhynchus mykiss*), which was fed two non-heated, pelletized diets: a control diet without melanoidins and an alternative diet containing 1.2% of glucose-glycine melanoidins. The results pointed to a small effect on gut pH in interaction with digestion time, and a lack of effect on gastric evacuation and digestive proteases. In addition, melanoidins were associated with a small increase in the digestibility of the diet dry matter, leaving unchanged nitrogen and phosphorus digestibility. On the other hand, melanoidins determined a complex increase in the postprandial antioxidant capacity of blood plasma, particularly apparent in the antioxidant components above 3 kDa.

1. Introduction

Melanoidins can be defined as high molecular weight, nitrogenous end products of the Maillard reaction (Mundt and Wedzicha, 2004). They usually appear in nutrient mixes containing amine groups and reducing sugars and/or lipid oxidation products (Hidalgo and Zamora, 2000) after undergoing one or several heating steps during the production process. Its presence is usually evidenced by a characteristic brown color of dietary matrixes as occurs, for example, in the so called DDGS (dry distilled grain with solubles), a heated by-product obtained from the bio-ethanol industry which has been evaluated as a protein source in aquafeeds (Magalhães et al., 2015; Welker et al., 2014; Prachom et al., 2013; Lim et al., 2011).

The structures of melanoidins are complex and still not well understood, but all of them are characterized by the presence of negative charges that actively interact to other positively charged feed compounds, like cationic minerals and proteins, when they are present together in the digestive tract. As an example, a browned Western-type diet has been recently associated with a reduction in the bioavailability of iron in adolescents (García et al., 2009), and Maillard products derived from bread-crust, after included in the diet, affected the distribution of calcium and magnesium in rat tissues but left the retention of these minerals unchanged (Roncero-Ramos et al., 2013a, 2013b). Not only the absorption process, but also the enzymatic hydrolysis of macronutrients is potentially affected by browning reaction products. Model melanoidins produced from a mixture of glucose and glycine or asparagine are known to exert a negative effect on digestive trypsin of vertebrates (Hirano et al., 1994, 1996a, 1996b; Ibarz et al., 2009) and, more recently, they were proven to decrease the *in vitro* activity of gastric proteases from the rainbow trout (Serrano et al., 2016). Notwithstanding these adverse effects of varied intensity, there are also a number of positive consequences associated with the inclusion of heated ingredients or melanoidins within the dietary matrix and hence in the digestive tract of animals. A number of these effects are related to their high antioxidant capacity of such compounds, which can improve the oxidative stability of fish meal during storage (Perrone et al., 2012; Mastrocola and Munari, 2000; Cho et al., 1988) or contribute to the antioxidant capacity of the diet (Pastoriza and Rufián-Henares, 2014).

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On the other hand, the intestinal absorption of melanoidins seems to be poor in mammals and decreases as the molecular weight of the compound increases, with values below 15% for molecules of 800 Da (Lee et al., 1992). This situation is reminiscent of the absorption of vegetal polyphenols and flavonoids in terrestrial animals, which is also low (Surai, 2014). Therefore, the gut is thought to be the primary target for the effects of these complex molecules (Tagliazucchi and Bellesia, 2015), but data on the potential bioavailability of melanoidins in fish are very scarce or lacking.

Taking into account all these facts, the aim of this study was to assess the potential effects of a glucose-glycine melanoidin model on different biological parameters related with the dynamics of the digestion process in the rainbow trout *Oncorhynchus mykiss*, including the postprandial gastric evacuation, gastrointestinal pH changes after feeding, activity of acid and alkaline digestive proteases, growth performance, nutrient digestibility, and the postprandial antioxidant capacity of plasma.

2. Material and methods

2.1. Preparation of melanoidins

A 1-l solution of glucose 1 M, glycine 1 M and $NaHCO_3$ 0.1 M was prepared, distributed in four 250 mL screw-capped flasks and heated at 103 °C for 24 h. The flasks were then left to cool at ambient temperature, acidified to pH 2.50 and stored at 6 °C for 24 h in a refrigerator to let melanoidins to precipitate, pH was then readjusted and the solutions maintained at 6 °C for another 24 h. After this time, solutions were centrifuged (4800*g*, 4 °C, 25 min), the supernatant was discarded and the obtained pellet suspended in 100 mL acidified (pH 2.50) distilled water, this step was repeated 5 times. Six similar cleaning steps were implemented, but suspending the pellet in 250 mL of acidified distilled water after centrifugation. The obtained pellets were stored at −20 °C overnight, then thawed and the exceeding water discarded. Pellets were now re-suspended in 250 mL of distilled water centrifuged at 4800*g*, 4 °C for 15 min, frozen at −20 °C, lyophilized and stored at −20 °C until use.

2.2. Preparation of diets

Two diets were prepared avoiding any heating step. The basal formulation was based on frozen Hoki hake (*Macruronus novaezelandiae*) muscle obtained from a local supermarket as the main protein source and cod liver oil (Fluka 74380) (Table 1). Fish muscle was freeze-dried, mechanically grinded and the resulting powder was stored at −80 °C until the preparation of the diets. The temperature of the muscle powder after the grinding process was in the range 21–29 °C. Dietary ingredients were then mixed, pelletized (Pelletizer Super Chacon MR 610) without extrusion (to avoid heating as much as possible) through a 2 mm die, freeze-dried and finally stored at −80 °C until being supplied to experimental animals.

No melanoidins were included in one of the diets and was defined as the control treatment. The second diet included glucose-glycine melanoidins at a level of 1.2%. Provided the absence of information in relation to aquafeeds, that inclusion rate was based on values reported in human diets, for which average daily intake of both macronutrients and melanoidins from solid foods is about 600–650 g and 8 g, respectively (Pastoriza and Rufián-Henares, 2014; Markwald et al., 2013), this suggesting a melanoidin content just above 1.0% for the solid component of the diet. This percentage was compatible with the potential use of melanoidins as a dietary additive in aquafeeds. Melanoidins were substituted by cellulose in the control diet. Both formulations were isonitrogenous (50.3 and 50.6% of Kjeldahl protein for control and browned diet, respectively), isoenergetic (23.5 and 23.6 MJ/kg, respectively) and with the same content of phosphorus (0.95 and 0.96%, respectively) (Table 1).

Table 1
Formulation and proximal analysis of experimental diets.

Ingredient/Nutrient	Control diet	Browned diet
Ingredients, g kg diet^{-1}		
Freeze-dried fish muscle[a]	410.0	410.0
Casein[b]	100.0	100.0
Gelatin[c]	40.0	40.0
Cod liver oil[d]	80.0	80.0
Sunflower oil[e]	80.0	80.0
Starch[f]	194.5	194.5
$Ca(H_2PO_4)_2{\cdot}H_2O$[g]	27.5	27.5
$CaHPO_4{\cdot}2H_2O$[h]	12.5	12.5
Macro-mineral mix[i]	10.0	10.0
Mineral/vitamin mix[j]	6.0	6.0
Cr_2O_3[k]	7.5	7.5
Cellulose[l]	32.0	20.0
Melanoidins	0.0	12.0
Proximal analysis, % (dry matter basis)		
Dry matter	98.5	98.0
Crude protein (Kjeldahl)	50.3	50.6
Crude lipid (Soxhlet)	19.3	19.4
Crude fiber	2.9	2.0
Ash	7.0	7.2
Non-nitrogenous extract	20.5	20.9
Phosphorus	0.95	0.96
Gross Energy (MJ/kg)	23.5	23.6

[a] Frozen Holi hake Jumbo™ (for human consumption, marketed by Jumbo, Temuco, Chile).
[b] Sigma-Aldrich C7078.
[c] Gourmet™ (for human consumption, marketed by Jumbo, Temuco, Chile).
[d] Sigma-Aldrich 74380.
[e] Miraflores™,(for human consumption; marketed by Jumbo, Temuco, Chile).
[f] Sigma-Aldrich S9765 + Chemix CX975706 (Chile) + Agrovet (Chile) in ratio 3:2.5:1.
[g] Sigma-Aldrich 21053.
[h] Veterquímica 100,775 (Chile).
[i] According to Satoh et al. (2003).
[j] Rovimix™ (mix supplied by SalmonFood, Chile).
[k] Sigma-Aldrich 393703.
[l] Sigma-Aldrich C8002.

2.3. Experimental design

Two experiments were design to explore the effect of melanoidin ingestion in rainbow trout from different points of view. The first one aimed at monitoring the immediate effects on protease activity in relation to gut pH and gastric evacuation; so that a fasting period was included in the experimental design. The second experiment was focused on digestibility coefficients and plasmatic antioxidant capacity after a longer period of feeding. Animal handling and sampling protocols were conducted according to Chilean legal requirements for the experimentation on live animals (Ley 20.380, Ministerio de Salud de Chile). Tissue samplings were implemented under accepted protocols for fish anesthesia and euthanasia, which are described below.

2.4. Experiment 1

Two levels of melanoidins were tested, 0% and 1.2% for the control and browned diet, respectively, in the first experiment. The basic design of experiment 1 consisted in feeding each diet to 3 tanks with juvenile trouts and implementing a subsequent sequential sampling to obtain data about the physiology of the digestive function. One hundred and fifty fish weighing 23.8 ± 0.4 g were stocked in six 100-L tanks (25 fish per tank). The Tanks were connected to a flow-through freshwater circuit at the aquaculture facilities of Universidad Católica de Temuco, and oxygen saturation was maintained at least at 80%. Fish were let to acclimate to the experimental diets for 5 days, and were fed (by hand) 2.0% of the initial biomass for 3 days, then 1% and 0.5% for the next

two days, respectively. After this time, animals were fasted for 72 h. Temperature was in the range 13.5–14.1 °C. The sampling day, each tank was fed to visual satiation in the morning (final ration of 2.0 ± 0.0% and 2.1 ± 0.2% for the control and browned diets), and three fish from each tank were sampled at random after 2, 4, 7, 10, 24 h of digestion. Sampled fish were anaesthetized with benzocaine, euthanized by cephalic concussion, and dissected to obtain the stomach and the anterior intestine. Gut pH was measured by inserting a narrow-tipped pH probe (Hanna Instruments HI 1083) calibrated at the water tank temperature into the pyloric stomach (the curved zone of the stomach), and into the anterior part of the intestine. Both organs were stored at −80 °C until further analyses. Stomachs plus contents were weighed, cut open while thawing and the content extracted and weighed. Gastric contents were then homogenized in ice, a portion of the homogenate was included into an Eppendorf tube and dried at 105 °C for 24 h to determine moisture. The rest of the gastric content homogenate was centrifuged at 15,700*g* for 15 min and 4 °C, and the supernatant was stored at −80 °C until assayed for acid protease activity. Because of the difficulty in separating the tissue of pyloric caeca from its contents, anterior intestines plus contents were homogenized in chilled water (1:3), the mix centrifuged at 15,800*g* for 15 min. at 4 °C, and the supernatants were aliquoted and stored at −80 °C until assayed. One fish per tank and temporal sampling were selected at random to assay the trypsin-like activity.

2.5. Experiment 2

The same diets tested in experiment 1 were used in the second experiment too. Prior to the digestibility experiment, the fish stock was fasted for two days and twelve fish were anaesthetized with benzocaine, euthanized by cephalic concussion and stored at −80 °C until being homogenized, freeze-dried and subject to proximal analysis. Then 198 rainbow trout juveniles weighing 27.8 ± 1.9 g were randomly distributed in six 100-L tanks connected to the same flow-through freshwater circuit of experiment 1. Three tanks were assigned at random to each diet, and water temperature was measured twice daily and fluctuated between 13.8 ± 0.5 °C at 8:00 to 14.4 ± 0.3 °C at 15:00. Animals were fed by hand twice a day (9:00 and 16:00) for 17 days, the average daily feeding rate being 2.35 ± 0.01% and 2.33 ± 0.02% (mean ± sem) for the control and browned diet, respectively. Fish were acclimated to the experimental conditions for the first 10 days. Faeces were collected in a sedimentation pipe (Guelph type) for 4 h after each meal, cleaned out from diet pellets (if any) and separately stored for each tank at −20 °C. Faeces collected from the 11th day onwards were considered for biochemical analysis. At the end of the experiment, faeces were freeze-dried and finely grinded prior to be analyzed.

At the 17th day of the experiment, tanks were supplied the whole daily ration in one meal in the morning, and 3 fish per tank were sequentially sampled at random 2, 4 and 10 h post feeding. Animals were subject to anesthesia with benzocaine. Blood samples were obtained with heparinized syringes from the caudal vein from the first 2 fish of each sample. Blood was immediately centrifuged at 1500 rpm and 4 °C for 10 min and the supernatant stored at −80 °C until analysis. The 3 fish of each sample were euthanized by cephalic concussion, dissected and gut pH was measured by inserting a clean, narrow-tipped electrode (Hanna Instruments HI 1083) at the pyloric stomach through the esophagus, and at the anterior intestine through the pylorus. Fish populations in experimental tanks were maintained for 72 h under fasting conditions; fish were similarly anaesthetized and euthanized, and weighed to obtain an estimation of the final mean weight per tank. At last, twelve fish were randomly sampled from each tank and stored at −80 °C, until being homogenized to a fine paste, freeze-dried, and subject to proximal analysis.

2.6. Measurement of the activity of digestive proteases

The activity of gastric proteases was measured based on the protocol of Anson (1938). The reaction medium consisted in 490 µL of a solution of bovine hemoglobin 0.5%, prepared in buffer glycine-HCl 100 mM and pH 2.5 and 10 µL of the gastric fluid phase diluted 1:6 in distilled water. After 15 min at 25.0 °C, the reaction was stopped by adding 250 µL of a solution of trichloro-acetic acid 20%. The reaction volume was then centrifuged at 15,800*g* for 15 min at 25 °C, and the supernatant absorbance read at 280 nm. Two experimental assays and one blank assay were carried out for each individual. The activity was calculated from the absorbance increment and the extinction coefficient for tyrosine, 1480 $M^{-1}\,cm^{-1}$ (Mach et al., 1992). One unit of acid protease activity was defined as the amount of gastric protease releasing 1 µmol of TCA-soluble tyrosine min^{-1}.

The activity of trypsin-like intestinal proteases was assayed using Nα-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as substrate. The reaction medium consisted in 272 µL of a solution of bovine hemoglobin 0.5%, prepared in buffer Tris-HCl 50 mM and pH 8.0 and 10 µL of intestinal crude extract diluted 1:60 in distilled water. The reaction was monitored at 253 nm and 25 °C in a microplate reader in kinetic mode. The activity was calculated from the slope of the rectilinear stretch of the increasing absorbance and the extinction coefficient for BAEE, 808 $M^{-1}\,cm^{-1}$ (Ashan and Watabe, 2001). One unit of acid protease activity was defined as the amount of gastric protease processing 1 µmol of BAEE min^{-1}.

2.7. Analysis of proximate composition of diets, faeces and fish

The content of macronutrients in diets, faeces and whole-body fish samples were determined according to AOAC (1995) methods. Dry matter (DM) was determined by oven-drying at 105 °C for 24 h. The content of nitrogen was determined following Kjeldahl protocol, and crude protein (CP) was calculated from the Kjeldahl nitrogen applying the factor 6.25 (Velp Heating Digester DK20 plus Velp Destillation Unit UDK 149). Crude lipid (CL) were determined gravimetrically in a Soxhlet extraction apparatus using petroleum benzine with boiling range 40–60 °C. The content of crude fiber (*CF*) was determined following the fritted glass crucible method for animal feeds (Welp Fiber Extractor FIWE 6). Ash content was determined gravimetrically after combustion in a muffle furnace at 550 °C for 3 h. Nitrogen-free extract (NNE) was calculated by difference: NNE = DM – CP – CL – CF – Ash. The content of phosphorus was determined according to the AOAC protocol 965.17. The content of Cr_2O_3 was estimated after oxidizing and digesting the samples of diets and faeces in accordance with the protocol of Furukawa and Tsukahara (1966).

2.8. Growth performance parameters

The average specific growth rate of fish per tank was estimated applying the formula:

$$SGR = \frac{\ln G_0 - \ln G_F}{\Delta t}$$

where G_0 and G_F are geometric means of initial and final fish weights, and Δt is the experimental period (Márquez et al., 2015).

2.9. Apparent digestibility coefficients (ADC's)

The apparent digestibility coefficient of dietary dry matter (ADC_{DM}) was calculated using the following expression:

$$ADC_{DM} = 100 \times \left(1 - \frac{[Cr_2O_3 \text{ in diet}]}{[Cr_2O_3 \text{ in faeces}]}\right)$$

The apparent digestibility coefficients of macronutrients or energy ($ADC_{Nut/E}$): crude protein (CP), crude lipids (CL), crude fiber (CF), non-

nitrogenous extract (NNE), ash (Ash), phosphorus (P) and energy (E), where calculated for each tank applying the formula:

$$ADC_{Nut/E} = 100 \times \left(1 - \frac{[\text{Nutrient or energy in faeces}]/[Cr_2O_3 \text{ in faeces}]}{[\text{Nutrient or energy in diet}]/[Cr_2O_3 \text{ in diet}]}\right)$$

2.10. Antioxidant capacity

The antioxidant capacity was estimated according to the ABTS (2, 2′-azino-di-[3-ethylbenz-thiazoline sulphonate]) (Pastoriza and Rufián-Henares, 2014). Measurements were made according to Merck "Total Antioxidant Status kit 615700" adapted for 96-well microplates read at 600 nm and 37 °C in kinetic mode (Thermo Scientific Multiskan GO microplate reader). Previous to the ABTS test, freeze-dried feed and faeces were grinded to a fine powder, then extracted for soluble antioxidant compounds by suspending 300 mg of powder in 3 mL of PBS buffer at pH 7.4 and stirring overnight at 600 rpm at 25–27 °C (IKA Multiposition magnetic stirrer RT10). Suspensions were then centrifuged at 14,000*g* for 15 min and the supernatant stored at −80 °C until analysis. Diet antioxidant capacity (AC) was measured in quadruplicate and AC for the faeces from each tank in duplicate. Plasmatic AC was assayed twice for each fish, once directly and once more after ultrafiltration through 3 kDa Amicon filters (14,000*g* for 30 min at 4 °C).

2.11. Statistics

In experiment 1, data on gastric contents, gastric content moisture, gastric and intestinal pH values, acid protease and trypsin-like activities were all analyzed by means of a repeated measures Anova with diet as a fixed factor and time as the repetition factor. In experiment 2, the potential differences in growth parameters (individual weights, feed ingestions, SGR and FCR shown in Table 2) and apparent digestibility coefficients (ADC's) for macronutrients (Table 3) were analyzed with a *t*-test with the diet as the experimental factor. Also in experiment 2, gastric and duodenal pH values were analyzed by means of a repeated measures Anova test with diet as a fixed factor and time as the repetition factor; in addition, postprandial antioxidant capacities of plasma was subjected to a repeated measures Anova by diet and ultrafiltration (fixed factors) and time (repetition factor). To localize differences among levels of the repetition factor Time, pairwise comparisons with a Bonferroni correction (type-I error kept at 5%) were performed; in the case of no localization of the differences, homogeneous subgroups were built taking into account that the lowest and highest values must be significantly different. Percentage variables were transformed according to the function arcsin(sqrt(x/100)) before being analyzed. The significance level was fixed at 5% in all the analyses.

Table 2
Growth performance of trout juveniles fed diets with and without glucose-glycine melanoidins in experiment 2. Figures indicate mean values ± SEM.

Growth parameter	Control diet	Browned diet	p
W initial, g fish^{-1}	27.6 ± 0.4	28.0 ± 0.3	0.476
W final, g fish^{-1}	40.3 ± 0.5	41.2 ± 1.1	0.499
Ingestion, g tank^{-1}	363.9 ± 4.4	366.0 ± 5.9	0.795
SGR[a], % d^{-1}	2.20 ± 0.06	2.25 ± 0.09	0.658
FCR[b], (g feed) (g wet weight gain)$^{-1}$	0.87 ± 0.03	0.84 ± 0.04	0.629

[a] SGR: Specific growth rate.
[b] FCR: Food conversion ratio.

Table 3
Apparent digestibility coefficients (ADC %) of macronutrients for trout juveniles fed diets with and without glucose-glycine melanoidins in experiment 2. DM: dry matter, CP: crude protein (Kjeldahl), CL: crude lipid (Soxhlet extraction), DF: crude fiber, NNE: non-nitrogenous extract calculated by difference, P: phosphorus, E: gross energy.

Nutrient fraction		Control diet	Browned diet	p
Dry matter	%	76.4 ± 0.2	77.4 ± 0.1	**0.010**
Crude protein	%	98.9 ± 0.1	99.0 ± 0.0	0.777
Crude lipid	%	99.5 ± 0.1	99.6 ± 0.0	0.455
Crude fiber	%	12.4 ± 1.4	17.2 ± 1.2	**0.062**
Ash	%	55.4 ± 1.0	57.4 ± 0.5	0.148
Non-nitrogenous extract	%	15.5 ± 0.8	17.3 ± 0.3	0.100
Phosphorus	%	61.9 ± 1.5	62.8 ± 1.3	0.688
Energy	%	85.0 ± 0.1	85.7 ± 0.1	**0.009**

Figures in bold are considered statistically significant or close to be significant.

3. Results

3.1. Experiment 1

Both experimental diets were very well accepted by fish and ingestion rates were not statistically different. Fish survival rate was 100% for all the tanks. No significant differences between diets were detected for the rate of gastric evacuation. Dry weight of gastric contents significantly dropped over time ($p = 0.003$). A minor part (less that 25%) of the ingested feed was evacuated before the 2nd postprandial hour; between the 2nd and the 7th hour of digestion no evacuation was detected and, after the 7th hour, a quasi-linear decay in the gastric contents was detected (Fig. 1A). Gastric moisture for the control diet tended to be higher in the period 2–10 h post-feeding, but no statistical significance was obtained for a period of 24 h; water content of digesta significantly rose with time ($p < 0.001$), this trend being particularly conspicuous from the 7th hour of digestion (Fig. 1B).

No significant difference in gastric pH was detected when comparing the control and the browned diet, but a significant change over time was detected ($p < 0.001$) (Fig. 2A). For both treatments, the mean gastric pH was near to 4.5 at the 2nd hour of digestion and it was kept above 4.0 during the period without evacuation (7th hour). After 24 h of digestion, pH reached the value 3.6–3.7, when > 75% of the gastric content dry weight was evacuated to the intestine. Duodenal pH also showed no statistical difference between diets, but a significant interaction time × diet was detected ($p = 0.043$). It fluctuated in a much narrower range (7.2–7.5) than gastric pH without an apparent effect of time. Duodenal pH for the control diet was minimal in the period 7th–10th hour (Fig. 2B), in coincidence with the onset of gastric evacuation (Fig. 1A), but this pattern is not apparent in the browned diet.

The presence of glucose-glycine melanoidins did not affect the activity of digestive proteases, neither pepsin-like nor trypsin-like activities, but they changed significantly over time ($p = 0.001$ and $p = 0.002$, respectively). Data showed the stability of pepsin-like activity while the stomach before 24 h postfeeding, and the increase of trypsin-like activity from the onset of gastric evacuation (after 7 h of digestion) onwards (Fig. 3A, B).

3.2. Experiment 2

Both diets were very well accepted, and fish of both diets displayed an active swimming behavior during feeding events. No significant effect of the inclusion of melanoidins was detected in values of growth performance, diet ingestion, weight gain, SGR or FCR (Table 2).

Gastric and duodenal pH patterns in the second experiments were shown in Fig. 4. Gastric pH was not significantly affected by the presence of melanoidins, but time ($p < 0.001$) and the interaction time diet ($p = 0.018$) exerted significant effects. The value of gastric

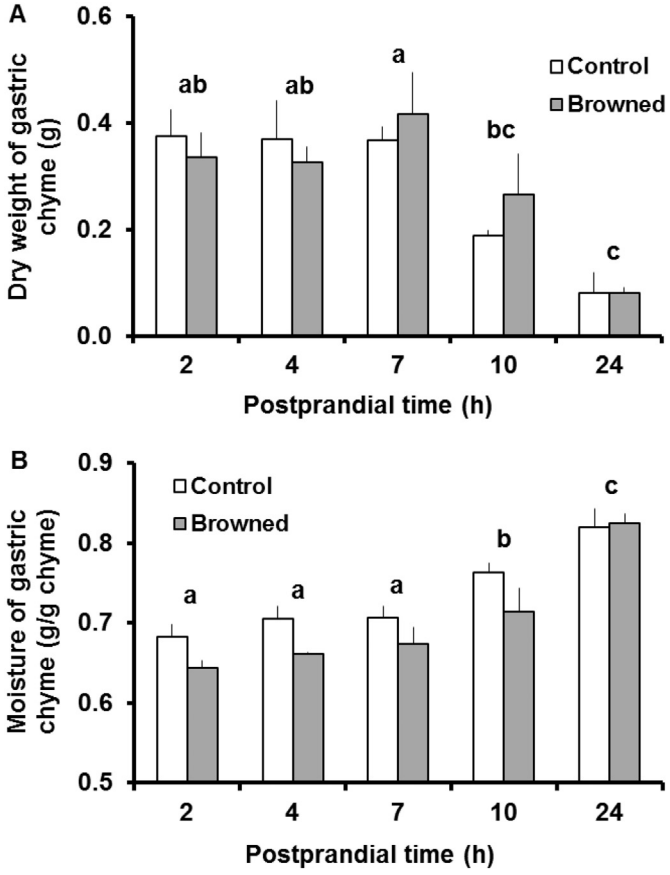

Fig. 1. Gastric content and moisture in in juvenile trouts fed diets with and without glucose-glycine melanoidins in experiment 1. (A) Dry weight of gastric contents. (B) Moisture of gastric contents. Letters on top of columns indicate significant differences with respect to time levels. Bars indicate SEM.

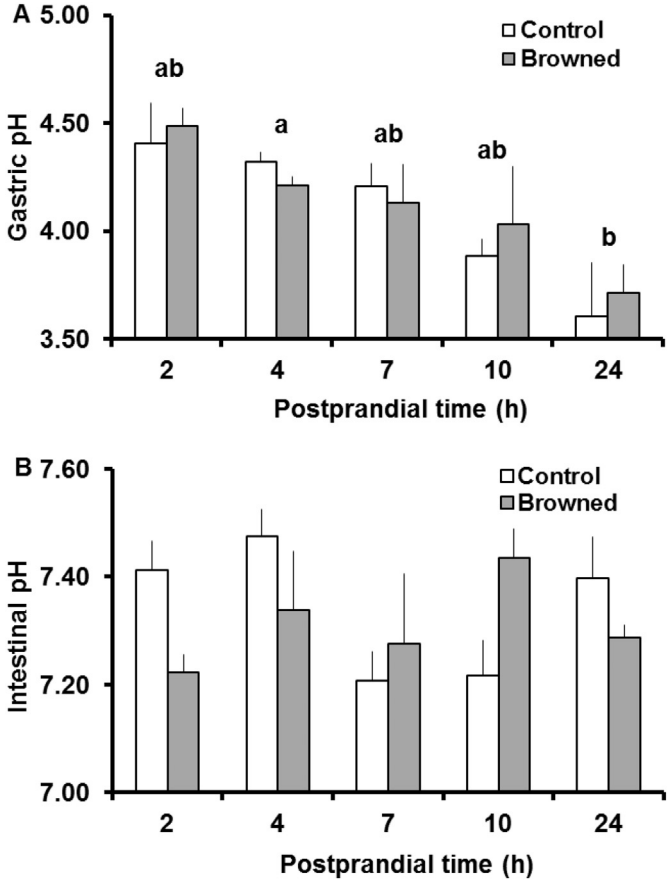

Fig. 2. Postprandial digestive pH values in juvenile trouts fed diets with and without glucose-glycine melanoidins in experiment 1. (A) Gastric pH: letters on top of columns indicate significant differences with respect to time levels. (B) Duodenal pH: only the interaction (Time × Diet) was significant, see details in the text. Bars indicate SEM.

pH dropped steadily from 2 h (pH values near 5.0 in both diets) to 10 h post-feeding, but this trend was faster in the control diet (pH 4.3 at the 10th hour) than in the browned diet (pH 4.6 at the 10th hour). Until the 4th hour post-feeding, the temporal pattern of duodenal pH mimicked that found in the stomach for both diets, but in the weakly alkaline range. As in experiment 1, duodenal pH was numerically higher for the browned diet in this experiment (about 0.3 units, see Fig. 4B), but no main effect of the diet nor interactive effect time × diet were detected.

Apparent digestibility coefficients of macronutrients are shown in Table 3. Protein and lipid apparent digestibility coefficients (ADC) were very high and similar in both diets, with values close to 99%. Lower ADC values were obtained for carbohydrates which presented values in the range 12–17%. The main observed difference was related to fiber ADC, which was about 5% higher for fish receiving the browned diet, although it was close to the threshold of statistical significance ($p = 0.062$). The ADC values dry matter ($p = 0.01$) and Energy ($p = 0.009$) showed statistical differences although the size of the effect was small: dry matter and energy ADC's were 1.0% and 0.7% higher, respectively, for fish fed the browned diet.

The antioxidant capacities (AC) of the diets were 94 ± 15 and 207 ± 14 mmol $(100\,g)^{-1}$ for the control and browned diet, respectively, showing a significance difference ($p < 0.05$). The factor most clearly affecting the plasmatic AC was the ultrafiltration through 3 kDa filters ($p = 0.001$), so that the total AC is close to double the AC due to molecules smaller than 3 kDa (Fig. 5A). In addition, the diet and the postprandial time also exert significant effects but only in complex combination with the ultrafiltration factor. The interactions (ultrafiltration × time) and (ultrafiltration × time × diet) showed *p*-values of 0.003 and 0.006, respectively. The double interaction ultrafiltration × time is explained by the differences in the rate of increase of AC over time, whose maxima are attained in the period 4–10 h for the control diet and, as a contrast, between 2 and 4 h post feeding for the browned diet (Fig. 5A). The triple interaction (ultrafiltration × time × diet) is disclosed after splitting the lines for ultrafiltration treatments in Fig. 1a into separate lines for each diet (Fig. 5B and C). In this way, it is observed that temporal patterns of the component of plasmatic AC related to molecules below 3 kDa are similar for both diets (Fig. 5C), and consisted in a stasis or slight decrease between 2 and 4 h after feeding, followed by a small increase of 0.074 mM Trolox equivalents in the period 4–10 h. On the other hand, the temporal pattern of total plasmatic AC showed clear differences in relation to diet. In the case of the control diet, the behavior of total plasmatic AC over time is similar to that recorded for molecules below 3 kDa, although the whole line is located 0.200 mM Trolox equivalents above the line for small molecules. On the contrary, the total plasmatic AC of the browned diet shows a very different pattern, with a steep increment in the 2–4 h period and a stasis between the 4th and the 10th hour post feeding. In addition, the total plasmatic AC for the browned diet showed the maximal recorded values for AC, amounting to > 0.500 mM Trolox equivalents in the period 4–10 h after feeding.

4. Discussion

The present work explores the effects of melanoidins on the ingestion rates, digestive physiology, and digestibility of nutrients in the rainbow trout *in vivo*. The high and similar ingestion of both diets indicates that glucose-glycine melanoidins do not affect diet palatability,

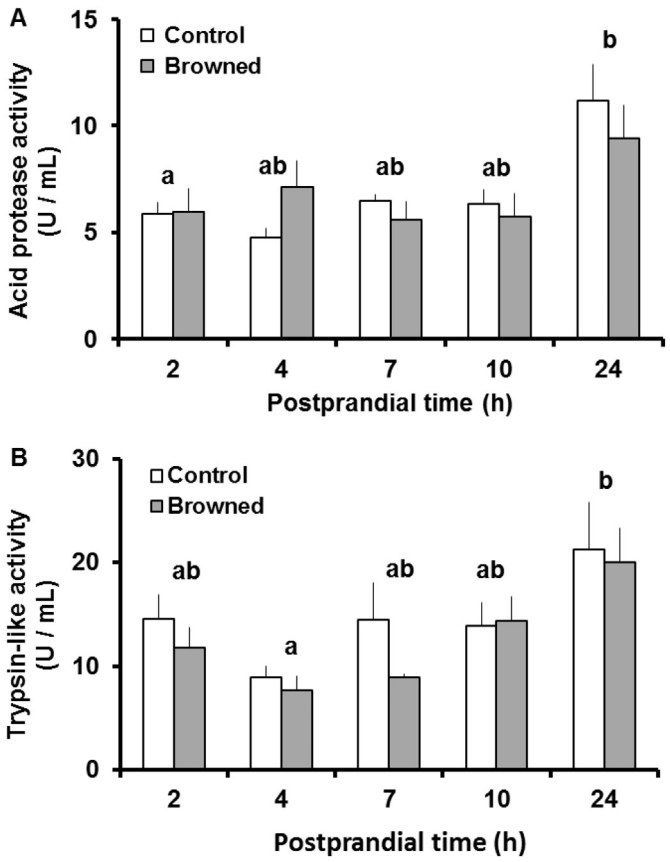

Fig. 3. Digestive protease activity in juvenile trouts fed diets with and without glucose-glycine melanoidins in experiment 1. (A) Activity of acid proteases in gastric chyme. (B) Activity of trypsin-like proteases in intestinal extracts. Letters on top of columns indicate significant differences with respect to time levels. Bars indicate SEM.

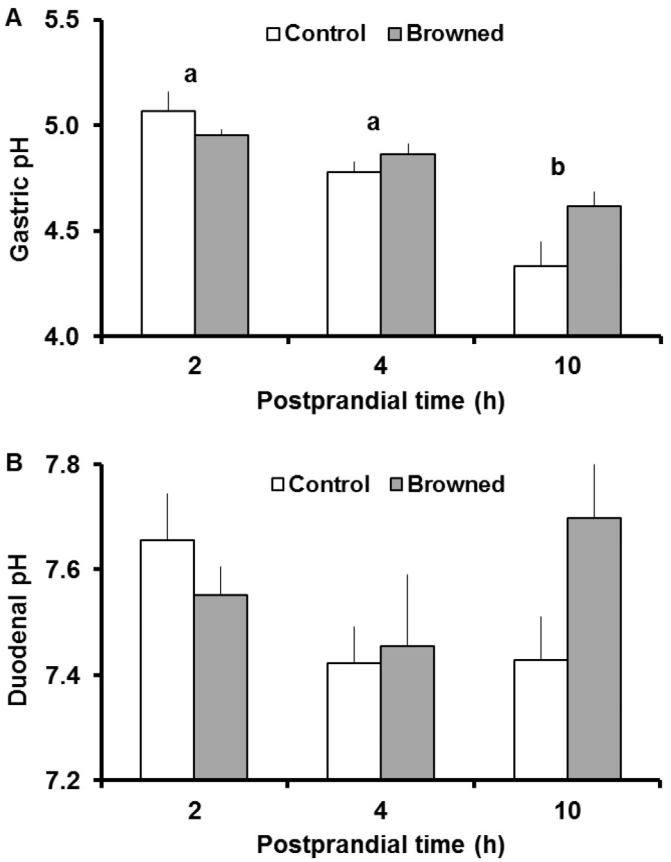

Fig. 4. Gastric and duodenal pH in juvenile trouts fed diets with and without glucose-glycine melanoidins in experiment 2. (A) Gastric pH: letters on top of columns indicate significant differences with respect to time levels; the interaction (Time × Diet) was also significant, see the text for details. (B) Duodenal pH: no significant effect was detected. Bars indicate SEM.

thus it is possible to interpret that the effects on the rest of physiological variables were solely affected by post-ingestion factors. Results also showed that feed ingestion and the main digestive parameters, such as gastric evacuation, gastric and duodenal pH, protease activities, as well as apparent digestibility coefficients of protein and lipids remained unchanged or nearly unchanged; nevertheless, the postprandial antioxidant capacity of blood plasma was modified in the presence of dietary melanoidins in the form of an interaction effect diet × time.

Maillard reaction products (MRPs) from the reaction between amino acids and monosaccharides have been shown to affect the motility of the stomach smooth muscles from rat *in vitro*, mainly those products with molecular weights above 3500 Da, although MRPs from glucose and glycine only exert a small effect (Argirova et al., 2010). This fact is compatible with the lack of effect of glu-gly melanoidins on the gastric evacuation of juvenile rainbow trout found in the present work.

Data here presented support the existence of interactive effects (diet × time) on gastric (Fig. 4A) and duodenal postprandial pH (Fig. 2B). The interpretation of these results is complex because there is not an effect of melanoidins by itself, but it can affect the temporal pattern of gut pH, *i.e.* its effect is intermingled with that of time. Thus, there is not a constant difference in gut pH between the control diet and the browned diet, but gut pH tended to be higher at the end of digestion of fish fed the browned diet (better observed in Fig. 4). These findings can be supported by the presence of dissociable groups in melanoidins, which are known to be molecules with low isoelectric points (Homma et al., 1982), probably due to carboxylic groups (Argirova et al., 2010) whose pKa values are in the range 3.0–4.0 and, hence, can interact with the physiological control of gut pH.

No significant effects of melanoidins on gastric or trypsin-like proteases were observed during digestion (Fig. 3), pointing to an equal secretion and/or synthesis of digestive enzymes in both dietary treatments. The potential effects of melanoidins on digestive enzymes during the *in vivo* digestion can be evaluated through the postprandial changes in gut pH. Glucose-glycine melanoidins have been proven to inhibit trout gastric proteases *in vitro*, probably by precipitation of the enzyme (Horikoshi and Gomyo, 1976), but this interaction was dependent on the pH so that inhibition rates are below 10% when pH is above 4.0 (Serrano et al., 2016). Since the postprandial pH in the gastric chyme of rainbow trout was in the range 4.1–4.5 between the 2nd and the 7th hour of digestion in the first experiment, a null or tiny effect of melanoidins on gastric protease activity can be expected *in vivo*. Gastric pH was even higher in the second experiment, standing above 4.2 after 10 h of digestion. Other authors have also recorded gastric pH of juvenile trout well above 4.0 for a long period after the ingestion of a commercial feed (Bucking and Wood, 2009), and the same is true for juveniles of the cultured marine fish *Sparus aurata* (Márquez et al., 2012). Similarly, trypsin-like activity did not show any significant change in relation to the presence of dietary melanoidins therefore, again, equal secretion/synthesis of the involved enzymes can be inferred. However, the inhibitory effect of sugar-amino acid melanoidins on the tryptic activity of terrestrial vertebrates is well documented (Hirano et al., 1994, 1996a; Ibarz et al., 2009) and, at pH values about 8.0, it does not depend on a precipitation mechanism (Hirano et al., 1996a). To our knowledge, no similar *in vitro* research on fish trypsins has been done to the moment, so that this inhibitory mechanism cannot be completely ruled out under *in vivo* conditions without further research.

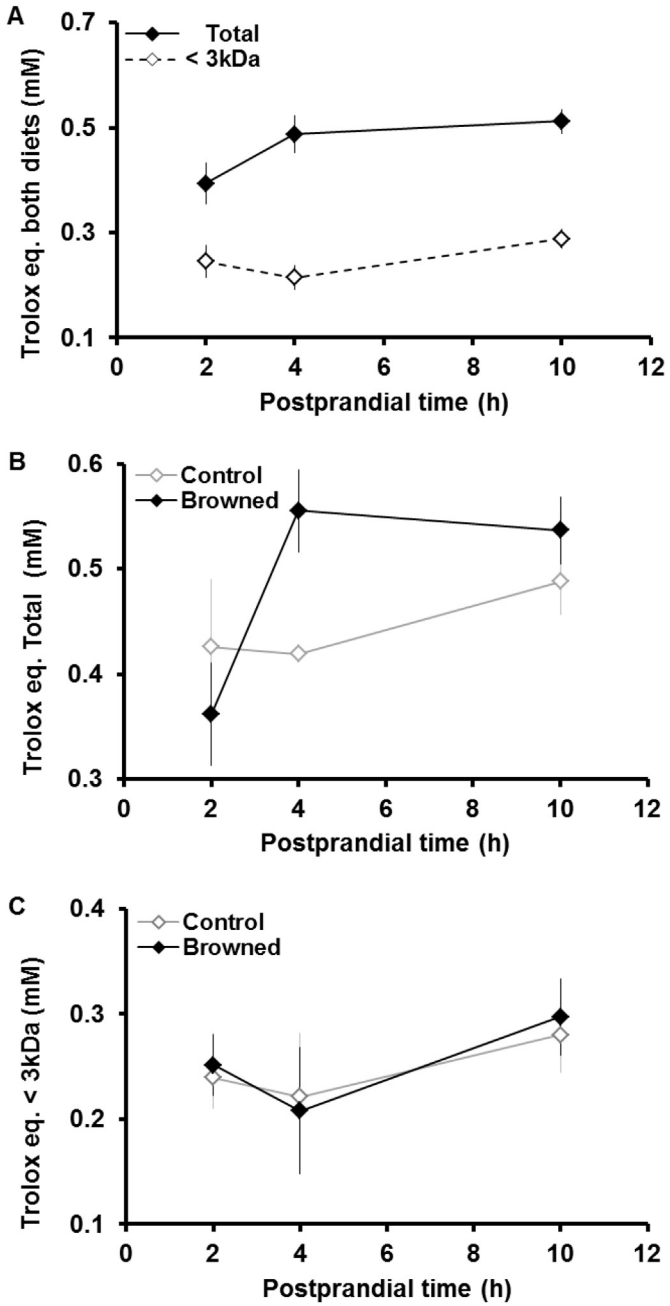

Fig. 5. Postprandial plasmatic antioxidant capacity in trouts fed diets with and without glucose-glycine melanoidins. (A) Total antioxidant capacity of blood plasma in comparison to the antioxidant capacity due to molecules above 3 kDa (both diets pooled). (B) Total plasmatic antioxidant capacity for both diets. (C) Antioxidant capacity from plasmatic molecules below 3 kDa for both diets. The factor Ultrafiltration, and the interactions (Ultrafiltration × Time) and (Ultrafiltration × Time × Diet) were significant, see the text for details. Bars indicate SEM.

Apparent digestibility coefficients of N and P did not seem to be affected by the presence of melanoidins (Table 2), although a small and significant improvement of DM/energy digestibility was detected (≤1%), probably due to an improvement in the digestibility of fiber. Interactions between diet browning and dietary melanoidins and the digestibility of nitrogen (Seiquer et al., 2006; Delgado-Andrade et al., 2014) and phosphorus (Delgado-Andrade et al., 2011; Roncero-Ramos et al., 2012) have been described, but the effects are usually small and/or not always clear. Thus, a conservative interpretation of our results made it possible to conclude that dietary glucose-glycine melanoidins exerted no deleterious effects on the digestibility of the main macronutrients in the rainbow trout, at least in the short run. This point warrants further research.

It is known that melanoidins derived from glucose-glycine aqueous mixtures show antioxidant capacity (Kim and Lee, 2009). However, the intestinal absorption rate of melanoidins in vertebrates is a decreasing function of the molecular weight and it is generally low (< 25%), but there is circumstantial evidence supporting a positive effect on the plasmatic antioxidant capacity in humans and rats (Morales et al., 2012). Similarly, data here presented support a postprandial and complex positive effect of the ingestion of melanoidins on the antioxidant capacity of plasma in juveniles of the rainbow trout, but the effect was evidenced as an interaction between the diet and the molecular weight of the plasmatic molecules (Fig. 5). The results are compatible with an effect of plasmatic molecules heavier than 3 kDa, but he mechanisms implied remain to be investigated. These preliminary findings point to a possible beneficial effect of the ingestion of melanoidins in fish, which is in keeping with recent research trends in human nutrition (Mesías and Delgado-Andrade, 2017).

In conclusion, ingested glucose-glycine melanoidins exerted minor effects on the digestive function, particularly in relation to gut pH, and did not impair the digestibility of the main macronutrients. On the other hand, the data here reported are compatible with an increase of the postprandial antioxidant capacity associated with plasmatic macromolecules in fish fed melanoidins. Further research is necessary to understand beneficial and deleterious effects derived from long-term ingestion of melanoidins and other Maillard-related compounds by fish. In this view, this study has only indirect implications for the aquafeed industry at the moment, but it suggests calling more attention to ingredients undergoing potential browning processes, dried distillers' grains with solubles being an interesting example.

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