Evaluation of the hypocholesterolemic effect and prebiotic activity of a lentil (*Lens culinaris* Medik) extract

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Abstract

Scope: the aim of our work was to produce a hydroalcoholic extract of lentils and to examine: a) the hypocholesterolemic action in an animal model, by studying the plasma cholesterol level and the concentration of bile acids in the faeces; b) the potential prebiotic effect, by conducting an *in vitro* culture fermentation experiment and assessing the level of short chain fatty acids (SCFAs) in the faeces of rats.

Methods and Results: Lentil extract (LE) was obtained by extracting lentils with a solution of H_20/EtOH (70/30 *v/v*) for 3 h, and the content of main nutrients was determined. After 71 days of diet-induced hypercholesterolemia in rats, LE reduced the cholesterol level of rats of 16.8% ($p<0.05$) and increased the level of bile acids (BAs) in the faeces of rats ($p <0.01$). LE revealed the same prebiotic activity of inulin and good bifidogenic activity, inasmuch as it enhanced the growth of *Bifidobacterium* spp. by 3 log ($p<0.05$). The concentration of SCFAs in the faeces of rats fed with LE increased during the time of the study.

Conclusion: This new hydroalcoholic extract obtained from lentils was shown to possess hypocholesterolemic and prebiotic properties, and could have interesting applications in the field of nutraceuticals.

Standard Abbreviation

SCFAs, Short Chain Fatty Acids; **LE**, Lentil extract; **BAs**, Bile Acid; **FAO**, Food and Agriculture Organization of the United Nations; **CHD**, coronary heart disease; **CVD**, cardiovascular disease; **WHO**, World Health Organization; **RFO**, raffinose-family oligosaccharides; **FOS**, fructooligosaccharides; **RS**, resistant starch; **HPLC-MS/MS**, high performance liquid chromatography-tandem mass spectrometry; **GC-FID**, gas chromatography-flame ionization detector; **FDP**, Freeze-dried powder.

Keywords

Cholesterol/ lentils/ nutraceutical/ prebiotic/ soyasaponins

1 Introduction

Pulses, which are a major component of the Mediterranean diet, are an important source of macronutrients such as proteins, carbohydrates, and dietary fibre [1]. Moreover, they provide micronutrients, vitamins, carotenoids, saponins and phenolic compounds, all of which are considered to be bioactive compounds [2]. Epidemiological and intervention studies indicate that legume consumption is associated with 22% and 11% lower risk of coronary heart disease (CHD) and cardiovascular disease (CVD), respectively [3]. Hypercholesterolemia is a problem faced by many societies and a cause of concern for health professionals, since it constitutes one of the major risk factors for the development of CVDs such as atherosclerosis and its complications, acute infarction of the myocardium or hypertension [4,5].

Among the various bioactive compounds of legumes, saponins appear to be able to reduce plasma cholesterol levels. For example, the review by Oakenfull et al. [6] reported that a study on 174 humans subjected to a diet rich in saponins [7] found ~~in some cases~~ around 20% ~~a convincing~~ reduction in plasma cholesterol levels. ~~(around 20%).~~ Soyasaponins, belonging to the family of saponins, are triterpenoidal glycosides, structurally divided into two groups, one called “A” (bidesmosidic) and the other “B” (monodesmosidic) [8]. Soybeans contain the highest level of soyasaponins (6000 mg kg^{-1}), but lentils (*Lens culinaris* Medik) are also a very good source (1600 mg kg^{-1}); in particular, they contain soyasaponins I and βg (group B), which are considered responsible for the hypocholesterolemic effect of this food [9]. In our laboratories, sensitive and specific analytical methodologies have been developed for evaluating the level of soyasaponins I and βg in lentils by using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) equipment [10, 11]. Literature reports that group B soyasaponins lowered plasma-cholesterol levels by a mechanism

involving the greater excretion of fecal bile acids [12]. According to the European Directive 90/496, saponins can be considered components of dietary fibre which are neither digested nor absorbed in the human small intestine [13]; these components together with other carbohydrates present in lentils may exert an important prebiotic action. In this regard, Johnson and collaborators studied the prebiotic potential of lentils by analyzing raffinose-family oligosaccharides (RFO), sugar alcohols, fructooligosaccharides (FOS), and resistant starch (RS) carbohydrates, and concluded that 100 g of lentils may provide over 13 g of prebiotics [14].

Prebiotics are not digested in the upper gastrointestinal tract and reach the colon intact, where they are selectively fermented by residential microbiota into short chain fatty acids (SCFAs) and lactate [15]. Based on all these findings, the aim of this work was to produce a hydroalcoholic extract of lentils and to test it for: a) the hypocholesterolemic action in an animal model, by studying the plasma cholesterol level and the concentration of bile acids (BAs) in faeces; b) the potential prebiotic effect in comparison with a fructooligosaccharide (inulin) using *in vitro* semi-continuous culture fermentation experiments, and the level of short chain fatty acids (SCFA) in the faeces of rats. To our knowledge, no published articles have described the formulation of similar food supplement and it may be hoped that this lentil extract will provide a base formulation for a new lentil-based nutraceutical.

2 Materials and Methods

2.1 Chemicals and Reagents

The analytical standards of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA) ursodeoxycholic acid (UDCA), and the short chain fatty acids (SCFAs) standards C2 (acetic acid), C3 (propionic acid), C4 (butyric acid), sulfuric acid, ethyl ether were purchased from Sigma-Aldrich (Milano, Italy). Pure standards of

soyasaponins I and βg were both purchased from Tauto Biotech Co. (Zhangjiang High-Tech Park, Shanghai, P.R. China).

2.2 Lentil extract (LE) preparation

Lentils (*Lens culinaris* Medik) (Colfiorito Selezione Oro) were kindly provided by Fertitecnica Colfiorito s.p.a (Colfiorito, Italy). 50 g of lentils were ground in a Jolly blender (Johnson, Elettrodomestici s.p.a., Italy) and the lentil flour thus obtained was extracted for 3 h under magnetic stirring with 500 ml of an H_20/EtOH solution (70/30 *v/v*) at room temperature. The mixture was then passed through a paper filter, and the solution was evaporated at < 30 °C. The ethanolic portion was evaporated by a Buchi R 200 rotary evaporator and water bath from Labortechnik (Flawil, Switzerland). At the end of the process, around 320 ml of ethanol free extract were obtained and stored in a glass bottle at -20 °C until the administration to animals. In order to quantify cholesterol-lowering soyasaponins in the stored liquid lentil extract, a previously developed HPLC-MS/MS method was used [11]. The separation of soyasaponins was achieved on a Gemini C18 (150 × 4.6 mm i.d., 5 µm) analytical column from Phenomenex (Chesire, UK) by using a mobile phase formed by a mixture of (A) water with 0.25% acetic acid (v/v) and (B) methanol with 0.25% acetic acid (v/v), flowing at 1 ml min^{-1} in isocratic conditions: 20% A, 80% B.

In order to obtain Freeze-dried powder (FDP) from lentil extracts and evaluate the nutritional properties of the extract (Table S1 supplementary information), after ethanol evaporation, the solution was divided into small flasks and then placed in a static state at -80 °C for 24 h. Next, samples were dried in a Zirbus freeze dryer (Zirbus Vaco 2; Bad Grund, Germany) with a condenser temperature of -50 °C and a chamber pressure P < 0.08 mbar for 48 h.

2.3 Experimental animals and diet

Nineteen male Sprague-Dawley rats (Charles River, Calco, Italy), weighing 225–250 g at the beginning of the experiments were used. Rats were housed under a 12-h light/dark cycle (lights off at 09:00 am) with free access to standard food pellets (4RF18 Mucedola, Italy) and water for a 2 week habituation period prior to the experiments. Rats were housed individually in a room at constant temperature (20–22°C) and humidity (45–55%). All experiments were carried out in accordance with the EC guidelines governing animal welfare and protection (EEC Council Directive 2010/63/UE), Italian legislation on animal experimentation (Decreto Legislativo n. 116, 27 January 1992) (n.1610/2013), and had the approval of the local Ethical Committee. With the beginning of the test, rats were fed with a commercially available high cholesterol diet (AIN-76A rodent diet with 1% cholesterol and 0.5 cholic acid, D04082702 Research Diet, New Brunswick, NJ 08901 USA). Body weight and food intake were recorded daily. To assess locomotor activity the same rats were used for open field tests (Figure S1 supplementary information).

2.4 Effect of lentil extract on diet-induced hypercholesterolemia in rats

The rats were given free access to this diet for 6 weeks, after which cholesterol levels were observed to have risen significantly (247 ± 24 mg/100 ml) over their initial values (68 ± 2 mg/100 ml).

The rats were divided in two experimental groups without significant differences in body weight ($p>0.05$), food intake ($p>0.05$), plasma cholesterol values ($p>0.05$), triglycerides ($p>0.05$), HDL ($p>0.05$) or LDL ($p>0.05$). The two experimental groups continued to receive the high cholesterol diet for the experimental period of 71 days, during which the 9 rats of group 1 received vehicle (water), while the 10 rats of group 2 received Lentil Extract (LE) containing soyasaponin (4 ml of Extract +16 ml of water/24 h) (n = 10). Blood samples were collected in a heparin tube (1 ml) and centrifuged at 3000 rpm for 10 minutes. They were

stored at 4 °C and delivered to the Fioroni laboratory (San Benedetto del Tronto, AP, Italy) within 24 hours for analysis.

2.5 Analysis of faecal BAs by HPLC-MS

Freeze dried faeces samples were weighed (100 mg) and placed in a vial to which 5 ml of methanol was then added. Samples were vortexed for 2 min, sonicated for 30 min and then filtered through a 0.22-µm PTFE filter before HPLC analysis. HPLC–ESI-MS studies were performed using an Agilent 1290 Infinity Series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA) equipped with an ESI source operating in both negative and positive ionization modes. Chromatographic separation was accomplished on a Symmetry C18 (4.6 mm ×250 mm, 5 µm) analytical column. The mobile phase for HPLC–MS/MS analyses was aqueous–formic acid (99.95–0.05%) (A) and methanol–formic acid (99.95–0.05%) (B) working in the gradient mode. We used the following elution program: 0–9 min, 80% B; 9–10 min, 97% B; 10–15 min, 97% B; 15–25 min, 80% B. The flow rate was set at 1 ml/min, and the column temperature was controlled at 30 °C. The injection volume was 5 µl. Operating ESI conditions were adjusted as follows: gas temperature 300 °C, nebulizer gas 60 psi, drying gas (nitrogen) flow rate 13 ml min^{-1}, and capillary voltage 4,000 V. The ESI interface and mass spectrometer parameters were optimised to obtain maximum sensitivity. Mass spectra were acquired in negative polarity and acquisition was performed by using single ion monitoring (SIM) mode, by selecting the ion *m/z* 407 $[M-H]^-$ for CA, the ion *m/z* 391.2 $[M-H]^-$ for CDCA, DCA and UDCA, the ion *m/z* 375.3 $[M-H]^-$ for LCA. A HPLC-MS chromatogram of the analyzed bile acids is reported in Figure S2 (supplementary information).

2.6 Faecal semi-continuous fermentations

An *in vitro* fermentation model, using a sterile glass autoclavable bioreactor (ADI Autoclavable Bio Reactor System 3 L, Applikon Biotechnology B.V., Schiedam, Netherlands) was developed to evaluate the prebiotic action of lentil extract on human intestinal microbiota. The fermentation experiments were performed using a semi-continuous culture system designed to closely mimic the *in vivo* human intestinal ecosystem. The complex culture medium used was formulated to mimic either ileostomy fluid and ileal chyme, and was based on that used by Zampa et al. [16]. The concentration of primary BA in the medium was 0.6 g/l for both CA and CDCA. Three groups of experiments were performed: (A) semi-continuous fermentations with the addition of glucose (1%) in the medium as the only source of carbohydrates (control test); (B) semi-continuous fermentations with addition of inulin (1%) in the medium as the only source of carbohydrates (positive control); (C) semi-continuous fermentations with LE (1%) in the medium as the only source of carbohydrates (test). Each fermentation was run for five days.

2.7 Bacterial counts

To estimate the bifidogenic activity of LE and to assess differences in bacterial composition, samples were collected during fermentation times. Total viable bacterial counts (aerobes and anaerobes) were estimated by spreading 10 fold dilutions of the original sample (10^{-1} – 10^{-9}) onto non selective Columbia Agar medium with +5% sheep blood (bioMerieux, Marcy l'Etoile, France). Rogosa Agar was used as a selective medium for the *Lactobacillus* count (OXOID Unipath Ltd. Basingstoke, Hampshire, UK); Beerens' agar [17, 18] for the *Bifidobacterium* spp. count; Mannitol Salt agar (MSA) (OXOID) for the *Staphylococcus* spp. count, MacConkey agar (OXOID) for the *Enterobacteriaceae* count, and Reinforced Clostridia Agar (OXOID) for the *Clostridium* spp. count. For the anaerobic counts the samples were immediately placed in an anaerobic cabinet (Concept 400, Ruskinn Technology

Limited, Leeds, West Yorkshire, UK), processed and then the plates incubated at 37°C aerobically and anaerobically for 24-72 hours.

2.8 SCFA extraction

The extraction of SCFAs was performed following the procedure reported in the article of Cresci et al. [19] with some modifications. Briefly, 250 mg of faecal sample was weighed in a 2 ml vial, 250 µl of aqueous sulfuric acid (50 % w/w) were added and the suspension was shaken by vortex for 2 minutes in order to homogenize it. Then a solution of isohexanoic acid in ethyl ether (50 mg l^{-1}, 500 µl) was added to extract SCFAs by means of a vortex (2 min). After centrifugation (5 min, 5000 rpm), the upper ethereal solution was injected directly into a gas chromatograph equipped with a flame ionization detector (GC-FID). The GC conditions used are reported in the article by Fiorini et al. [20].

~~Results are expressed as means ± S.E.M.~~ All data were submitted to one-way analysis of variance (ANOVA) in order to assess significant differences ($p < 0.05$) between the contents of individual results in the different rats groups, using the Paleontological Statistics Software Package (PAST) [21]. For behavioural experiments, data were analyzed by one-way ANOVA, using SYSTAT version 10.0 (Systat Software, San Jose, CA, USA).

3 Results and discussion

3.1 Soyasaponin content in LE and in FDP

LE tested on rats and FDP tested for prebiotic activity were previously analysed for soyasaponin content by using an HPLC-MS/MS analytical method [11]. The total concentration of soyasaponins I and βg in LE was 103.66 mg l^{-1}(% RSD= 7.1, n=3), while in FDP it was 1935 mg/kg (% RSD= 5.5, n=3). Twenty days later, the analysis was repeated to evaluate the FDP stability of soyasaponins, and it was found that the content remained almost

similar. The choice of dose of LE used for the study on rats is based on our previous work [9] in which we have determined the concentration of soyasaponins in lentils, and in general in legumes, by assuming the fact that in Mediterranean area the consumption of legumes is around 8 and 23 g/capita [22].

3.2 Effect of LE on diet-induced hypercholesterolemia in rats

For the entire duration of treatment, body weight and food intake (Table 1) were unaffected by the administration of LE.

As shown in Table 1, at the end of treatment, the blood cholesterol levels of the rats fed with LE were significantly lower than those of the vehicle group ($p<0.05$). Furthermore, a non-significant trend in the reduction of LDL levels ($p>0.05$) and in the increase of HDL levels ($p>0.05$) was observed.

Table 1 shows that a lowering of total cholesterol levels was observed in both the rats fed with the vehicle and those fed with LE, partially due to the physiological adaptation of their organisms to a cholesterol-enriched diet (exogenous), so that they were no longer capable of synthesizing endogenous cholesterol at the hepatic level, with a consequent physiological decrease in total cholesterol. However, after 71 days of treatment, there was a marked decrease in the total cholesterol level of the rats fed with LE (T0 319.2 ± 41.2 mg/100g, T fin 224.8 ± 12.5 mg/100g, -29.6%), while those fed with the vehicle exhibited a less marked decrease (T0 318.5 ± 39.7 mg/100g, T fin 277.7 ± 20.6 mg/100g, -12.8%): the difference in the decrease observed in rats treated with LE compared to rats given vehicle (16.8%) is statistically significant. These data are in agreement with literature that reports the hypocholesterolemic effect of group B soyasaponins in golden syrian hamsters (decreasing of total cholesterol around 20%) [12]. This new LE showed to possess interesting ability to decrease the cholesterol level, due both to the presence of soyasaponins and to the presence

of other bioactives molecules soluble in ethanol-water as isoflavones, phytosterols, polyphenols (phytocomplex) that could have a synergistic effect on cholesterol level decreasing.

3.3 Quantification of faecal BAs in rats

In this study, we have investigated the level of most abundant BAs in faeces [23] for understanding if the cholesterol-lowering effect obtained by using LE soyasaponins rich can be related with the mechanism that involves a greater excretion of BAs as reported in literature [12].

As expected (Table 2), there was no statistically significant change in the excretion of BAs in rats of the vehicle group from the beginning (T 0) to the end of the study (T final). On the other hand, for the group that received LE, there were significant treatment effects on BA excretion in rat faeces. In rats fed with LE, the mean value of total faecal BAs at the end of the 71 days was significantly greater than it was at the beginning ($p<0.01$). In fact, the total amount of BAs in faeces changed from 3463.6 mg kg^{-1} (T 0) to 4474.2 mg kg^{-1} (T final) in rats treated with LE, a conspicuous increase of total BAs of 1010.6 mg kg^{-1}. Thus, the cholesterol-lowering effect of soyasaponin-rich LE was mediated by increased faecal output of BAs, indicating the inhibition of intestinal reabsorption of BAs.

These results are in agreement with the work of Lee et al. [12], in which is reported a cholesterol lowering effect mediated by increased faecal output of BAs in animals fed with group B soyasaponins.

3.4 Faecal semi-continuous fermentation

The results obtained by semi-continuous fermentation systems showed good prebiotic activity for LE. In the control test (Figure 1A), the total anaerobes showed values over 10^9 CFU/ml during the whole fermentation time. Also the *Clostridium* spp. and the *Enterobacteriaceae* maintained values around 10^9 CFU/ml during the entire fermentation time. *Staphylococcus*

spp. presented stable values up to the end, ~~albeit with a decrease at T2~~. *Bifidobacterium* spp. showed values around 10^4 CFU/ml for the entire time, while *Lactobacillus* spp. values decreased from 10^8 CFU/ml (T1) to 10^6 CFU/ml (T4).

The effect of inulin on the bacterial groups studied overlapped with that of LE in the respective fermentations, and the corresponding graphs (Figure 1B and C) are similar. The total anaerobes decreased significantly ($p<0.05$) in response to the presence of the two substrates in comparison to the control fermentation. The *Enterobacteriaceae* showed similar trends for both substrates and comparable to the control test. *Staphylococcus* spp. had a similar decrease in the two fermentations even if they presented higher values than those of the control test (Figure 1B and C). The values of *Bifidobacterium* spp., which are considered beneficial bacterial species, increased during the time of fermentation and remained stable at the end.

There was a statistically significant increase ($p<0.05$) during the two fermentation cycles with inulin (positive control) and LE, compared to the control fermentation (Figure S3 supplementary information). The observation that this bacterial group increased by 3 log provides evidence that LE has the same activity as inulin in specifically enhancing the growth of *Bifidobacterium* spp., that is, it has similar bifidogenic activity. LE showed the same prebiotic ability of inulin, and its components exhibited good bifidogenic activity.

3.5 Level of SCFAs in faeces

The single levels of SCFAs (acetic, propionic, butyric) and total found in the faeces of rats expressed in mg kg^{-1} are reported in Figure 2. While no significant differences in SCFAs from T0 to T final were recorded in the faeces of rats receiving vehicle, the faeces of rats fed with LE had a significant increase of the total concentration of SCFAs in this time period ($p<0.05$). In particular, in the LE group, the concentration of acetic acid increased from 1777 ± 416 (T 0) to 2680 ± 553 mg kg^{-1} (T fin) ($p<0.05$), that of propionic acid from 668 ± 204 (T

0) to 985 mg kg^{-1} (T fin) ($p < 0.05$), and that of butyric acid from 202 (T 0) to 440 mg kg^{-1} (T fin) ($p<0.05$). According to the literature [19], the increased level of SCFAs in the faeces of LE-treated rats confirms the prebiotic activity of LE.

4 Concluding remarks

A new hydroalcoholic lentil extract (LE) rich in soyasaponins was produced, and its prebiotic properties as well as its ability to reduce plasma cholesterol levels were evaluated. In an animal model, LE was shown to reduce the plasma cholesterol levels of rats by 16.8% ($p<0.05$) with an increase of HDL levels ($p>0.05$) and a decrease of LDL levels ($p>0.05$). In rats fed with LE, the total content of faecal BAs was significantly greater ($p<0.01$) at the end of the 71 days than it was at the beginning, confirming that the cholesterol-lowering effect of soyasaponin-rich LE was mediated by the inhibition of intestinal reabsorption of BAs. Not only did LE show the same prebiotic ability of inulin, but it also had good bifidogenic activity: the growth of *Bifidobacterium* spp in the LE animals was enhanced by 3 log ($p<0.05$) compared to that in the control animals given only vehicle. The level of SCFAs found in the faeces of rats fed with LE was higher than that found in the faeces of vehicle group, confirming the prebiotic activity of LE, especially related to the increase of butyric acid. Finally, this new extract of *Lens culinaris* Medik could be the base formulation for a new food supplement with hypocholesterolemic and prebiotic activity.

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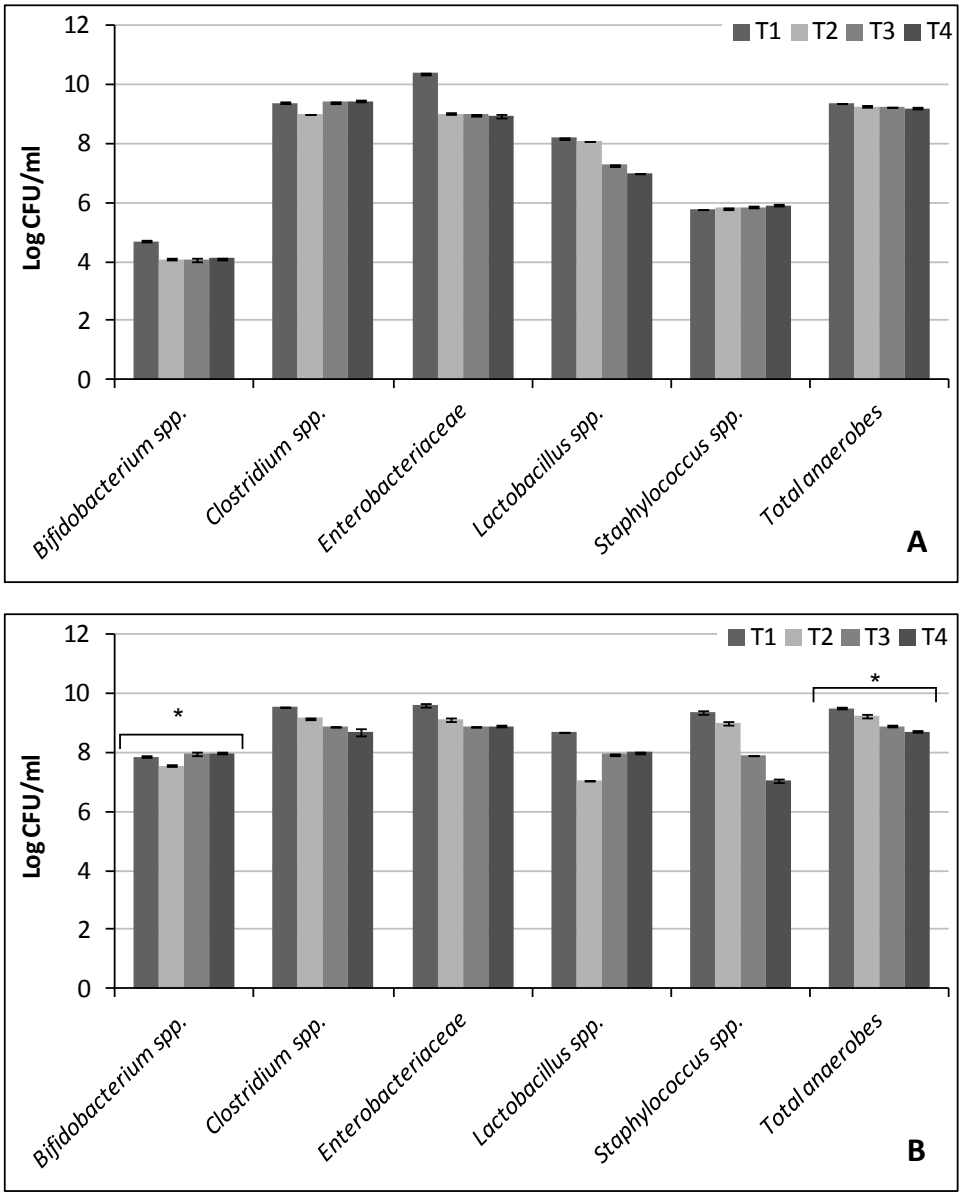
Figure captions



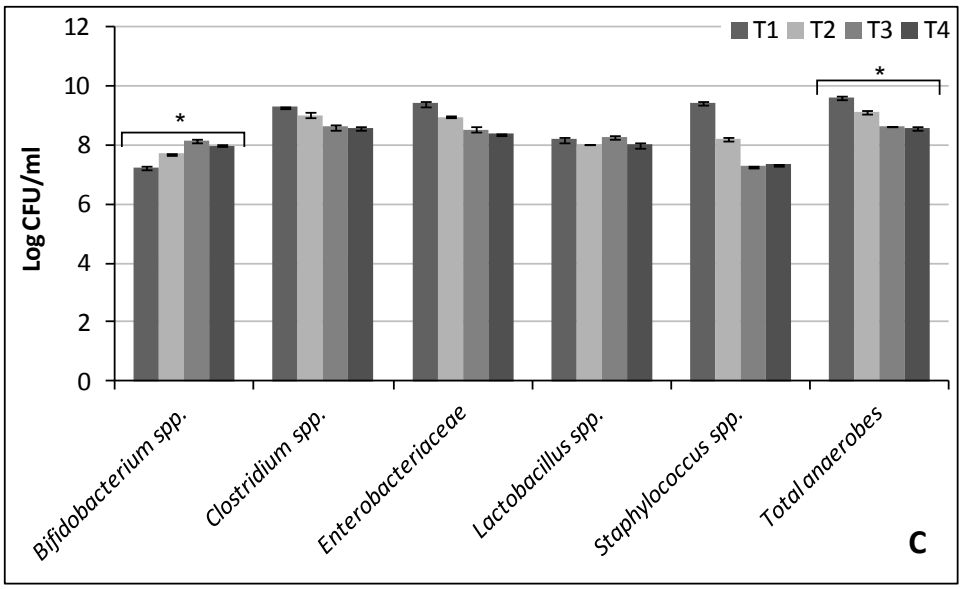


Figure 1. Bacterial counts (log CFU/ml) of main groups of intestinal microorganisms during each feeding day in A) control fermentation, B) inulin fermentation, C) LE fermentation. *T1 (first feeding day), T2 (second feeding day), T3 (third feeding day), T4 (fourth feeding day). *p<0.05 statistically different from the control (A).*

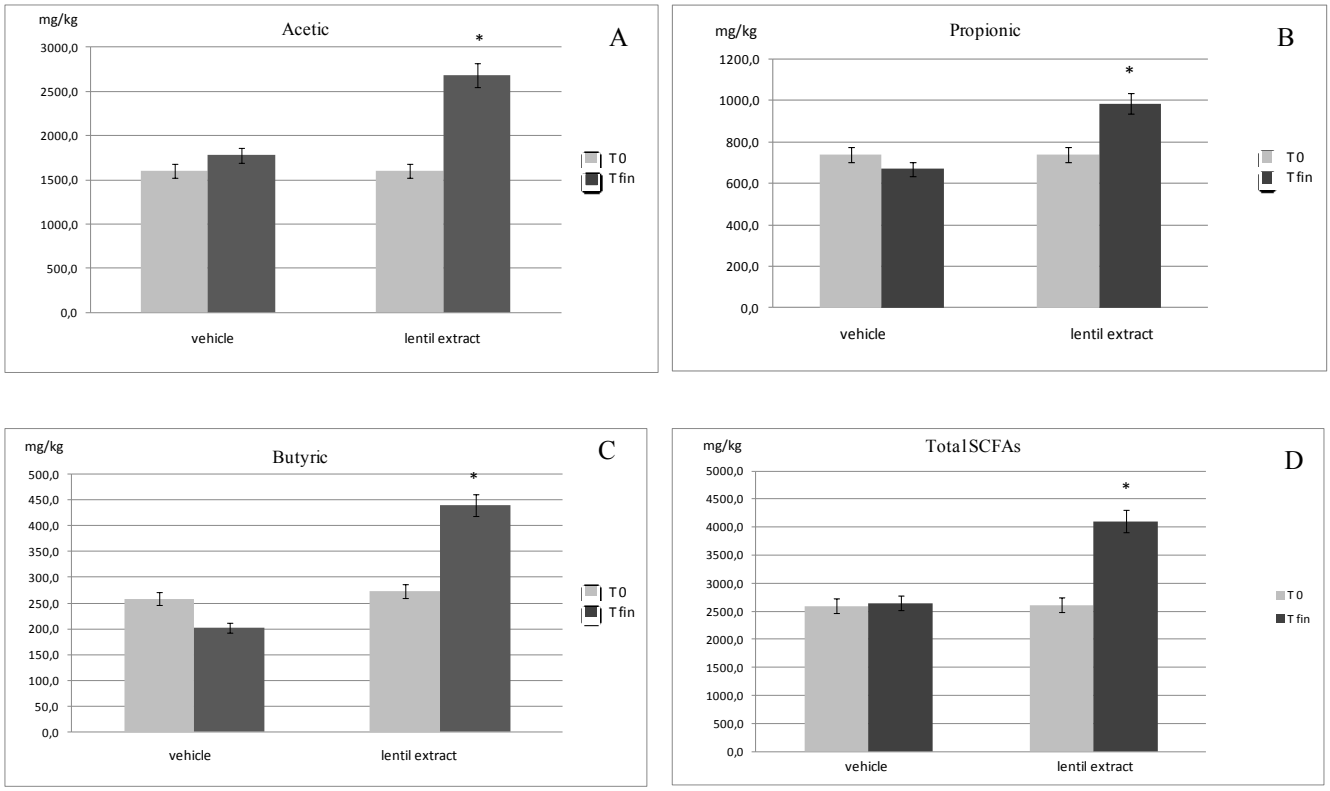


Figure 2. Level of SCFAs A) acetic, B) propionic, C) butyric and D) total expressed in mg/kg in the faeces of rats fed with LE in comparison with vehicle and control. *$p<0.05$ statistically different from the control and vehicle according to the one way analysis of variance (ANOVA).

Table 1. Results of cholesterol lowering activity of Lentil Extract in an animal model.

	Vehicle		Lentil Extract (LE)	
	T 0	T final	T 0	T final
Body weight (g)	548.7 ± 11.4	637.8 ± 13.4	552.9 ± 12.6	640.4 ± 18.9
Food intake (g/day)	23.4 ± 1.2	21.9 ± 1.0	20.4 ± 1.0	22.6 ± 1.2
Total cholesterol (mg/100 ml)	318.5 ± 39.7	277.7 ± 20.6	319.2 ± 41.2	224.8 ± 12.5*
Total HDL cholesterol (mg/100 ml)	24.2 ± 2.5	37.8 ± 3.0	20.4 ± 1.6	31.4 ± 2.2
Total LDL cholesterol (mg/100 ml)	101.1 ± 14.0	66.4 ± 7.2	105.6 ± 14.0	± 5.1

The results are reported as mean ± SEM. One-way ANOVA was performed and statistical significance was set at *p<0.05 Lentil Extract (LE) T 0 vs LE Final

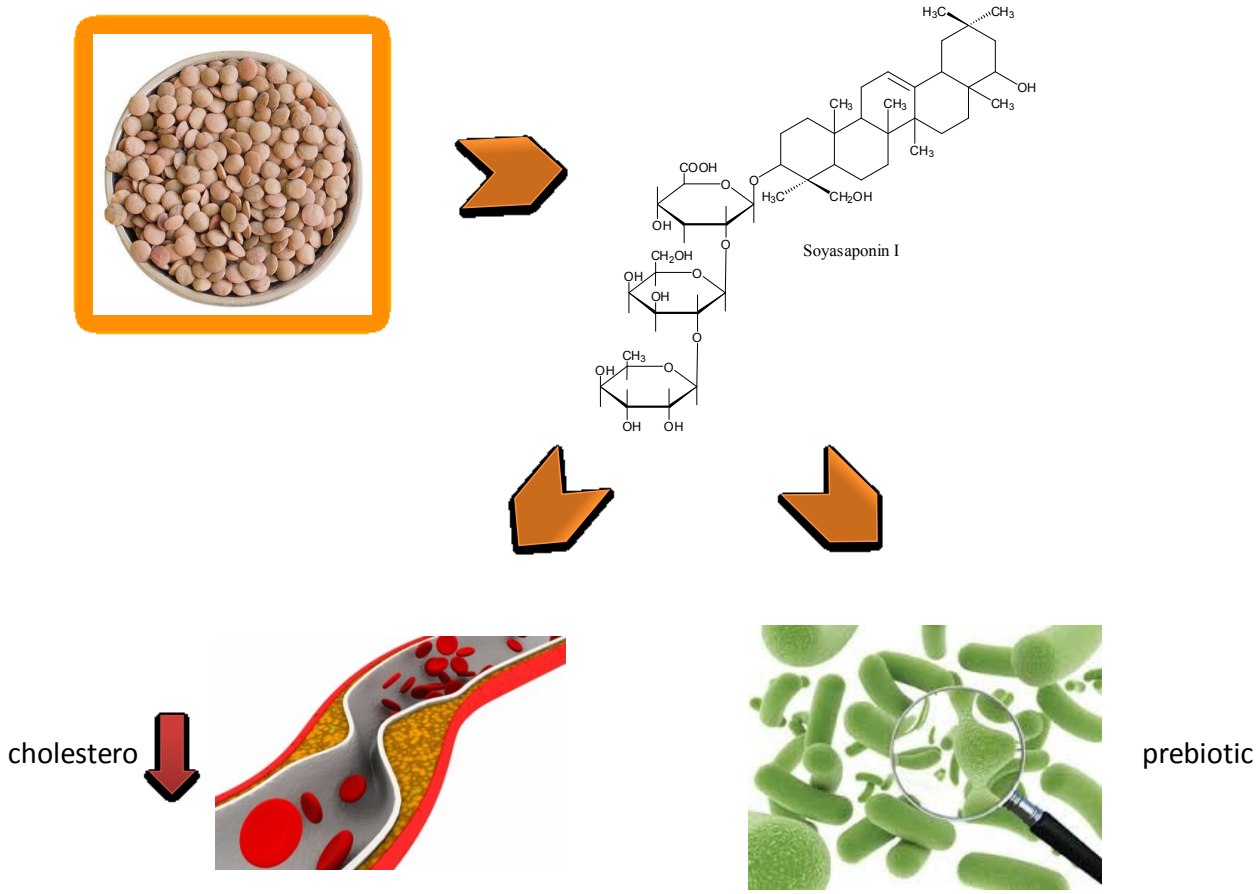
Table 2. Faecal excretion of bile acids in the groups of rats (data are expressed in mg kg^{-1}).

	Group vehicle		Group Lentil Extract (LE)	
	T 0	T final	T 0	T final
Total bile acids[a]	3818.6[b]±1428.1	3549.9[b]±667.7	3463.6[b]±608.3	4474.2[c]±587.8

[a]The values are the mean of 8 different rats ± standard deviation.

[b,c]Different letters indicate significant differences ($p < 0.01$)

GRAPHICAL ABSTRACT



Lentils are a source of bioactive compounds such as soyasaponins that are responsible of hypocholesterolemic and prebiotic activity.