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α-Galactosides present in lupin flour affect several metabolic parameters in Wistar rats

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The aim of this study is to evaluate the influence of α-galactosides present in a lupin diet on metabolic parameters in growing Wistar rats. Lupin (*Lupinus albus*) is a rich source of non-digestible carbohydrates called α-galactosides whose beneficial health effects have been widely reported. For our investigation, twenty male Wistar rats were fed with two different diets: control (C) and lupin flour (L) for a 60-day period. The fermentation of these α-galactosides was monitored by measuring the following indicators: changes in the dynamic lactobacilli and enterobacteriaceae population, cecal content pH, generation of short chain fatty acids (SCFA), Ca and P absorption, and serum lipid (triglycerides and cholesterol) contents. The body weight was lower in the L diet than that in the control with significant differences ($p < 0.05$). The microbial analyses showed an increase of total lactobacilli counts and a decrease of enterobacteriaceae counts in the L diet when compared to the control, with significant differences ($p < 0.05$). The fermentations of α-galactosides were also confirmed by decrease in pH levels and the formation of relevant SCFA in the cecal content. The total content of SCFA (μmol per g) at the end of the assay period reached values of 13.58 and 3.64 for L and C rats, respectively. This significant increase of total SCFA in the L diet is responsible for a low pH of the cecal content that in turn prevents the growth and development of potentially pathogenic bacteria ($p < 0.05$). Regarding lipids, serum triglycerides and cholesterol levels were significantly reduced in the case of animals fed with lupin flour with respect to C animals ($p < 0.05$). Finally, the L rats showed higher values of an apparent Ca absorption percentage and bone volume percentage with respect to the C animals ($p < 0.05$). All these results confirm that lupin flour which contains α-galactosides could possibly be considered as a functional ingredient with health promoting properties.

Introduction

Lupin (*Lupinus albus*) is a legume plant, which belongs to the Fabaceae family. Lupin seeds are a valuable source of nutrients for human and animal nutrition. This is mainly due to its high content of proteins, minerals, dietary fiber and unsaturated fatty acids,[1] as well as being a good source of α-galactosides, to which several beneficial health properties have been attributed.[2–4] These α-galactosides are soluble low-molecular-weight oligosaccharides represented by raffinose, stachyose, verbascose, and ajugose formed by an α-(1→6)-galactoside linked to C-6 of the glucose moiety of sucrose[5] (Fig. 1). α-Galactosides have been the subject of growing interest in the field of nutrition as prebiotics, due to the fact that they modify the composition of the colon microflora and positively affect the gut metabolism and function.[6] Prebiotics are non-digestible but fermentable oligosaccharides that are specifically designed to change the composition and activity of the intestinal microbiota with the prospect to promote the health of the host.[7] In this intention, inulin and fructooligosaccharides are the most studied and most commonly applied prebiotics, and they are added as ingredients in a large number of products.[2,8,9] α-Galactosides have received much attention as functional ingredients for the design of functional foods because of their prebiotic properties. These compounds are widely distributed in legume seeds, with lupin (*Lupinus albus*) being the species with one of the highest α-galactoside

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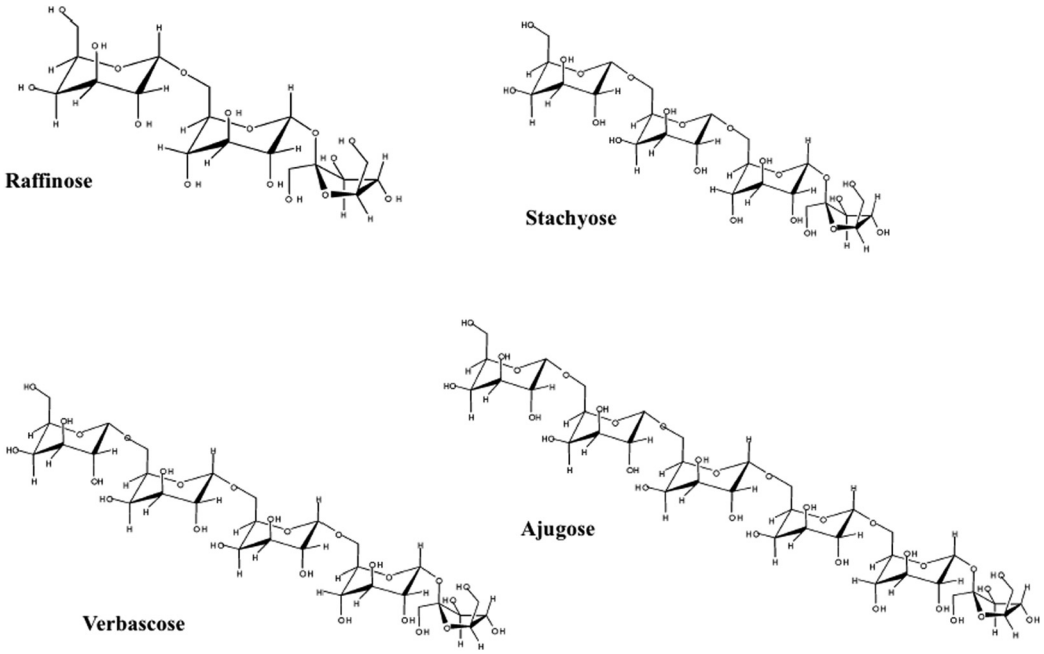

Fig. 1 Chemical structure of α-galactosides.

contents,[10,11] so this legume represents a potential candidate to be used as a functional ingredient. Humans and animals do not possess the enzyme α-galactosidase required to hydrolyze the linkage present in these oligosaccharides, so, once ingested, they arrive intact in the colon where they are preferably fermented by bifidobacteria and lactobacilli, promoting the establishment and development of these bacteria.[12] α-Galactosides are metabolized by these types of beneficial colonic anaerobic bacteria, producing end products such as short chain fatty acids (SCFA), acetate, propionate and butyrate.[13] These fermentation products exhibit cholesterol lowering effects, increase mineral bioavailability and stimulate the growth of bifidobacteria, thus showing a prebiotic action that confers a number of beneficial effects for the host.[14–18] We have previously demonstrated that α-galactosides extracted from lupin seeds were fermented by the probiotic strain *Lactobacillus paracasei* BGP1 and not fermented by an enteric mixture under *in vitro* conditions.[19] By promoting the growth of these health beneficial microorganisms, α-galactosides are considered compounds with prebiotic activity, enabling their use as an ingredient for functional food formulations.

Growing interest in plant species that can be used for obtaining flour mainly arises from the finding and promotion of nutritionally relevant attributes.[20] These products can also gain value as functional ingredients. So, we hypothesize that the inclusion of lupin flour in a diet (with a fermentable fiber such as α-galactosides) may stimulate the growth and proliferation of beneficial species of intestinal microflora and the resultant fermentation end-products may improve colon function. Therefore, the aim of this study is to evaluate if lupin flour containing α-galactosides responds as an ingredient with prebiotic activity in growing Wistar rats.

Materials and methods

Lupin seeds and chemical reagents

Lupin seeds (*Lupinus albus*) were purchased from a local market (Tandil, Argentina) and stored in polyethylene bags until used. Lupin flour was obtained by milling seeds to a fine powder (mesh 32) in a cereal mill (Mod. TDMC, TecnoDalvo, Argentina). HPLC-grade water and acetonitrile (J.T. Baker, USA) were used for HPLC. Sucrose, raffinose, stachyose and verbascose standards were purchased from Sigma-Aldrich (USA). All the other chemicals used were of reagent grade.

Extraction and purification of α-galactosides from lupin seeds

Hydroalcoholic extraction of α-galactosides was performed following the procedure described by Gulewicz *et al.* (2000)[8] with slight modifications. Defatted lupin flour (Soxhlet) was extracted with ethanol 70 : 100 v/v in a screw-capped tube by orbital shaking at room temperature overnight (1 : 10 w/v). After paper filtration, activated carbon was added to the filtrate (1 : 100 w/v) and, after carbon removal, the discolored extract was evaporated on a rotavapor (Vaccum-System B-169, Büchi, Switzerland). About 3 g of the resulting α-galactoside extract was dissolved in tri-distilled water (10 mL) and the slurry was applied to a Dowex 50WX8 column (15 × 1.5 cm i.d.) of 20–50 mesh (Fluka, USA), and eluted with 50 mL of tri-distilled water. The pH of the eluate was adjusted to pH 7.0 using freshly prepared $Ca(OH)_2$ (4 : 100 w/v). The resulting solution was boiled (2 minutes) and centrifuged and the supernatant was rotaevaporated and finally dried in a vacuum oven (FAC, Argentina) at 40 °C to a constant weight. The α-galactosides thus obtained were resuspended in tri-distilled water 1 : 1 w/v and subjected to ultrasound for 5 minutes to improve their

dissolution. The resulting suspension was filtered through a 0.22 μm membrane filter (MSI, USA) and kept in a sterile container at −18 °C until use.

Analysis of carbohydrates by high performance liquid chromatography (HPLC)

The identification and quantification of α-galactosides was performed according to the method of Muzquiz *et al.* (1992)[21] by liquid chromatography (UHPLC Dionex UltiMate 3000 Liquid Chromatography System, Thermo Scientific, USA) equipped with a refractive index detector (Shodex RI-101, Japan). A Luna-5-NH_2 column (250 × 4.6 mm i.d.) (Phenomenex, USA) was used, which was maintained at 40 °C. The mobile phase was used under isocratic conditions with a flow of 1 mL min^{-1}, and consisted of CH_3CN–H_2O 60 : 40 v/v to assess disaccharides and α-galactosides. The injected sample volume was 20 μL. Quantification was performed by comparison of areas through the external standard method, using standard sugars at different concentrations ranging from 5 to 50 mg mL^{-1}. The retention times of the standards for sucrose, raffinose, stachyose and verbascose (Sigma-Aldrich, USA) were 4.3, 4.7, 5.3 and 5.8 min, respectively. Total sugar was calculated by the sum of raffinose, stachyose, verbascose, and sucrose, respectively.

In vivo experimental design: rats and diets

A total of twenty male Wistar rats (44.4 ± 2.9 g) were obtained from the Animal Service Laboratory of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. Throughout the experiment, the animals were allowed free access to deionized water and food and were housed in individual stainless steel cages in a temperature (21 ± 1 °C) and humidity (60 ± 10%) controlled room, with a 12 h light–dark cycle. The two groups of rats (n = 10 per group) were fed *ad libitum* for 60 days with the following diets (Table 1):

- Control group (C): semi-synthetic diet prepared according to the American Institute of Nutrition Diet (AIN 93)[22] containing 5% of cellulose.

Table 1 Composition of control (C) and lupin (L) diets

Ingredient (g per kg diet)	C diet	L diet
Lupin flour	—	425.5
Casein	170.0	42.4
Soybean oil	69.0	21.3
Mineral mix (AIN-93-M-MX)	35.0	19.9
Vitamin mix (AIN-93-VX)	10.0	10.0
L-Cystine	3.0	3.0
Choline	7.1	7.1
Cellulose	50.0	—
Dextrin[a]	655.9	470.8

Considering the contribution of lupin flour to the total content of proteins, lipids, carbohydrates and minerals, both diets provided equivalent amounts of these nutrients, with final values in accordance with the requirements AIN 93 for rats. [a] Dextrin was added as a carbohydrate source to achieve 1 kg of diet.

- Lupin group (L): semi-synthetic diet prepared according to the AIN 93 containing 10% of total fiber from lupin flour.

The analyses of both diets confirmed that they were isocaloric and supplied a similar amount of macronutrients, calcium (Ca) (0.5 g per 100 g diet) and phosphorus (P) (0.3 g per 100 g), respectively.

This study was approved to be carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Committee of Health Guide for the Care and Use of Laboratory Animals of the FFyB, UBA. All experiments comply with the current laws of Argentina.

Sampling methods

Body weight (BW) was recorded every two weeks throughout the study. Food intakes were recorded every three days throughout the experiment and total intake, daily intake and daily Ca intake (Ca I) were calculated. Also, individual fresh feces samples were collected and weighed at 3, 20, 45 and 60 days of the experiment and microbial assays were analysed on the same day.

At the end of the experiment, the rats were anesthetized with intraperitoneal injection of 0.1 mg per 100 g BW of ketamine hydrochloride + 0.1 mg per 100 g BW of acepromazine maleate. An abdominal incision was made and blood samples were withdrawn from the abdominal aorta and centrifuged at 3500 rpm for 20 minutes at 4 °C. Serum was separated and stored at −80 °C until use.

The cecum from each animal was excised, weighed and split open, and the pH of the cecal content was measured using a portable digital pH meter (HANNA HI-98103, USA) previously calibrated. The cecal content samples obtained from each animal were weighed and collected in tubes and phosphoric acid was added (1 : 4 w/v) for SCFA analysis.

Microbial methods

To analyze the relationship between the benefic and pathogenic microflora of both groups of rats, total lactobacilli and enterobacteriaceae were evaluated. Individual fresh feces samples collected at different times of the assay, were diluted (1 : 10 w/v) with physiological solution (9 : 1000 w/v NaCl) and homogenized under sterile conditions. Serial dilutions of the homogenized samples were obtained and aliquots (0.1 mL) of the appropriate dilution were spread onto the surface of two agarized media (Britania, Argentina): Mann–Rogosa–Sharpe (MRS) for total lactobacilli, and MacConkey for enterobacteriaceae counts. These analyses were carried out twice for each sample. The MRS media culture plates were anaerobically incubated at 37 °C for 48 h, while MacConkey media culture plates were aerobically incubated at 37 °C for 24 h. All media were prepared using sterile distilled water. The results are expressed as log CFU g^{-1} feces.

Analytical methods

Bone measurements. At the end of the experiment (t_{60}) right femurs were excised and cleaned of any adhering soft tissues

and dried at 100 °C for 72 h, and fat was extracted by immersion of bones for 15 days in chloroform–methanol (3 : 1 v/v), which was removed and replaced with fresh solvent every 3 days. Finally, the femurs were dried for 48 h at 100 °C. The defatted and dried femurs were weighed and subjected to 700 °C until white and crystalline ashes were obtained. Subsequently, the ashes were dissolved in concentrated HCl and they were properly diluted for Ca and P analysis. The amounts of Ca and P were calculated as the total content and percentage content of dried defatted bone and the femur Ca/P ratio was also calculated.

Also, at t_{60}, the right tibia was resected, fixed by immersion in buffered formalin for 48 h, and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) (pH 7) for 25 days and embedded in paraffin. An 8- to 10-μm-thick longitudinally oriented section of the subcondral bone was obtained at the level of the middle third, including primary and secondary spongiosa. It was stained with haematoxylin-eosin and microphotographed (AXIOSKOP, Carl Zeiss) to perform bone volume percentage (%BV) on the central area of the metaphyseal bone displayed on the digitalized image.[23]

Mineral analysis. The Ca concentration in diets, feces and femurs was determined using an atomic absorption spectrophotometer (Perkin Elmer, AAnalyst 400, Argentina).[24] Lanthanum chloride (6500 mg per L in the final solution) was added to avoid interferences. The P concentration was measured by the Gomori method.[25]

Feces. Feces samples were dried under infrared light and weighed. Diets and feces were wet-ashed with nitric acid using Parr bombs.[26]

Apparent calcium absorption. Food intake and feces collected during the last 3 days of the experiment were used to calculate the apparent Ca absorption percentage (%Ca-Abs) with the following equation (eqn (1)):

$$\%\text{Ca-Abs} = [(\text{Ca I} - \text{fecal Ca})/\text{Ca I}] \times 100. \quad (1)$$

SCFA analysis by gas chromatography (GC). To analyze the effect of prebiotic compounds on the gut microbiota metabolic activity, the SCFA profile in the cecal contents of both experimental groups was measured. The cecal content samples kept with phosphoric acid (1 : 4 w/v) were deproteinized with methanol (0.8 : 1 v/w), centrifuged at 9000 rpm for 10 minutes at 5 °C, and filtered through nylon membrane 0.45 μm pore (MSI, USA) and finally internal standard and methanol were added (5 : 1 : 4 v/v/v) to analyze by GC (Shimadzu GC-17A, Kyoto, Japan) using a FID detector according to Jouany.[27] A Stabilwax-DA column, 30 m × 0.32 mm i.d. (Restek, USA) was used. A standard mixture of 10 mM-SCFA C_1–C_7 from Supelco (Fluka, USA) and a solution of 2-ethyl butyric acid 0.001% (Fluka, USA) as an internal standard were used.

Triglycerides (TG) and cholesterol determination. Serum TG and cholesterol were determined by enzymatic methods using the commercial kits purchased from Wiener Lab, Argentina: TG Color GPO/PAP AA and Colestat Enzimatico, respectively.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD) for each treatment group (n = 10). Differences were tested by one-way analysis of variance (ANOVA) followed by the LSD (least significant difference) test to determine significant differences ($p < 0.05$) using Infostat version 2011. Different letters were used to label values with statistically significant differences among them.

Results and discussion

HPLC of α-galactosides extracted from lupin seeds

Fig. 2 shows a chromatogram of water-soluble sugars (sucrose and α-galactosides) extracted from lupin seeds which was obtained as previously described. The presence of sucrose, raffinose, stachyose and verbascose was identified. The corresponding peaks were quantified, and the results are shown in Table 2. Considering the total sugar content, sucrose and stachyose were found to be the main carbohydrates with similar amounts (38.5% and 37.7%, respectively) followed by raffinose (14%), while the lowest content corresponded to verbascose (5.1%). These values are within the average values found for different varieties of lupin species.[11,28]

Effects of diets on intakes and body weight gain (BWG)

Table 3 shows the daily intake (g per day), total intake (g per 60 days), initial and final rat BW (g), and BWG (g per 60 days) for both groups. Significant differences between C and L rats were observed for daily intake and total intake, being lower for L, which was reflected in a lower BWG for this group of rats compared to C ($p < 0.05$). Animals fed with L diet showed a decrease in the total food intake by 27% compared to those fed with a diet having cellulose as the source of fiber. The significant differences in these values obtained for both animal groups were probably due to the bitter taste of lupin flour,[11] as well as the higher content of the dietary fiber (specifically

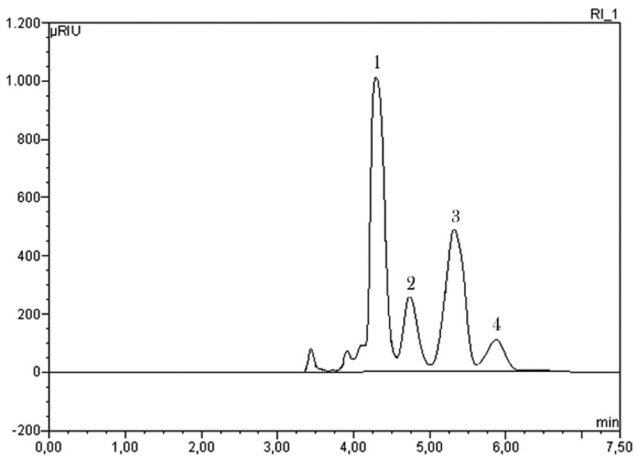

Fig. 2 HPLC chromatogram of oligosaccharides from lupin seeds: (1) sucrose, (2) raffinose, (3) stachyose, and (4) verbascose.

Table 2 Content (mg per g flour) of soluble oligosaccharides in dry seeds of lupin[a]

Seed	α-Galactosides					
	Raffinose	Stachyose	Verbascose	Total	Sucrose	Total sugar[b]
Lupin (*Lupinus albus*)	0.9 ± 0.01	2.4 ± 0.02	0.33 ± 0.01	3.63 ± 0.05	2.48 ± 0.03	6.11 ± 0.08

[a] Data are expressed as mean ± SD of three replicates. [b] Total sugar (raffinose + stachyose + verbascose + sucrose).

Table 3 Daily intake, total intake, initial and final body weight (BW), and body weight gain (BWG)[a]

Diets	Daily intake (g per day)	Total intake (g per 60 days)	Initial BW (g)	Final BW (g)	BWG (g per 60 days)
C	16.15 ± 0.40[b]	933.62 ± 16.87[b]	44.08 ± 0.87[a]	261.63 ± 13.16[b]	215.78 ± 11.32[b]
L	11.34 ± 0.44[a]	677.47 ± 18.65[a]	44.98 ± 1.07[a]	188.79 ± 10.74[a]	142.54 ± 9.19[a]

[a] Data are expressed as mean ± SD (n = 10 per group). Results with the same superscript within a column are not significantly different when analysed by the LSD test ($p < 0.05$).

soluble fiber) present in the L diet with respect to C. Porres *et al.* (2006)[1] and Paturi *et al.* (2012)[29] had established that high-fiber foods produce a higher grade of satiety, compared to those poor on this component, thus lowering the total food intake. In spite of the lower food intake and BWG, no signs of malnutrition or negative effects on animal health were observed. Satiation produced by dietary fiber accompanied by a subsequent reduction in food intake, is the basis of many dietary treatments aimed at weight control.[30,31]

Effects of diets on fecal counts of total lactobacilli and enterobacteriaceae

One criterion for classifying a food ingredient as a prebiotic is its ability to be fermented by the intestinal microbiota and its capacity to selectively stimulate the growth and/or the activity of intestinal bacteria associated with health and wellbeing.[32] Regarding the intestinal microbiota, the counts obtained in this study for total lactobacilli and enterobacteriaceae are presented in Table 4. Enterobacteriaceae population was selected as an indicator of pathogenic flora, while lactobacilli population was considered as an indicator of the beneficial flora. The ratio between the two populations was used to indicate the intestinal balance of rats.[33]

The results showed a significant increment in the lactobacilli number (log CFU g^{-1}) and a significant decrease in the counts of enterobacteriaceae, within the 60 days of the feeding period, in feces from rats fed with L diet with respect to C rats ($p < 0.05$). According to these results, it can be considered that α-galactosides from lupin flour present in the L diet stimulated a selective growth of beneficial flora and, at the same time, inhibited or delayed the growth of pathogenic flora in the gut of these animals. These results are consistent with those obtained in a previous study carried out under *in vitro* conditions.[19] In that work, we found a positive prebiotic activity score for α-galactosides extracted from lupin seeds, which was assessed as the ability to stimulate the growth of the probiotic strain *L. paracasei* compared to that obtained for a pathogen mixture of *E. coli* strains.

Table 4 Total lactobacilli and enterobacteriaceae fecal counts (log CFU g^{-1}) of Wistar rats fed for 60 days with control (C) and lupin (L) diets[a]

Time (days)	Diet	
	C	L
	Lactobacilli	
t_3	8.98 ± 0.37[a]	9.14 ± 0.12[a]
t_{20}	8.52 ± 0.10[a]	9.02 ± 0.10[b]
t_{45}	8.51 ± 0.15[a]	9.31 ± 0.11[b]
t_{60}	8.55 ± 0.13[a]	9.54 ± 0.10[b]
	Enterobacteriaceae	
t_3	8.03 ± 0.26[b]	3.42 ± 0.29[a]
t_{20}	8.74 ± 0.17[b]	3.65 ± 0.15[a]
t_{45}	9.06 ± 0.23[b]	3.96 ± 0.17[a]
t_{60}	9.44 ± 0.26[b]	3.98 ± 0.25[a]
	Intestinal balance[b]	
t_{60}	−0.89 ± 0.44[a]	5.56 ± 0.41[b]

[a] Data are expressed as mean ± SD (n = 10 per group). Results with the same superscript within a file are not significantly different when analysed by the LSD test ($p < 0.05$). [b] Intestinal balance = ratio between lactobacilli and enterobacteriaceae populations.

Effects of diets on cecum, fecal weight and pH of cecal content

Table 5 shows the cecum weight (g), cecum weight relative to BW (g per 100 g BW), fecal weight (g) and the pH of cecal content. The rats fed with L diet showed a significant increase of the cecum weight relative to BW compared to C ($p < 0.05$).

Table 5 Cecum weight, fecal weight and pH of cecal content[a]

Diets	Cecum weight (g)	Cecum weight (g per 100 g BW)	Fecal weight (g)	Cecal content pH
C	1.19 ± 0.09[a]	0.41 ± 0.07[a]	4.81 ± 0.22[b]	7.42 ± 0.18[a]
L	1.11 ± 0.10[a]	0.88 ± 0.07[b]	1.83 ± 0.17[a]	5.27 ± 0.20[b]

[a] Data are expressed as the mean ± SD (n = 10 per group). Results with the same superscript within a column are not significantly different when analysed by the LSD test ($p < 0.05$).

Considering fecal weight, significant differences among the diets were observed ($p < 0.05$). The lupin-fed rats had a lower fecal weight and those fed with the C diet, a higher one. Dietary fiber affects bowel function by the bulking effect (increase of the fecal volume and weight), improving stool consistency, decreasing transit time, and increasing stool frequency, all of which ease defecation and prevent upset in the stomach and gut.[31] Fermentable water-soluble fiber has been associated with an increase of bacterial mass and stool frequency, while insoluble non-fermentable fiber has been mainly related to the bulking effect. In our work we observed that the rats fed with diets containing α-galactosides (considered fermentable fiber) from lupin flour presented a significantly higher weight of the cecum and lower fecal weight compared to the rats fed with diets supplemented with cellulose (insoluble fiber). Furthermore, the cecal content was significantly acidified in lupin-fed rats compared to cellulose-fed rats (pH 5.27 *vs.* 7.42) ($p < 0.05$). This result can be attributed directly to the fermentation of α-galactosides by beneficial intestinal microflora discussed previously as a result of their use as an energy substrate.

Effects of diets on SCFA production of cecal content

Table 6 shows the concentrations of relevant SCFA on the cecal content (μmol per g) of rats fed with both diets for 60 days. The differences observed in the SCFA profile for L rats were consistent with the variation on the counts of lactobacilli *vs.* enterobacteriaceae obtained. These high levels of SCFA also correlated with the final-pH obtained, as shown in Table 5.

In the cecal content of both groups of animals, the presence of decreasing amounts of acetic, propionic and butyric acids was recorded. Except for i-butyric acid, all the levels of SCFA resulted higher in the case of lupin-fed rats than those in control animals ($p < 0.05$). Thus, in the cecal content of the L rats respective increases of 5.88-fold, 4.49-fold and 3.26-fold for propionic, butyric and acetic acid relative to the control animals were registered. These acids are responsible for a low pH of cecal content that prevents the growth and development of potentially pathogenic bacteria.

In summary, the reduced pH in the cecal content combined with significant increases in SCFA production strongly suggest that the α-galactosides present in the L diet were fermented by the beneficial microbiota. The increase in the SCFA content was due mainly to acetic acid production, the main fermentation end product of lactobacilli.[34–36] This was in turn confirmed by an increase in total lactobacilli numbers.

Effects of diets on TG and cholesterol content

Table 7 shows total cholesterol content, high density lipoprotein-cholesterol (HDL-Col), non-high density lipoprotein-cholesterol (non-HDL) and TG in serum, as mg per dL relative to the final BW. Because rats have no cholesterol ester transfer proteins (CETP), it is useful to evaluate both Col-HDL and non-HDL to include lipoprotein remnants. Significant differences were observed in serum total cholesterol, Col-HDL and non-HDL, between the two groups of animals ($p < 0.05$). The inclusion of lupin flour in the diet of L rats caused a significant decrease in TG and non-HDL serum contents with respect to the values obtained for the rats fed with the C diet (18% and 38.7%, respectively), while a significant increase in the HDL-Col content compared to the C rats (43%) was found. In addition, the mean values of serum TG were significantly higher in the C rats compared to the animals fed with the L diet (76%) ($p < 0.05$). Considering that the lupin-fed rats had a lower food intake compared to the C rats, the effects of diets on TG and cholesterol were calculated relative to the final BW, so the results obtained could be related to the intestinal fermentation of α-galactosides present in the L diet that generates SCFA, specially butyric acid, that may act in the reduction of

Table 7 Cholesterol contents, HDL-cholesterol (HDL-Col), non HDL-cholesterol (non-HDL) and triglycerides (TG) from serum (mg per dL relative to BW)[a]

Diets	Total cholesterol	HDL-Col	Non-HDL	TG
C	93.22 ± 4.08[b]	16.13 ± 4.54[a]	77.72 ± 2.78[b]	97.87 ± 16.64[b]
L	75.76 ± 4.47[a]	28.46 ± 6.42[b]	47.63 ± 14.53[a]	22.84 ± 3.20[a]

[a] Data are expressed as the mean ± SD ($n = 10$ per group). Results with the same superscript in a column are not significantly different when analysed by the LSD test ($p < 0.05$). BW = body weight.

Table 8 Daily Ca intake (Ca I), daily fecal Ca excretion (Ca E) percentage of apparent Ca absorption (%Ca-Abs), and bone volume percentage (%BV)[a]

Diets	Ca I (mg per day)	Ca E (mg per day)	%Ca-Abs	%BV
C	309.99 ± 100.60[b]	101.88 ± 11.53[b]	63.66 ± 11.61[a]	20.46 ± 2.56[a]
L	121.23 ± 16.13[a]	11.41 ± 4.54[a]	90.09 ± 4.43[b]	37.16 ± 2.56[b]

[a] Data are expressed as the mean ± SD ($n = 10$ per group). Results with the same superscript in a column are not significantly different when analysed by the LSD test ($p < 0.05$).

Table 6 SCFA concentrations (μmol g^{-1}) on cecal content[a]

Diets	Acetic	Propionic	i-Butyric	Butyric	Ratio A/P	Total
C	2.31 ± 1.26[a]	0.69 ± 0.19[a]	0.25 ± 0.04[a]	0.39 ± 0.02[a]	3.35	3.64
L	7.55 ± 1.60[b]	4.06 ± 1.42[b]	0.22 ± 0.05[a]	1.75 ± 0.95[b]	1.85	13.58

[a] Data are expressed as the mean ± SD ($n = 10$ per group). Results with the same superscript within a column are not significantly different for the same acid when analysed by the LSD test ($p < 0.05$). A/P = ratio between acetic and propionic.

Table 9 Effects of diets on mineral content of right femur at the end of the experiment[a]

Diets	Ash (mg per 100 g BW)	OC[b] (mg per 100 g BW)	Ash/OC	Ca (mg per 100 g BW)	P (mg per 100 g BW)	Ca/P
C	52.57 ± 0.97[a]	47.44 ± 0.98[a]	1.2 ± 0.04[a]	14.88 ± 0.46[a]	9.11 ± 0.13[a]	1.63 ± 0.08[a]
L	50.56 ± 1.37[a]	49.43 ± 1.38[a]	1.03 ± 0.06[a]	15.58 ± 0.65[a]	9.38 ± 0.19[a]	1.73 ± 0.06[a]

[a] Data are expressed as the mean ± SD (n = 10 per group). Results with the same superscript in a column are not significantly different when analysed by the LSD test ($p < 0.05$). [b] Organic content (OC).

cholesterol in serum. These results are in agreement with various studies. Daubioul *et al.* (2002)[37] observed a decrease in serum lipids of rats fed with a diet supplemented with non-digestible carbohydrates (fructan type) compared to cellulose-fed rats (control) and Adam *et al.* (2001)[38] showed that the consumption of whole cereals had an influence on the lipid metabolism of rats with respect to animals fed with a control diet, proposing that the generation of SCFA (specially butyrate) influenced the lowering of serum cholesterol.

Furthermore, it is well known that substrates that can decrease the acetic/propionic (A/P) ratio may be involved in the reduction of serum lipid levels.[39,40] We can observe in Table 6 that the ratio of A/P shows a lower value for the lupin-fed rats compared to C animals (1.85 *vs.* 3.35 μmol g^{-1}) ($p < 0.05$), confirming the above.

Taking into consideration the numerous factors which may affect the serum lipid content discussed above, additional trials related to bioactive peptides present in a lupin diet are needed to obtain further knowledge on the subject. *In vivo* studies on lupin peptides to evaluate biological effect would add further support to the application of lupin flour as a functional ingredient.

Effects of diets on daily Ca intake, fecal Ca, apparent Ca absorption, mineral content and bone volume

Table 8 shows the values corresponding to the daily Ca intake (Ca I), daily fecal Ca excretion (Ca E) and %Ca-Abs. In rats fed with the L diet, both the Ca I and the fecal losses resulted significantly lower compared with the values corresponding to C rats ($p < 0.05$). These results led to higher %Ca-Abs in the case of L animals with respect to C ($p < 0.05$). As it is already shown in Table 5, a significant decrease of the cecal content pH was also registered for these animals. Some authors confirmed that a low pH maintains Ca and other minerals in solution which in turn improves their absorption.[41] According to Cashman (2003),[42] the decrease of pH promotes the growth of both *Bifidobacterium* spp. and *Lactobacillus* spp. and also improves absorption of some minerals. In addition, numerous studies have repeatedly shown that prebiotics, such as inulin, oligofructose and galactooligosaccharides, stimulate Ca absorption in rats.[43–45] In agreement with the values of %Ca-Abs levels, the higher %BV in L rats was observed ($p < 0.05$) (Table 8). Although the lupin-fed rats showed a lower food intake and BW gain compared to the C rats (Table 3), the fermentation of α-galactosides from lupin flour by benefic flora produced an increase in Ca absorption and retention (L rats).

Table 9 shows the mineral content of the right femurs of L and C rats at the end of the experiment (60 days): the ash content, organic content (OC), ash/OC ratio, Ca and P content and, Ca/P ratio. This bone was selected because it is considered a representative bone tissue of the rest[46] and is even considered a representative parameter for bone strength against fracture.

Ca and P represent two of the main constituents of bone minerals, giving rise to hydroxyapatite crystals with different degrees of maturity and/or amorphous calcium phosphate. Both minerals should be available in sufficient quantities for an adequate bone mineralization. Zaichicka and Tzaphlidou (2002)[47] confirmed that the ratio of the Ca/P bone in healthy human adults reaches an approximate value to 2. In the present study, the normal values of this ratio were found without significant differences for both groups of animals ($p > 0.05$).

Conclusions

In this work, we first described a method for the extraction of α-galactosides from lupin seeds suitable for food applications using green solvents. Also, we evaluated some potential health benefits associated with the prebiotic effects of these oligosaccharides when experimental animals were fed with lupin flour. Several beneficial health effects were observed in Wistar rats (21 days age) fed for 60 days with a diet containing 10% of dietary fiber of lupin flour compared to a control group. These benefits included: a positively improved intestinal microbiota by the development of benefic flora with a decrease in cecal content pH and an increase of SCFA content (being acetic the major SCFA followed by propionic and butyric acids); a decrease in serum lipids; and an increase in Ca absorption, Ca retention and bone volume percentage. In conclusion, the results allow us to consider lupin flour, which contains α-galactosides, as a potential functional ingredient with prebiotic activity and with interesting health properties. This study represents a contribution for selecting plant species as feasible sources of prebiotics. However, further trials on humans are needed to establish the clinical relevance of this potential prebiotic with an aim to its inclusion in functional food development.

Abbreviations

%BV	Bone volume percentage
%Ca-Abs	Apparent Ca absorption percentage

A/P	Ratio acetic/propionic
ANOVA	One-way analysis of variance
BW	Body weight
BWG	Body weight gain
Ca	Calcium
Ca I	Ca intake
Ca E	Ca excretion
CETP	Cholesterol ester transfer proteins
C	Control
EDTA	Ethylene diamine tetraacetic acid
GC	Gas chromatography
HDL-Col	High density lipoprotein-cholesterol
HPLC	High performance liquid chromatography
L	Lupin
LSD	Least significant difference
MRS	Mann–Rogosa–Sharpe
Non-HDL	Non high density lipoprotein-cholesterol
OC	Organic content
P	Phosphorus
SD	Standard deviation
SCFA	Short chain fatty acids
TG	Triglycerides
t_{60}	Day 60

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