Effect of the Incorporation of Amaranth (*Amaranthus Mantegazzianus*) into Fat- and Cholesterol-Rich Diets for Wistar Rats

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Abstract: The hypocholesterolemic effect of amaranth was studied in male Wistar rats fed a high-fat diet that was supplemented with amaranth flour, AF, or isolated protein, AI. Likewise, an *in vitro* test was carried out, in which the capacity of the AI, AF, the digested isolate, DAI, and the digested amaranth flour, DAF, to displace the cholesterol of the model micelles was evaluated. The *in vivo* results showed an increase in the excretion of cholesterol through feces (77% for AF7; 23% and 108% for AI30 and AF30, respect control) and a decrease in the content of hepatic cholesterol (98% for AF7; 96% and 53% for AI30 and AF30 respect control); whereas *in vitro* it was shown that both AF and DAF have greater power to displace cholesterol than the AI and DAI (IC_{50} 0.1, 0.71, 0.2, and 2.1 for AF, DAF, AI, and DAI, respectively). These evidences show that the proteins and fibers of amaranth have an effect on cholesterol metabolism.

Keywords: amaranth, cholesterol, hypocholesterolemia, fiber, peptides

Practical Application: Nowadays, consumers give great importance to the effect that food has on health. The results shown in this work evidence the potential hypocholesterolemic activity presented by amaranth, this is of great importance due to the increase in the incidence of dyslipidemia in the world population and the importance of amaranth as a nonextensive crop of excellent agronomic, nutritional, and bioactive properties suitable for preparation of functional foods.

Introduction

Nontransmissible diseases (NTD) are the main cause of mortality worldwide. More than 40 million deaths due to NTD are reported yearly, with these diseases affecting people of all ages, regions, and countries (World Health Organization, 2018). The main NTD are the cardiovascular and respiratory diseases, cancer, and diabetes. NTD are all influenced by genetic, physiological, environmental, and behavioral factors. Smoking, a sedentary lifestyle, excessive alcohol ingestion, and unhealthy diets are habits that increase the risk for NTD (Ministerio de Salud de la Nación, 2011). A sedentary behavior and the ingestion of unhealthy diets can cause hypertension, overweight and obesity, hyperglycemia, and hyperlipidemia, which are risk factors for metabolic syndrome. It is estimated that hypercholesterolemia, the most frequent type of hyperlipidemia, causes 2.6 million deaths yearly, which represents 2% of the total number of deaths globally (Ministerio de Salud, 2009; Ministerio de Salud de la Nación, 2011; World Health Organization, 2018).

Food proteins are not only an essential amino acids source, they also give rise to peptides with biological activity, biopeptides, encrypted in their sequences and released during gastrointestinal digestion and/or food processing (Sánchez & Vázquez, 2017). Many studies carried out in animal models and humans have demonstrated that proteins also have a strong impact on health by decreasing the lipid levels in individuals with dyslipidemia (Boachie, Yao, & Udenigwe, 2018; Howard & Udenigwe, 2013). In addition to proteins, other components such as phenolic compounds, free or bound to carbohydrates, lipids, or proteins have shown has hypolipidemic, antihypertensive, antiinflammatory, and antitumor activities (Alu'datt et al., 2017).

The consumption of soy, amaranth, hemp, and lupine proteins helps prevent hypercholesterolemia by either hampering the absorption of exogenous cholesterol (Cho) or by modulating its biosynthesis. It has been demonstrated that the former effect is the result of a decrease in micellar solubility of Cho and/or a modulation of enterocyte membrane transporters (Boachie et al., 2018).

Amaranth seeds are high in protein, near to 15% (w/w), and their essential amino acid composition is close to the optimum amino acid balance required in the human diet (Ogrodowska, Zadernowski, Czaplicki, Derewiaka, & Wronowska, 2014). They are a very good source of healthy lipids, like unsaturated fatty acids, and bioactive compounds like tocopherols, squalene, and biopeptides, between others (Ogrodowska et al., 2014; Tovar-Pérez, Lugo-Radillo, & Aguilera-Aguirre, 2019; Velarde-Salcedo, Bojórquez-Velázquez, & Barba de la Rosa, 2019). Different studies show that amaranth biopeptides in addition to presenting cho-lowering activity, present antithrombotic, antioxidant, and antihypertensive activity, which is why they have a potential application in foods as a functional component (Chávez-Jáuregui et al., 2010; Orsini-Delgado et al., 2016; Lado, Burini, Rinaldi, Añón, & Tironi, 2015; Plate & Arêas, 2002; Quiroga, Aphalo, Nardo, & Añón, 2017; Sabbione, Rinaldi, Añón, & Scilingo, 2016).

Although there is an extensive literature on the relationship between diet and lipid metabolism (Escudero, Zirulnik, Gomez, Mucciarelli, & Giménez, 2006; Frota, Mendonça, Saldiva, Cruz,

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& Arêas, 2008; Grajeta, 1999; Lado et al., 2015), there is still no a clear knowledge about the action exerted by different components present in seeds—proteins and fibers among others—nor a relation between their possible mechanisms of action, evaluated by means of *in vitro* assays, and the effect exerted *in vivo*. For that reason, the aim of this work was to assess the potential Cho-lowering capacity of amaranth *in vivo* in male Wistar rats fed with fat and Cho-rich diets supplemented with an amaranth isolate (AI) or with amaranth flour (AF), during 7 and 30 days. The capacity of AI and AF to displace Cho from model micelles before and after subjecting the preparations to a simulated digestion process (DAI and DAF) was also evaluated.

Materials & Methods

Plant material, sample preparation, and chemical characterization

Amaranthus mantegazzianus seeds, line AMAN-G1/3 2015 harvested in Río Cuarto, Córdoba, Argentina, were used. The AF was obtained with a cyclone mill (1 mm mesh). To prepare the AI, the flour was defatted with *n*-hexane (10 g flour/100 mL *n*-hexane) for 24 hr at room temperature, with constant stirring during the first 5 hr and then with overnight contact. The protein isolate was prepared according to Martinez and Añón (1996). Briefly, defatted flour was suspended in water and pH was adjusted to 9. The suspension after 30 min stirring at 25 °C was centrifuged (9000 × *g*, 20 min at 10 °C). The supernatant was harvested and storage proteins were precipitated at the isoelectric point (pH 5). After centrifuging (10000 × *g*, 20 min, 4 °C), the sediment was separated, suspended in water, and adjusted to neutral pH, freeze dried, and stored at 4 °C, until use. The procedure was repeated several times. All the preparations were pooled to perform the experiments. The protein content was determined by micro-Kjeldahl method (Nkonge & Ballance, 1982) using a conversion factor of 5.85 (Paredes Lopez, 2000). The following methods were also applied: 2 hr hydrolysis with 1.2 M hydrochloric acid at boiling temperature followed by the anthrone method for carbohydrates, heating in a muffle at 550 °C for the determination of total ashes, drying in a stove at 105 °C until constant weight for water content, and sequential enzymatic digestion by heat stable α-amylase, protease, and amyloglycosidase according to AOAC methods 923.03, 925.09, and 991.43, for quantification of total dietary fiber (Association of Official Analytical Chemists, 2006). Every determination was performed at least in duplicate.

In vivo experiments

Diets were prepared at the Nutrición y Bromatología, Facultad de Farmacia y Bioquímica, Univ. de Buenos Aires, Buenos Aires, Argentina, and were formulated according to the American Institute of Nutrition, using AIN-93 as base diet (Table 1, BD; Reeves, Nielsen, & Fahey, 1993). All diets were prepared with casein (Dilsa, Lanús, Argentina), L-cystine (Anedra, San Fernando, Argentina), soybean oil (Molinos Río de La Plata, Victoria, Argentina), choline chloride (Sigma), micro-crystalline cellulose (Cofem), dextrin (Cofem), vitamins, and minerals.

A total of 36 male 11 to 12 weeks old Wistar rats (345–377 g of body weight), provided by Instituto de Investigaciones Bioquímicas de La Plata bioterium, Facultad de Ciencias Médicas, Univ. Nacional de La Plata, Argentina, were used in this assay. The present protocol (T04-02:2015) was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL, Facultad de Ciencias Exactas, Univ. Nacional de La Plata, Argentina).

Animals were divided into six groups of six animals each. After 1 adaptation week with the AIN-93 diet, five groups began to be fed the diets under study. The control group received the control diet, prepared by adding pork fat (10% [w/w]; Dinamarg SA, Loma del Mirador, Buenos Aires, Argentina) and Cho (2% [w/w]; Sigma, St. Louis, MO, USA) to base diet (Table 1 and 2). The remaining four groups were divided into two feeding schemes. Animals subjected to one of the schemes were fed for 30 days with a diet in which 25% of the casein was replaced by amaranth protein as either protein isolate (AI30) or as amaranth flour (AF30). In the other scheme, animals were fed for 7 days with a diet in which 50% of the casein was replaced by amaranth protein (AI7 and AF7) (Table 1 and 2). The sixth group of animals, BD group (Table 2, BD basic diet), was fed with the AIN-93 diet throughout the experiment.

Animals were placed in stainless steel cages, three rats per cage, in a room with controlled lighting (12 hr of light, 12 hr of darkness) and temperature (18 and 20 °C). Food and water were given *ad libitum*. During the last week of the trial, animals were placed individually in metabolic cages in order to collect the feces. The fecal matter was collected, weighed, lyophilized, milled, and stored at 20 °C until used. Before sacrifice, animals were fasted for 12 hr and anaesthetized with pentobarbital/diazepam. The whole blood volume was collected by cannulation of the abdominal aorta into heparin-coated tubes. The plasma was obtained by centrifugation of blood samples at 1500 × *g* for 10 min at room temperature. The liver of each animal was resected, weighed, fractionated, and immediately frozen in liquid nitrogen.

The content of triacylglycerides (TAG) was determined by TG color GPO/PAP AA, Wiener Laboratorio SAIC, Rosario, Argentina. The content of total Cho, and low- and high-density lipoproteins (LDL and HDL) were determined, by Enzymatic Colestat, LDL precipitating reactive, and HDL precipitating reactive, Wiener Laboratorio SAIC, Rosario, Argentina.

The total lipid (TL) content was determined in livers. Briefly, 1 g of liver was homogenized in 20 mL of the Folch, Lees, and Sloane Stanley (1957) reagent (chloroform/methanol, 2/1). The homogenate was then quantitatively transferred to a ground glass test tube and 2 mL of water were added. The preparation was mixed by inversion and left to settle overnight for phase separation. The upper aqueous phase was then separated, and the extract was filtered through filter paper containing anhydrous sodium sulfate to retain the aqueous residues. The lipids fraction was obtained and weighed after evaporating the chloroform phase. The lipids extract was suspended with Triton X-100 (5 g/L) and aliquoted to determine the content of Cho and TAG. The content of phospholipids, PL, was determined colorimetrically by measuring the phosphorus content in the suspension after wet digestion with perchloric acid (Chen, Toribara, & Warner, 1956). Freeze-dried feces (0.2 g) were mixed with 2 mL of a 10 M NaOH and 96% ethanol (1/2, v/v) mixture. Suspensions were homogenized and incubated at 70 °C for 45 min. After cooling at room temperature, samples were centrifuged (5 min, 1000 × *g*, room temperature). The supernatant was extracted twice with 1 mL of hexane. Hexane phases were pooled and washed with 70% (v/v) ethanol to reach neutrality. The solvent was then evaporated. The solid extract obtained was suspended in acetonitrile/isopropanol (70/30 v/v), stirring for 75 min at 600 rpm and 23 °C, and analyzed by RP-HPLC using a C18 Symmetry column (150 mm × 4.4 mm ID, 5 μm). The flow rate was set at 1 mL/min at 35 °C. The absorbance of eluates was monitored at 210 nm. A

Table 1–Formulation and Energy provided by diets.

	Diets composition					
	Control	**AI30**	**AF30**	**AI7**	**AF7**	**AIN-93/BD**
Ingredients (g/kg)						
Casein	140	104	105	68	70	140
Dextrin	640.7	640.7	492.7	640.7	344.7	720.7
Soy oil	–	–	–	–	–	40
Fiber	50	50	50	50	50	50
Choline	2.5	2.5	2.5	2.5	2.5	2.5
L-Cystine	1.8	1.8	1.8	1.8	1.8	1.8
Mix of vitamins[a]	10	10	10	10	10	10
Mix of minerals[a]	35	35	35	35	35	35
Cholesterol	20	20	20	20	20	–
Pork fat	100	100	100	100	100	–
Amaranth isolate	–	36	–	72	–	–
Amaranth flour	–	–	183	–	366	–
Energy (kcal/kg)	4202.8	4171.12	4200.97	4139.44	4199.14	3802.8

[a]According to recommendations of AIN-93 for rodents (Reeves et al., 1993).

Table 2–Design of the experiment with animals. Time of feeding and diets used.

Animals groups		**Feeding time (in weeks)**			**Source of amaranth protein**	**Percentage of casein replacement (%)**
Names	**Number of rats**	**AIN-93**	**Control diet**	**Specific diet**		
BD	6	6	–	–	–	–
Control	6	1	5	–	–	–
AI30	6	1	–	4	Isolate	25
AF30	6	1	–	4	Flour	25
AI7	6	1	4	1	Isolate	50
AF7	6	1	4	1	Flour	50

calibration curve with pure Cho solutions (0.1–0.5 mg/mL) was used.

In vitro assay

Preparation of amaranth samples. The DAI and DAF were prepared according to the protocols described by Gawlik-Dziki, Dziki, Baraniak, and Lin (2009), Minekus et al. (2014), and Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005) with some modifications. Briefly, 300 mg of AF or lyophilized AI were suspended in 6 mL of salivary-like fluid (2.38 g/L Na_2HPO_4, 0.19 g/L KH_2PO_4, 0.3 g/L NaCl, pH 6.8, 60 mg of α-amylase) and shook at 37 °C for 5 min. Then 12 mL of gastric-like fluid (2.5 g/L pepsin in 0.03 M NaCl, pH 2) were added at 37 °C and stirred for 60 min. Later, 12 mL of duodenal-like fluid (3 g/L pancreatin in 0.1 M $NaHCO_3$ pH 7) were added at 37 °C, and stirred for 60 min. Finally, all enzymes were inactivated by heating the mixtures for 10 min at 80 °C. The digests obtained were lyophilized and stored at 4 °C. The degree of hydrolysis (DH%) was determined by quantifying free amino groups by the *o*-phthaldialdehyde method (OPA; Sigma) according to Nielsen, Petersen, and Dambmann (2001). The DH% was calculated according the following expression:

$$GH\% = [(NH_{2t} - NH_{2t0}) / NH_{2\,total}] \times 100$$

where NH_{2t}, NH_{2t0} are the free amino groups at time t and zero time of hydrolysis; $NH_{2\,total}$ is the total amino groups present in the protein calculated as:

$$NH_{2\,total} = NH_{2\infty} - NH_{2t0} \text{ and}$$

$$NH_{2\infty} = (1/MW_{aa}\ \text{average}) \times \left(1 + f_{Lys}\right) \times C_{prot}$$

where $NH_{2\infty}$ corresponds to the free amino groups of a completely hydrolyzed sample; MW_{aa} is the average of the molecular weights of the amino acids in the sample protein. For amaranth proteins 130 g/mol is considered; f_{Lys} is the proportion of lysine in the samples (1/20) (Bressani & Garcia-Vela, 1990) and C_{prot} is the concentration of the sample used in the test (g/L).

Displacement of cholesterol in model micelles. The exclusion of Cho from model micelles was assessed as described by Nagaoka et al. (2001) with slight modifications. Micelles were prepared as follows: 5 mL of a solution containing sodium taurocholate (6.6 mM), Cho (0.5 mM), oleic acid (1 mM), phosphatidylcholine (0.6 mM), NaCl (132 mM), and sodium phosphate (15 mM), pH 7.4 were sonicated with pulses of 10 s (with 10 s in between) for 2 min at 50% intensity in either the presence or absence of amaranth samples (AI, DAI, AF, and DAF at 0.01–5.00 mg protein/mL). Micelles were incubated for 2 hr at 37 °C with shaking and then centrifuged at 70000 × *g* for 1 hr at 20 °C. Free Cho in the supernatant (ChoS) and micellar Cho in the pellet (ChoP) were quantified by HPLC. To calculate the percentage of Cho displaced from the micelles, the following formula was used, where the blank corresponds to the micelles formed in the absence of amaranth:

$$\%\ \text{of displacement} = \left(\frac{ChoS}{ChoS + ChoP} - \frac{ChoS\ blank}{(ChoS + ChoP)\ blank}\right) 100 \qquad (1)$$

Measurement of model micelle size. DLS determinations of model micelles prepared with and without amaranth were made at a constant scattering angle in all cases using a photon correlation spectrometer which allows size measurements of particles with

sizes between 0.6 nm and 6 μm from INIFTA Facultad de Ciencias Exactas, UNLP, or from Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, UBA (Zetasizer NanoZS, Malvern Instruments Ltd., UK). The Zetasizer NanoZS uses a laser at the light wavelength of 633 nm. All measurements were carried out in triplicate at 37 °C after 5 min of equilibration; the values reported are the average of at least three independent samples (Rupp, Steckel, & Müller, 2010).

Statistical analysis

Results showed were evaluated using one-way analysis of variance (ANOVA) with the *post hoc* Dunnett's test for *in vivo* experiments or using ANOVA with the *post hoc* Tukey's test for *in vitro* assays. IC_{50} values were calculated using GraphPad Prism program.

Results and Discussion

Diets and animal weight gains

Table 1 shows the composition of the hypercholesterolemic diets Control, AI30, AF30, AI7, and AF7, used to feed the different groups of male Wistar rats. The diets contained the same amount of Cho and pork fat, but they differed in the origin and amount of added protein, as well as in the time of consumption (Table 2).

The AF contained 13.6 ± 0.4% proteins, 7.3 ± 0.2% lipids, 69 ± 3% carbohydrates, 2.4 ± 0% ashes, and 8.2 ± 0.2% water. In addition to proteins, the AF added other components to the diet, such as fiber, starch, triglycerides, squalene, and salts. Due to its composition, AI mainly contributed proteins to the diets (80.7 ± 0.6%), with carbohydrates as soluble fiber, lipids, ashes, and water being minor components (8.3 ± 0.2%, 1.2 ± 0.1%, 2.6 ± 0.1%, and 8.55 ± 0.06%, respectively).

The lipid-rich diets employed had similar energy densities, in the order of 4100 to 4200 kcal/kg, and equivalent contents of carbohydrates, fibers, ashes, fat and Cho (Table 1). All diets differed from the AIN-93 diet in the Cho and fat contents, and therefore, in the energy value.

Table 3 shows the daily consumption of feed, the weight gains, and the food efficiency for the different groups. All the animals ingested similar amounts of food, except for those belonging to group AF7, which ingested a significantly lower amount of feed than the rest of the animals (35% lower, $P < 0.05$). However, this difference in the amounts of feed ingested was reflected neither in the weight gain, nor in the food efficiency values.

Although the animals of the BD group consumed the same amount of food as those of the other groups, and though not significant, they showed a difference in the weight gain and the daily weight gain. This finding could be attributed to the consumption of lower amounts of fat.

Lipid profiles in plasma, liver, and fecal matter

Animals fed for a long time with a diet containing low quantities of amaranth proteins. The consumption of the control diet increased total Cho plasma levels (12%; Figure 1A) and decreased those of HDL (13%; Table 4), as compared to the BD group. The liver weights of animals fed with the control diet were markedly increased (35%). The contents of PL, TL, and Cho were also increased (70%, 125%, and 20%, respectively), as compared to animals of the BD group (Table 4; Figure 1B). Importantly, animals of the BD group excreted 0.36 ± 0.07 mg of Cho/day/g of food, whereas the excretion of Cho in feces in the Control group was 9.5 times higher (Figure 1C).

The AI30 group, fed for a prolonged period with the diet containing casein and AI (75/25), presented the same content of plasma HDL, LDL, and TAG (Table 4) and a slightly higher Cho level (7%) than animals belonging to Control group (Figure 1A). The content of TL (Table 4) and Cho (Figure 1B) in the liver of these animals was 36% and 96% lower, respectively, than that of Control group, whereas the levels of TAG and PL were similar in both groups (Table 4). The amount of Cho in the feces of the animals of this group was 23% higher, as compared to Control group (Figure 1C).

Animals belonging to group AF30 showed a different behavior. The levels of plasma HDL, LDL, TAG (Table 4), and Cho (Figure 1A) did not differ from those corresponding to Control group. The same phenomenon was observed for liver TL, TAG, and PL (Table 4). Furthermore, the amount of Cho in the liver was nearly half of that detected in Control group, (53% lower, Figure 1B), whereas the excretion of Cho in feces doubled that obtained in Control group (108%, Figure 1C).

There was a small difference in the content of amaranth protein between the AI30 and AF30 diets (2.9 and 2.5 g/100 g of diet, respectively); however, the fiber content was roughly 1.2% and only 0.03% for AF30 and AI30, respectively. These diets also differed in the lipidic contribution from amaranth, which was 0.035% and 0.18% for AI30 and AF30, respectively. When comparing the results obtained in the animals fed AI30 and AF30, we observed differences in the quantities of plasma and liver Cho, as well as in the amount of Cho in feces. The data obtained clearly indicate that the presence of fiber promotes the excretion of Cho in feces, because the animals belonging to group AF30 excreted 68% more Cho than those belonging to AI30. The products resulting from the gastrointestinal digestion of amaranth proteins, that is, peptides of different size and composition, were capable of altering the lipid metabolism and slightly affecting the excretion of Cho, as observed in the AI30 group. It can be speculated that peptides resulting from the digestion process enhance the effect exerted by fiber in animals fed AF. Although an effect of the components of the amaranth lipid fraction cannot be ruled out, it should be minimal if we consider that the animals ingested 16 to 18 g of food per day, contributing less than 0.001 g of amaranth lipids/day.

Although the Cho-lowering capacity of cereal and pseudocereal proteins added to Cho-rich diets has been extensively studied, the results are difficult to compare, because different protein sources, animal models, and different diets were used.

The studies carried out by Lado et al. (2015) and Escudero et al. (2006) with Wistar rats with diets containing isolated or amaranth protein concentrates have been shown a modification of the lipid profile in animals, particularly in liver, and increases in the excretion of Cho in feces. Grajeta (1999) has shown that Buffalo rats fed for 28 days on a diet containing 4% amaranth fiber from whole seed, 15% fat, 20% protein provided by casein and amaranth seed, and 0.5% Cho reduced the content of plasma and hepatic Cho whereas decrease TAG in the liver. This author attributed the lipid-lowering effect to the fiber present in the amaranth seed and considered that proteins, tocotrienols, unsaturated fatty acids, and vitamin E could play a role in the Cho-lowering effect.

Although the results obtained by these authors show the same tendency as those of the present work, it should be noted that we have obtained these results feeding the animals with a much smaller amount of proteins from amaranth, AI replaced only 25% of the casein present in AIN-93. Besides, under our experimental conditions, the hypercholesterolemic effect of casein was not counteracted by the addition of AI.

Table 3–Amount of food, weight of animals, and food efficiency in the groups of rats studied.

	Animals groups					
	Control (mean ± SEM)	AI30 (mean ± SEM)	AF30 (mean ± SEM)	AI7 (mean ± SEM)	AF7 (mean ± SEM)	BD (mean ± SEM)
Daily intake (g/day)	17.4 ± 0.5	16^{ns} ± 0.8	18.2^{ns} ± 0.8	15.2^{ns} ± 0.9	11.7* ± 0.6	19^{ns} ± 1
Total diet intake (g)	3136	2883	3276	638	492	3504
Body weight (g)						
Initial	377 ± 18	345 ± 9	328 ± 16	346 ± 11	322 ± 5	369 ± 17
Final	432 ± 16	395 ± 9	373 ± 9	374 ± 8	368 ± 7	399 ± 22
Weight variation (g)	55 ± 28	50^{ns} ± 13	45^{ns} ± 26	28^{ns} ± 16	46^{ns} ± 12	29^{ns} ± 13
Weight gain per day (g/day)	1.8 ± 0.8	1.6^{ns} ± 0.4	1.5^{ns} ± 0.8	0.9^{ns} ± 0.5	1.5^{ns} ± 0.4	0.6^{ns} ± 0.3
Food efficiency (%)[a]	11 ± 5	10^{ns} ± 3	8^{ns} ± 4	6^{ns} ± 3	13^{ns} ± 3	5^{ns} ± 2

ns, nonsignificative differences compared with Control.
*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$ significant differences compared with Control (one-way analysis of variance [ANOVA], with the *post hoc* Dunnett's test).
[a]Food efficiency % = (weight gain/food intake) × 100.

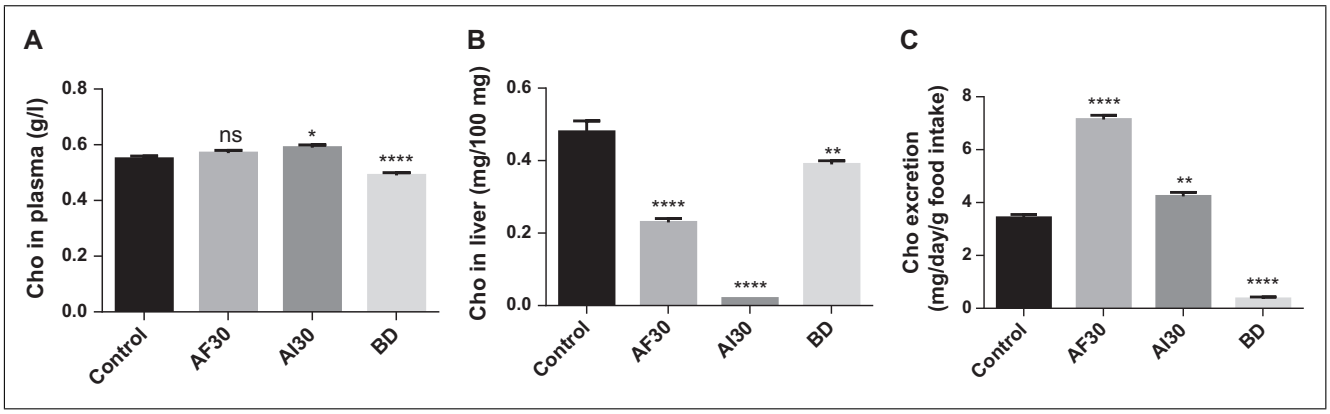

Figure 1–Cholesterol quantification in animals fed during 30 days with diets containing amaranth flour (AF30) or amaranth protein isolate (AI30), replacement 25% of casein of the base diet. Cho in feces (A), in liver (B), and in plasma (C). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$), ****$P < 0.0001$ significant differences compared with Control (analysis of variance [ANOVA] with the *post hoc* Dunnett's test).

Table 4–Lipids in liver and in blood of rats. Liver weight and total blood volume.

	Animals groups					
	Control (mean ± SEM)	AI30 (mean ± SEM)	AF30 (mean ± SEM)	AI7 (mean ± SEM)	AF7 (mean ± SEM)	BD (mean ± SEM)
Liver						
Liver weight (g)	12.4 ± 0.8	11.2^{ns} ± 0.3	11.8^{ns} ± 0.8	11.3^{ns} ± 0.5	11.1^{ns} ± 0.9	8.9^{**} ± 0.6
Phospholipids - PL (mg/100 mg of liver)	1.93 ± 0.09	1.7^{ns} ± 0.06	1.86^{ns} ± 0.07	1.42^{***} ± 0.02	1.73^{ns} ± 0.12	1.13^{****} ± 0.07
Triacylglycerides (TAG) (mg/100 mg of liver)	1.1 ± 0.1	1.02^{ns} ± 0.07	0.95^{ns} ± 0.05	1.05^{ns} ± 0.06	0.80^{ns} ± 0.05	1.08^{ns} ± 0.07
Total lipids (TL) (mg/100 mg of liver)	14 ± 1	9^{**} ± 1	13^{ns} ± 0.8	15.4^{ns} ± 0.9	12.2^{ns} ± 0.9	6.2^{****} ± 0.5
Blood						
Volume (mL)	9.4 ± 0.3	8.7^{ns} ± 0.4	9.1^{ns} ± 0.7	9.9^{ns} ± 0.2	6.7^{***} ± 0.3	7.7^{ns} ± 0.8
HDL (g/L)[a]	0.34 ± 0.01	0.37^{ns} ± 0.01	0.37^{ns} ± 0.01	0.33^{ns} ± 0.01	0.34^{ns} ± 0.00	0.39^{**} ± 0.01
LDL (g/L)[b]	0.33 ± 0.01	0.35^{ns} ± 0.01	0.33^{ns} ± 0.00	0.37^{ns} ± 0.01	0.3^{**} ± 0.01	0.33^{ns} ± 0.01
TAG (g/L)[c]	1.38 ± 0.03	1.41^{ns} ± 0.03	1.29^{ns} ± 0.02	1.31^{ns} ± 0.03	1.2^{***} ± 0.03	1.4^{ns} ± 0.1

ns, nonsignificative differences compared with Control.
*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$ significant differences compared with Control (one-way analysis of variance [ANOVA], with the *post hoc* Dunnett's test).
[a]HDL, high-density lipoproteins.
[b]LDL, low-density lipoproteins.
[c]TAG. triacylglycerides.

Studies carried out with other animal models and proteins have shown similar results to those obtained in this work as concerns the modification of the lipid profile in the liver and the excretion of Cho in feces, whereas the modifications detected in the lipid profile of the plasma were variable (Frota et al., 2008; Mendonça, Saldiva, Cruz, & Arêas, 2009; Takao et al., 2005; Villanueva, Yokoyama, Hong, Barttley, & Rupérez, 2011).

Animals fed for a short period with diets containing high amounts of amaranth protein. The AI7 group, fed with a high proportion of AI (casein/AI, 50/50), presented increased

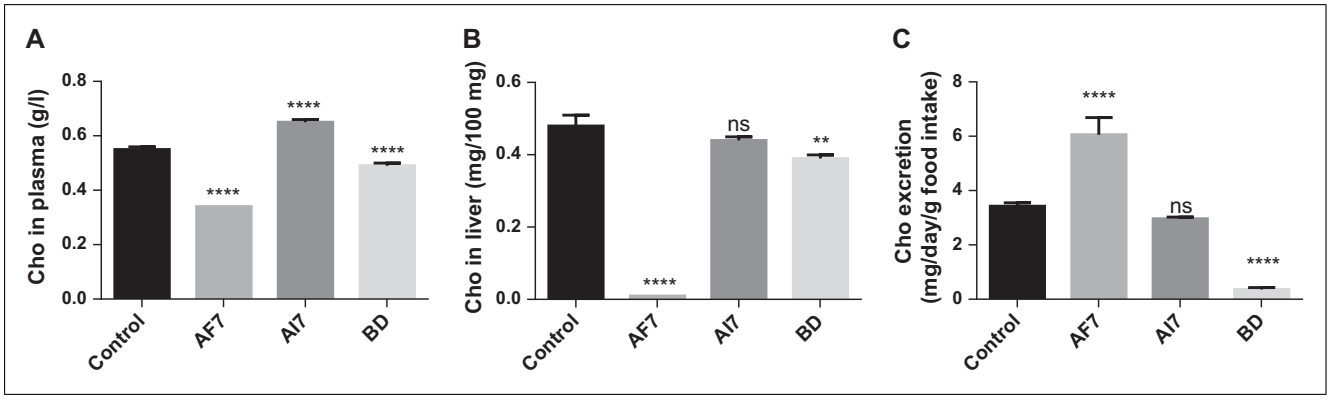

Figure 2–Cholesterol quantification in animals fed during 7 days with diets containing amaranth flour (AF7) or amaranth protein isolate (AI7), replacement 50% of casein of the base diet. Cho in feces (A), in liver (B), and in plasma (C). *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$), ****$P < 0.0001$ significant differences compared with Control (analysis of variance [ANOVA] with the *post hoc* Dunnett's test).

plasma Cho levels, as compared to Control group (18%, Figure 2A) and a minor content (27%) of PL in the liver (Table 4). The levels of LDL, HDL, and TAG in plasma (Table 4) and of Cho in liver (Figure 2B) were equivalent to those of Control group. No differences were detected in the excretion of Cho in feces (Figure 2).

The inclusion of AF caused different effects in the animals. The levels of plasma LDL, TAG, and Cho were lower (reductions of 12%, 14%, and 38%, respectively; Table 4; Figure 2A), whereas the content of Cho in the liver decreased dramatically (reduction of 98%) with respect to Control group (Figure 2B). These animals also excreted higher amounts of Cho than animals of Control group (approximately 77%, Figure 2C). The animals that were fed with the AF7 diet received a greater amount of fiber and a smaller quantity of amaranth protein than those fed with the AI7 diet (approximately 2% and 0.06% of fiber; 5% and 5.8% of protein from amaranth, respectively). Animals from groups AF7 and AI7 also received 2.7 and 0.08 g/100 g of amaranth lipids. In agreement with previous works, the results obtained herein suggest that the amaranth fiber has a high capacity to interfere with the absorption of dietary Cho and that after digestion, the amaranth proteins lose part of the Cho absorption-blocking capacity; however, their effect on lipid metabolism is more specific. Although an effect exerted by components of the amaranth lipid fraction cannot be ruled out, their contribution is negligible considering the amounts of feed ingested by the animals daily (12–15 g of food/day).

Recent studies carried out by Wang, Nagaoka, Kusada, Shimada, and Kato (2014) in Wistar rats fed for a short period with a Cho-rich diet containing casein and rice bran protein as a protein source, and fiber, have shown a reduction of plasma Cho, as compared to animals fed only with casein, and an increase in the level of neutral and acid steroids in feces, respectively. The results obtained in the AF7 group showed the same trend described by Wang et al. (2014), though in our study, animals received lower protein and higher fiber amounts than the ones used by Wang et al. (2014). Furthermore, our results are supported by a level decrease of LDL and TAG in plasma (Table 4).

In vivo experiments demonstrated that the amaranth fiber would interfere with the absorption of dietary Cho and might also affect the enterohepatic circulation of bile salts. This effect is currently being analyzed in our laboratory. A complete characterization of the fiber present in the amaranth seed has not been performed. However, recent work has shown that amaranth fiber is constituted by 78% of insoluble fiber rich in pectin, and 22% of soluble fiber consisting mainly of arabans and xylans (Lamothe, Srichuwong, Reuhs, & Hamaker, 2015).

To date, no studies have been performed to assess the hypocholesterolemic properties of pseudocereal proteins added to Cho-rich diets for short periods.

The studies carried out herein also suggest that the hydrolysis products of the amaranth proteins generated during the digestion process in animals have a much lower capacity to increase the excretion of Cho. Furthermore, some aspects cannot be ruled out, such as an interaction between these peptides and bile salts, their capacity to modify the activity of some key enzymes of Cho metabolism for example, HMGR, and/or the capacity to modulate Niemann-Pick C1-like transporter, as demonstrated for amaranth and other peptides of food origin (Matsuoka et al., 2014; Nardo, 2017; Soares et al., 2015).

Micellar solubility. The incorporation of dietary Cho into micelles is a critical step in the absorption of Cho by enterocytes. Given the greater excretion of Cho found in the feces of the animals that consumed amaranth, particularly during a relatively long period, we carried out *in vitro* tests to estimate the potential effect of amaranth on the incorporation or exclusion of Cho from micelles. Initially, the formation of the micelles was monitored by measuring the average size of the particles obtained by sonication of the mixtures of Cho, PL, sodium taurocholate, oleic acid, and amaranth samples (AI, AF, and DAI and DAF). Figure 3 shows the micelle size (mean ± standard error) obtained in the absence of amaranth proteins. A nonnormal distribution of particles with an important degree of polydispersity (10–200 nm) was observed, with a maximum frequency of 53 ± 0.01 nm. It is noteworthy that the micellar size remained stable for at least 3 hr (data not shown).

When the micelles were formed in the presence of AI, a displacement of the particle size distribution was detected towards larger diameter values, with a maximum size of 71 ± 0.03 nm. In the presence of AF, a polymodal distribution was detected with a first smaller peak of 46 ± 0.02 nm and more abundant micelle population with sizes greater than 150 ± 0.01 nm, which could correspond to micelle aggregates and/or aggregates of some of the components present in the mixture (Figure 3 and insert). These results suggest that amaranth proteins can interact with some of the components that participate in the formation of the micelle, that is, Cho, bile acids, and/or PL, thus modifying its size. This effect was enhanced when, in addition to proteins, fibers from amaranth were present in the mixture, suggesting an additional

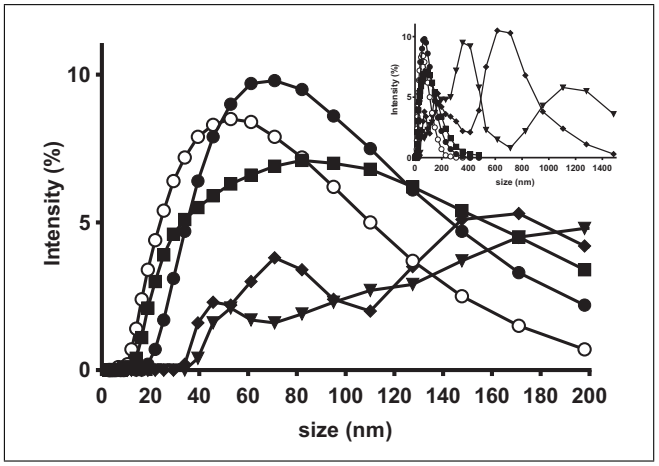

Figure 3–Micelles size (in nm) prepared without amaranth (empty circles), with amaranth protein isolate (full circles), with amaranth flour (full triangles), with amaranth protein isolate or amaranth flour submitted to simulated gastrointestinal digestion (full squares and full rhombus, respectively). All the maximum frequencies were significantly different respect to the micelle without amaranth protein ($P < 0.05$, analysis of variance [ANOVA] with the *post hoc* Tukey's test), every determination was performed at least in triplicate.

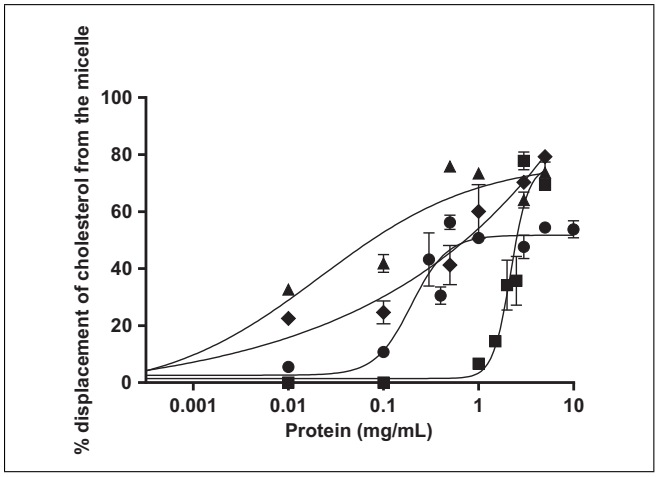

Figure 4–Displacement of cholesterol (%) from the micelles prepared with amaranth flour (full triangles), with amaranth flour submitted to simulated gastrointestinal digestion (full rhombus), with amaranth protein isolate (full circles), and with digested amaranth protein isolate (full squares) in function of protein concentration. Protein concentrations (mg/mL) that promote 50% displacement (IC_{50}, mean ± standard error) were 0.1 ± 0.3, 0.71 ± 0.07, 0.2 ± 0.1, and 2.1 ± 0.03 all the values are significantly different except the amaranth protein isolate with respect to the amaranth flour ($P < 0.05$, analysis of variance [ANOVA] with the *post hoc* Tukey's test), every determination was performed at least in triplicate.

interaction of these components with the rest of the components of the micelle.

AI and AF were subjected to a simulated gastrointestinal digestion process, and the products obtained, DAI (44% DH) and DAF (48.4% DH) were evaluated for their capacity to influence the formation of micelles. The results obtained show, in both cases, a larger micellar size with respect to the respective undigested samples, with the maximum particle size of 82 ± 0.01 and 71 ± 0.02 nm for each distribution, respectively. The presence of fiber in the DAF sample induced the formation of more than one population of particles larger than 100 nm, as observed for AF.

During the simulated gastrointestinal digestion, proteins decrease their molecular size while leaving the fibers present in the AF intact. It is therefore evident that the modification of the size of the proteins/peptides affects their ability to interact with the other components of the micelle and that the fibers also have the ability to bind to micelles and are responsible for the formation of aggregates of different molecular sizes.

As far as we know, no studies have been conducted on the effect of simulated gastrointestinal digestion products of seed flours and protein isolates in the solubility tests.

Figure 4 shows that the interaction of the proteins and the amaranth fibers with micelles is dose-dependent, that both components have the capacity to interfere in the formation of micelles, and that the size of the proteins is crucial in such interaction. The IC_{50} values calculated were 0.2, 0.1, 2.1, and 0.71 mg/mL for AI, AF, DAI ,and DAF, respectively. On the other hand, the amounts of Cho displaced during the formation of the micelle in the presence of 1 mg/mL of proteins were 50.7 ± 0.5%, 73.4 ± 1%, 6.6 ± 1%, and 60 ± 6% (mean ± standard error all the values are significantly different $P < 0.05$) for AI, AF, DAI, and DAF, respectively. This finding would indicate that the proteolysis that occurs during the artificial gastrointestinal digestion has a negative effect on the ability of proteins to hamper the incorporation of Cho into the micelle. However, a concentration 3 mg/mL DAI protein induced a displacement of 80% of the initial Cho present in the mixture, a value that was significantly higher than the one displaced by the same amount of AI, which shows a maximum competitive power of 50%. For all micellar solubility tests, we obtained a cholesterol recovery of around 80% to 90%.

Many studies (Howard & Udenigwe, 2013; Ruiz, Ancona, & Campos, 2014; Yust, Millán-Linares, Alcaide-Hidalgo, Millán, & Pedroche, 2012; Zhang, Yokoyama, & Zhang, 2012) have shown that hydrophobic peptides have greater Cho-lowering capacity because they are stronger competitors and displace Cho from the micelle more efficiently. Zhang et al. (2013) have also shown that the presences of cationic amino acids in the peptide composition, which can interact with the carboxylic groups of bile acids, are important to accomplish the lipid-lowering effect. Interestingly, some peptides can interact with both Cho and bile acids being its hypocholesterolemic activity equivalent to that of the cholestyramine compound that limits the absorption of cholesterol, reduces its concentration in plasma and possesses a high affinity for bile acids (Nagaoka, Nakamura, Shibata, & Kanamaru, 2010).

Our results are in agreement with those obtained with egg albumin and cowpea protein hydrolysates (Marques et al., 2015; Matsuoka et al., 2014; Nagaoka, Masaoka, Zhang, Hasegawa, & Watanabe, 2002), in terms of the ability of dietary proteins to reduce the incorporation, and possibly, the displacement, of Cho into/out of micelles, as well as with authors showing the importance of size and composition of bioactive peptides.

To our knowledge, no studies have been reported on the effect of fibers in micellar solubility tests.

Conclusions

In *in vivo* tests, we have demonstrated that both fibers and hydrolysis products of the amaranth proteins interfere with Cho absorption at the intestinal level. Amaranth fiber, proteins, and the peptides released in the digestion process are also capable of interfering with the formation of micelles and of reducing the amount of Cho that is incorporated. In a case of peptides, a greater amount of them is required to initiate the displacement of Cho. This mechanism would account for the greater excretion of dietary Cho in

feces. Fiber components and digested products, in turn, modify the lipid profile, particularly when half of the casein present in the diet is replaced by AF during a short period of time. The ingestion of AI during short periods after a longer intake of the Cho-rich diet yielded negative results, because not only was it unable to increase the excretion of Cho, but it elevated its plasma levels.

According to these results, the consumption of foods prepared with flour and/or amaranth protein isolates would contribute to the reduction of cholesterol in consumers.

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Authors' Contributions

M.S. Sisti, A. Scilingo, and M.C. Añón designed this study. M.S. Sisti performed experimental analyses and discussed all data. M.S. Sisti, A. Scilingo, and M.C. Añón wrote and corrected this manuscript.

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