*Journal of Animal and Feed Sciences, 25, 2016, 326–334 https://doi.org/10.22358/jafs/67804/2016*
*The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna*

# Effects of fermentation of narrow-leafed lupine (*L. angustifolius*) seeds on their chemical composition and physiological parameters in rats

**A. Zaworska[1], M. Kasprowicz-Potocka[1,3], A. Frankiewicz[1], Z. Zduńczyk[2] and J. Juśkiewicz[2]**

[1] Poznań University of Life Sciences, Department of Animal Nutrition and Feed Management
Wołyńska 33, 60–637 Poznań, Poland
[2] Institute of Animal Reproduction and Food Research, Polish Academy of Sciences
Tuwima 10, 10–747 Olsztyn, Poland

**KEY WORDS:** lupine, fermentation, yeast, bacteria, intestinal parameters, rats

Received: 4 January 2016
Revised: 12 October 2016
Accepted: 10 December 2016

[3] Corresponding author:
e-mail: malgokas@poczta.onet.pl

**ABSTRACT.** The aim of the study was to determine the effects of fermentation of *Lupinus angustifolius* seeds with yeasts and multibacterial preparation on their chemical and microbial composition, protein digestibility and selected gut and lipid blood parameters in rats. Contents of nutrients, fibre fractions, alkaloids, oligosaccharides, amino acids and phytate in raw (RL) and fermented (FL) lupine seeds were determined. The RL and FL seeds and soyabean oil meal were used as the only protein supplements in wheat diets and fed for 4 weeks to 3 groups of 8 male Wistar rats, approximately 7-week-old. Apparent total tract digestibility of protein (ATTD), intestinal tissue and digesta weights, caecal metabolites concentrations and lipid profile in blood were determined. Fermentation did not affect or induced small changes in crude protein, ash, fat, total alkaloids and phytate P concentrations whereas it eliminated oligosaccharides, reduced considerably true protein and increased crude fibre, NDF and ADF contents. The ATTD of dietary protein was higher in rats fed FL than RL diet. Feeding FL vs RL did not affect intestinal tissue weight, decreased caecal and colon digesta weights, decreased pH and activity of α-galactosidase, and increased that of xylosidase in caecal digesta. The short chain fatty acids (SCFA) caecal concentration and profile were not affected by fermentation whereas pools of acetate, butyrate and total SCFA were lowered. Total triacylglycerols blood level did not differ between FL and RL group, nevertheless total cholesterol and HDL concentration were higher in FL fed rats.

## Introduction

Each year, fermented feed is gaining more interest as a diet component in monogastric animals feeding. Fermentation of lupine seeds contributes to the improvement of nutritional value increasing the availability of minerals and leads to more effective degradation of phytates, oligosaccharides and alkaloids (Jiménez-Martínez et al., 2007; Suliburska et al., 2009; Kasprowicz-Potocka et al., 2016). Narrow-leafed lupine (*Lupinus angustifolius*) is the most popular among lupine species cultivated in Poland because of better growth condition, lower antinutrients content and lower fibre content than

other species. Fermentation of lupine was successfully carried out with the use of bacteria *(L. sakai, P. pentosaceus*), fungi (*A. oryzae*) and yeast (*S. cerevisiae, C. utilis*) (Sulibursk a et al., 2009; Bartkiene et al., 2013; Fritsch et al., 2015; Kasprowicz-Potocka et al., 2015). The nutrient contents of products fermented by these microorganisms are different. Bacteria generally reduce pH and decompose structural carbohydrates, whereas yeast increase protein and reduce antinutritive factors (ANF) (Fritsch et al., 2015). Some bacterial strains are capable of using lupanine, the predominant quinolizidine alkaloid in lupine species, as a sole carbon source (Santana et al., 2002). There are no studies about simultaneous fermentation of lupine seeds by yeast and bacteria. There is also only few studies focused on fermented lupine products usage in animal nutrition (Bartkiene et al., 2013; Kasprowicz-Potocka et al., 2015). Fermentation of diets can be a strategy to improve feeding effects in pigs but it should be investigated (Lyberg et al., 2006; Canibe and Jensen, 2012). We hypothesized that combined fermentation of lupine seeds would allow to obtain new products with higher nutritional value and better microbial status in comparison with unprocessed seeds.

The aim of the study was to: 1. determine the effect of fermentation on the chemical composition and nutritive value of lupine seeds and 2. assess the impact of lupine components as a substitute for soyabean meal (SBM) on the protein digestibility and intestinal ecosystem, activities of selected enzymes and concentrations of volatile products in digesta sampled from various parts of the intestines of rats as the model animals.

# Material and methods

## Fermentation process

The seeds of *Lupinus angustifolius* var. Neptun were obtained from the Plant Breeding Station Przebędowo (Przebędowo, Poland). Soyabean meal was obtained from the market. The seeds were decontaminated with 0.07% sodium hypochlorite solution (POCH, Gliwice, Poland) for 15 min, repeatedly washed with distilled water, dried at 55 °C and ground in a laboratory grinder (Retsch, Haan, Germany). Two kg of lupine meal was mixed vigorously with 8 l of distilled water in the plastic fermentation buckets. The fermentation of lupine seeds was carried out using dry baker's yeast – *Saccharomyces cerevisiae* (Dr Oetker, Gdańsk, Poland) and commercial multi-bacterial preparation (Polmass, Bydgoszcz, Poland) containing strains of *Enterococcus faecium, Lactobacillus plantarum, L. Buchneri* and *L. casei*. Lupine meal was inoculated with yeast (1%, by lupine mass) and bacterial preparation (0.1%, by lupine mass) and mixed. Fermentation was conducted for 24 h at 25 °C under aerobic conditions. Next, the bacterial enzymes were deactivated for 15 min at 70 °C, and the material was dried at 55 °C. Fermented material for experiment was produced as one part in the amount necessary for experiment on rats.

## Experiment on animals

The Local Ethical Committee in Poznań approved the tests (Resolution No. 43/2011 of 15th May 2011). The experiment was carried out on 24 male Crl:WI(Han) Wistar rats aged approximately 7 weeks. The experimental diets were administered for 4 weeks to 8 rats per group housed individually in Plexiglas cages. The diets (Table 1) had a similar content of protein (approximately 18.5%), fat (soyabean oil), minerals and vitamins. Diets and tap water were available *ad libitum*. The animals were kept in the room at a temperature of 21–22 °C, relative air humidity of 50–70%, with

**Table 1.** Composition of diets, %

| Indices | Diet | | |
|---|---|---|---|
| | SBM[1] | RL[2] | FL[3] |
| Soyabean meal | 18.5 | - | - |
| Narrow-leafed lupine seeds | - | 27.8 | - |
| Fermented narrow-leafed lupine | - | - | 20.9 |
| Wheat | 72.3 | 63.0 | 69.9 |
| Soya oil | 5 | 5 | 5 |
| Mineral premix[4] | 3 | 3 | 3 |
| Vitamin premix[5] | 1 | 1 | 1 |
| Choline chloride | 0.2 | 0.2 | 0.2 |

[1]SBM – soyabean oil meal; [2]RL – raw lupine seeds; [3]FL – fermented lupine seeds; [4]Mineral premix AIN-93G, per kg of mix: g: anhydrous calcium carbonate 357 (40.04% Ca), monobasic potassium phosphate 196 (22.76% P, 28.73% K), potassium citrate and tripotassium monohydrate 70.78 (36.16% K), sodium chloride 74 (39.34% Na, 60.66% Cl), potassium sulphate 46.6 (44.87% K, 18.39% S), magnesium oxide 24 (60.32% Mg), ferric citrate 6.06 (16.5% Fe), zinc carbonate 1.65 (52.14% Zn), sodium metasilicate 9 $9H_2O$ 1.45 (9.88% Si), manganous carbonate 0.63 (47.79% Mn), cupric carbonate 0.3 (57.47% Cu), powdered sucrose 221026, chromium potassium sulphate 0.275 × $12H_2O$ (10.42% Cr); mg: boric acid 81.5 (17.5% B), sodium fluoride 63.5 (45.24% F), nickel carbonate 31.8 (45% Ni), lithium chloride 17.4 (16.38% Li), anhydrous sodium selenite 10.25 (41.79% Se), potassium iodate 10 (59.3% I), ammonium paramolybdate 7.95 × $4H_2O$ (54.34% Mo), ammonium vanadate 6.6 (43.55% V); [5]Vitamin premix AIN-93G, per kg of mix: g: nicotinic acid 3.0, Ca pantothenate 1.6, pyridoxine-HCl 0.7, thiamin-HCl 0.6, powdered sucrose 974655, riboflavin 0.6, folic acid 0.2, biotin 0.02, vit. $B_{12}$ 2.5 (cyanocobalamin, 0.1% in mannitol); IU: vit. E 15.0 (all-rac-α-tocopheryl acetate, 500), vit. A 0.8 (all-trans-retinylpalmitate, 500 000), vit. $D_3$ 0.25 (cholecalciferol, 400 000), vit. $K_1$ 0.075 (phylloquinone)

intensive ventilation (20 times · $h^{-1}$) and a 12 h light regimen. Individual body weight and feed intake were recorded.

In the last week of the experiment, the rats were transferred to balance cages (Techniplast Spa, Buguggiate, Italy), and faeces were quantitatively collected in order to calculate the protein digestibility coefficients. After 4 weeks of the experiment, the rats were anaesthetized with sodium pentobarbitone (14 mg per kg of body weight). Blood samples were collected from the caudal *vena cava*. Serum samples were prepared by centrifugation at 1500 *g* for 15 min at 4 °C, and stored at −40 °C for further analyses. After laparotomy, small intestine, caecum and colon were removed and weighed. Directly after euthanasia (approximately 10 min) ileal, caecal and colonic pH values were measured, digesta samples were collected in order to determine dry matter, ammonia and short-chain fatty acids (SCFA) contents and the remaining material was frozen at −70 °C for the determination of protein content and microbial enzyme activity. The ileal, caecal and colonic walls were flushed, cleaned with ice-cold saline, blotted on filter paper and weighed to determine tissue mass.

## Chemical and microbial analysis

For chemical analysis, all samples were ground to pass through a 0.5-mm sieve. Raw seeds, fermented products and digesta samples were analysed twice for dry matter, crude protein, ether extract, crude fibre, crude ash, acid detergent fibre and neutral detergent fibre using AOAC International (2006) methods: 934.01, 976.05, 920.39, 978.10, 942.05, 973.18, respectively. The true protein was determined by extraction of protein with 20% trichloroacetic acid. Protein was determined using Kjeldahl method. Lupine alkaloids were extracted with trichloroacetic acid and methylene chloride (Sigma-Aldrich, St. Louis, MO, USA) and determined using gas chromatography method (Shimadzu GC17A, Kyoto, Japan) with a capillary column (Phenomenex, Torrance, CA, USA). Raffinose family oligosaccharides were extracted and analysed using high-resolution gas chromatography as described by Zalewski et al. (2001). Phytate content was analysed according to the AOAC International (2006) method 986.11. Amino acid (AA) content was determined using an AAA-339 Mikrotechna amino acid analyser (Prague, Czech Republic) with ninhydrin for post-column derivatization. Before analysis, the samples were hydrolysed using 6 M HCl for 24 h at 110 °C (procedure 994.12; AOAC International, 2006).

Samples for bacteriological analysis were prepared by adding 27 ml of buffered peptone water (Oxoid, Hampshire, UK) to 3 g of samples and homogenized for 30 s in a laboratory stomacher. Microbial counts were determined using a decimal dilution series of homogenised samples. Total bacteria count and lactic acid bacteria count were determined after 72 h incubation at 30 °C using plate standard methods with plate count agar (PCA) and MRS broth (Oxoid, Hampshire, UK), respectively. *Salmonella* count was determined at 37 °C using pre-suplemented dichloran Rose Bengal chlorcamphenicol (DRBC) and agar Salmonella Chromogen (Oxoid, Hampshire, UK), after 18 h and 24 h incubation, respectively. Yeast content was calculated using pre-supplemented DRBC (Oxoid, Hampshire, UK) after incubation at 25 °C for 3–5 days. Coliform bacteria were determined using Violet Red Bile lactose agar (Oxoid, Hampshire, UK) after 24 h of incubation at 30 °C.

Caecal pH was measured using a microelectrode and pH/Ion meter (model 301, Hanna Instruments, Vila do Conde, Portugal). Ammonia was extracted and trapped in a boric acid solution, and then analysed through direct titration with sulphuric acid (POCH, Gliwice, Poland). Short-chain fatty acids in fresh caecal contents were determined using gas chromatography (Shimadzu GC-14A with a 2.5 mm × 2.6 mm glass column containing 10% SP – 1200/1% $H_3PO_4$ on 80/100 Chromosorb WAW (Shimadzu Corp., Kyoto, Japan); column temperature 110 °C, flame ionization detector (FID) temperature 180 °C, injector temperature 195 °C). Aliquots of caecal digesta were mixed with 0.2 ml of formic acid (POCH, Gliwice, Poland), diluted with deionized water and centrifuged at 10 000 *g* for 5 min. Samples of the supernatant were subjected to gas chromatography analysis. Caecal short-chain fatty acids pools were calculated as the product of acids concentrations per caecal digesta mass. SCFA profile was calculated as the proportion of respective fatty acid (C2: C3: C4) in sum. Activity of microbial enzymes (α- and β-glucosidase, α- and β-galactosidase, β-glucuronidase and xylosidase) was measured on the basis of the rate of *p*-nitrophenol and *o*-nitrophenol release from nitrophenyl-glucosides, and was expressed in micromoles of the product formed per h per g of caecal digesta at 10 000 *g* for 10 min. Cholesterol, triacylglycerol and cholesterol concentrations in the serum were determined using Alpha Diagnostics (Warsaw, Poland) and Pointe Scientific (Warsaw, Poland) commercial kits.

## Statistical analysis

The composition of raw and fermented seeds (Tables 2 and 3) were presented as mean vaues ± standard deviation. The results of the animal experiment were analysed by one-way ANOVA method. The significance of differences between groups were calculated using the detailed Duncan's test (for comparing all the three groups) at $P \leq 0.05$ or Student's t-test (for comparison in pairs, fermented and raw) at $P \leq 0.05$. Statistical analysis was performed using the computer programme SAS Enterprise Guide 5.1 (Cary, NC, USA).

**Table 2.** Chemical composition of raw (RL) and fermented lupine (FL) seeds

| Indices | RL | FL |
|---|---|---|
| Dry matter, % | 87.57 ± 0.02 | 97.02 ± 0.04 |
| Components, % in dry matter | | |
| crude protein | 38.23 ± 0.69 | 39.37 ± 0.03 |
| true protein | 32.41 ± 0.92 | 24.12 ± 0.04 |
| crude ash | 4.07 ± 0.01 | 4.38 ± 0.02 |
| crude fat | 6.12 ± 0.02 | 4.66 ± 0.01 |
| crude fibre | 13.65 ± 0.05 | 18.81 ± 0.03 |
| ADF | 20.46 ± 0.23 | 25.62 ± 0.03 |
| NDF | 21.59 ± 0.11 | 27.72 ± 0.02 |
| total alkaloids | 0.053 ± 0.001 | 0.052 ± 0.010 |
| total RFOs | 11.12 ± 0.23 | 0.00 ± 0.00 |
| phytate phosphorus | 0.43 ± 0.08 | 0.35 ± 0.01 |
| Indispensable amino acid, % of protein | | |
| arginine | 10.43 ± 0.08 | 7.50 ± 0.02 |
| histidine | 2.59 ± 0.03 | 2.50 ± 0.01 |
| isoleucine | 3.65 ± 0.02 | 4.10 ± 0.02 |
| leucine | 6.27 ± 0.04 | 6.77 ± 0.04 |
| lysine | 4.20 ± 0.02 | 4.43 ± 0.02 |
| phenylalanine | 3.93 ± 0.02 | 3.49 ± 0.03 |
| threonine | 3.11 ± 0.05 | 2.73 ± 0.02 |
| valine | 3.48 ± 0.03 | 3.87 ± 0.02 |

ADF – acid detergent fibre; NDF – neutral detergent fibre; RFO – raffinose family oligosaccharides; results are expressed as mean ± standard error

**Table 3.** Microbial composition of raw (RL) and fermented lupine (FL) seeds

| Indices | RL | FL |
|---|---|---|
| Total bacteria, CFU · $g^{-1}$ | $5.4\times10^5 \pm 1.1\times10^2$ | $8.6\times10^5 \pm 1.8\times10^2$ |
| Lactic acid bacteria, CFU · $g^{-1}$ | $2.4\times10^4 \pm 7.1\times10^2$ | $1.6\times10^6 \pm 1.2\times10^3$ |
| Coliform bacteria, CFU · $g^{-1}$ | $5.5\times10^4 \pm 2.4\times10^2$ | $1.8\times10^4 \pm 2.2\times10^2$ |
| *Salmonella,* CFU · $g^{-1}$ | 0 ± 0.0 | 0 ± 0.0 |
| Yeast, CFU · $g^{-1}$ | $2.3\times10^4 \pm 9.9\times10^2$ | $9.55\times10^4 \pm 5.1\times10^2$ |
| pH | 5.5 ± 0.1 | 3.8 ± 0.1 |

CFU – colony forming units; results are expressed as mean ± standard error

# Results

## Chemical composition and microbial status of raw and fermented lupine meal

Fermentation by bacteria and yeast slightly increased crude protein content but lowered the level of true protein, increased crude fibre, acid detergent fibre and neutral detergent fibre, but decreased crude fat content in comparison with unprocessed seeds (Table 2). Due to the fermentation raffinose family oligosaccharides (RFO) were eliminated, and phytate phosphorus content was decreased by about 20%. Amino acids composition of lupine protein differed between RL and FL to a small extent, except arginine content which was lower in FL (Table 2). Fermentation reduced the pH of lupine products (Table 3). Total bacteria and yeast number were slightly higher and number of coliform bacteria was slightly lower in the fermented than raw seeds. The fermentation process significantly increased the number of the lactic acid bacteria.

## Feed intake, body weight and protein digestibility and utilization

The consumption of the soyabean meal (SBM) and experimental diets was similar (Table 4). Body weight gain and final weight were greater in the group fed SBM diet than RL diet ($P = 0.007$), but there was no effect of lupine fermentation. Body weight gain of rats per g of protein intake for animals offered RL diet were significantly lower than in other groups ($P = 0.004$). Between the experimental and control groups there were no differences in the digestibility of protein. Fermentation improved the

**Table 4**. Feed intake, coefficients of apparent protein digestibility, body weight gain and body weight gain per g of protein intake in rats fed diets containing different protein supplements

| Indices | Diet[1] | | | *P*-value | Fermentation effect, *P*-value |
|---|---|---|---|---|---|
| | SBM | RL | FL | | |
| Feed intake, g · $day^{-1}$ | 19.7 | 19.8 | 19.7 | 0.978 | 0.851 |
| Initial body weight, g | 182 | 182 | 182 | 0.995 | 0.930 |
| Final body weight, g | 313[a] | 300[b] | 306[ab] | 0.014 | 0.212 |
| Body weight gain, g | 131[a] | 118[b] | 124[ab] | 0.007 | 0.148 |
| Body weight gain per g of protein intake, g · $g^{-1}$ | 1.29[a] | 1.15[b*] | 1.26[a**] | 0.004 | 0.019 |
| Apparent protein digestibility, % | 84.9 | 83.9[*] | 85.4[**] | 0.128 | 0.033 |

[1] see Table 1; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$ (ANOVA); *,** – data significantly different between lupine groups at $P < 0.05$ (Student's t-test); results are expressed as mean ± standard error

apparent total tract digestibility (ATTD) of dietary protein ($P$ = 0.033) and body weight gain per g of protein intake in rats ($P$ = 0.019).

## Gastrointestinal parameters

The use of different high-protein components in the diets had an impact on most of the analysed parameters of the rat caecum and colon, but not of the small intestine (Table 5). In rats fed RL diet higher ($P$ = 0.007) pH and ceacal digesta weight ($P$ = 0.039) were measured in comparison with other groups. Caecum tissue weight ($P$ = 0.012) and the concentration of ammonia in the ceacal digesta ($P$ = 0.049) were higher in both lupine than in the SBM group.

**Table 5.** Parameters of gastrointestinal functioning of rats fed diets containing different protein supplements

| Indices | Diet[1] | | | *P*-value | Fermentation effect, *P*-value |
|---|---|---|---|---|---|
| | SBM | RL | FL | | |
| Issue weight, g · 100 g$^{-1}$ body weight | | | | | |
| small intestine | 2.64 | 2.64 | 2.70 | 0.838 | 0.687 |
| caecum | 0.32$^{b}$ | 0.38$^{a}$ | 0.39$^{a}$ | 0.012 | 0.897 |
| colon | 0.42$^{b}$ | 0.55$^{a}$ | 0.52$^{ab}$ | <0.001 | 0.194 |
| Digesta weight, g · 100 g$^{-1}$ body weight | | | | | |
| caecum | 1.19$^{b}$ | 1.46$^{a**}$ | 1.14$^{b*}$ | 0.039 | 0.036 |
| colon | 0.31$^{c}$ | 0.73$^{a**}$ | 0.54$^{b*}$ | <0.001 | 0.006 |
| pH of digesta | | | | | |
| small intestine | 6.82 | 6.74 | 6.86 | 0.423 | 0.238 |
| caecum | 6.09$^{b}$ | 6.38$^{a**}$ | 6.17$^{b*}$ | 0.007 | 0.019 |
| colon | 5.90 | 5.87 | 5.94 | 0.615 | 0.400 |
| Ammonia, mg · g$^{-1}$ | | | | | |
| caecum | 0.20$^{a}$ | 0.16$^{b}$ | 0.16$^{b}$ | 0.049 | 0.969 |

[1] see Table 1; $^{abc}$ – means with different superscripts within a row are significantly different at $P$ < 0.05 (ANOVA);*,** – data significantly different between lupine groups at $P$ < 0.05 (Student's t-test); results are expressed as mean ± standard error

The weight of caecum and colon tissue in rats fed FL or RL diets did not differ significantly, however, in the FL group content in both sections and lower pH in the colon was found ($P$ < 0.007). Rats from RL group had also higher mass of colon and caecum digesta than rats from SBM group. The ammonia content in the caecum digesta did not differ between lupine groups, but was significantly lower than in the SBM group. In comparison with SBM group both lupine groups had increased caecum and colon tissue weight.

## Microbial enzyme activity and short-chain fatty acids in the caecal digesta

Microbial activity of α- and β-glucosidase and β-galactosidase did not differ among groups (Table 6). Activity of α-galactosidase was lower in FL group and of xylosidase higher than in RL group,

**Table 6.** Microbial enzyme activity in the caecal digesta of rats fed diets containing different protein supplements

| Enzyme activity, IU · g$^{-1}$ of digesta | Diet[1] | | | *P*-value | Fermentation effect, *P*-value |
|---|---|---|---|---|---|
| | SBM | RL | FL | | |
| α-glucosidase | 26.81 | 29.23 | 26.77 | 0.638 | 0.477 |
| β-glucosidase | 29.04 | 27.40 | 30.96 | 0.667 | 0.446 |
| α-galactosidase | 99.03$^{a}$ | 101.10$^{a**}$ | 70.92$^{b*}$ | 0.006 | 0.011 |
| β-galactosidase | 143.9 | 168.4 | 137.4 | 0.265 | 0.177 |
| β-glucuronidase | 72.96$^{a}$ | 40.47$^{b}$ | 42.49$^{b}$ | 0.002 | 0.808 |
| Xylosidase | 12.19$^{b}$ | 10.33$^{c*}$ | 14.65$^{a**}$ | 0.012 | 0.014 |

[1] see Table 1; $^{abc}$ – means with different superscripts within a row are significantly different at $P$ < 0.05(ANOVA); *,** – data significantly different between lupine groups at $P$ < 0.05 (Student's t-test); results are expressed as mean ± standard error

whereas activity of β-glucuronidase in both lupine groups was lower than in SBM. Also α-galactosidase activity was lower in FL than in SBM group.

Concentrations of particular and total SCFA in caecal digesta did not differ between FL and RL groups whereas concentrations of acetate and valerate were lower and of butyrate higher in rats fed both lupine diets than SBM (Table 7). Caecal pool (product of concentration and relative weight of caecal digesta) of acetate, butyrate and total SCFA was lower in FL than RL rats (Table 8) and only pool of butyrate was greater in digesta of RL than in SBM groups. SCFA profile did not differ between FL and RL whereas proportion of acetate was lower and of butyrate – higher in both lupine groups than in SBM.

**Table 7.** Content of short-chain fatty acids (SCFA) in the caecal digesta of rat

| SCFA content, μmol · g$^{-1}$ | Diet | | | | *P*-value | Fermentation effect, *P*-value |
|---|---|---|---|---|---|---|
| | SBM | RL | FL | SEM | | |
| Acetate | 122.8$^{a}$ | 96.6$^{b}$ | 91.8$^{b}$ | 4.0 | 0.002 | 0.526 |
| Propionate | 22.18 | 19.71 | 22.42 | 1.15 | 0.591 | 0.428 |
| Isobutyrate | 1.29 | 1.54 | 1.09 | 0.14 | 0.466 | 0.240 |
| Butyrate | 68.59$^{b}$ | 80.01$^{a}$ | 85.10$^{a}$ | 2.72 | 0.032 | 0.476 |
| Isovalerate | 1.29 | 0.78 | 1.04 | 0.14 | 0.319 | 0.420 |
| Valerate | 5.41$^{a}$ | 2.47$^{b}$ | 2.23$^{b}$ | 0.52 | 0.014 | 0.592 |
| Total SCFA | 220.6 | 201.1 | 203.7 | 5.1 | 0.242 | 0.606 |

[1] see Table 1; $^{ab}$ – means with different superscripts within a row are significantly different at $P$ < 0.05 (ANOVA); results are expressed as mean ± standard error

## Lipids profile of blood serum

Rats fed FL diet had a higher total and high-density lipoprotein (HDL) cholesterol levels than RL animals whereas triacylglycerol (TAG) concentrations did not differ. The TAG levels were lower in both lupine groups than in SBM group and also total

**Table 8.** Pool and profile of short-chain fatty acid (SCFA) in caecal digesta of rats fed diets containing different protein supplements

| Indices | Diet | | | *P*-value | Fermentation effect, *P*-value |
|---|---|---|---|---|---|
| | SBM | RL | FL | | |
| Pool SCFA, µmol per 100 g of caecal digesta | | | | | |
| acetate | 144.2$^{a}$ | 140.0$^{a**}$ | 102.8$^{b*}$ | 0.007 | 0.010 |
| propionate | 26.40 | 28.73 | 25.25 | 0.679 | 0.445 |
| butyrate | 81.5$^{b}$ | 117.8$^{a}$ | 94.6$^{b}$ | 0.009 | 0.075 |
| total SCFA | 262$^{ab}$ | 294$^{a**}$ | 227$^{b*}$ | 0.004 | 0.020 |
| % of total SCFA in digesta | | | | | |
| acetate | 55.05$^{a}$ | 47.90$^{b}$ | 45.29$^{b}$ | <0.001 | 0.251 |
| propionate | 10.03 | 9.92 | 10.88 | 0.681 | 0.503 |
| butyrate | 31.29$^{b}$ | 39.78$^{a}$ | 41.70$^{a}$ | <0.001 | 0.323 |

[1] see Table 1; $^{ab}$ − means with different superscripts within a row are significantly different at $P < 0.05$ (ANOVA); *, ** − data significantly different between lupine groups at $P < 0.05$ (Student's t-test); results are expressed as mean ± standard error

**Table 9.** Blood serum lipid profile of rats fed diets containing different protein supplements

| Indices | Diet[1] | | | *P*-value | Fermentation effect, *P*-value |
|---|---|---|---|---|---|
| | SBM | RL | FL | | |
| Triacylglycerols, mmol · l$^{-1}$ | 2.20$^{a}$ | 1.76$^{b}$ | 1.62$^{b}$ | 0.024 | 0.438 |
| Total cholesterol, mmol · l$^{-1}$ | 1.84$^{a}$ | 1.46$^{b*}$ | 1.75$^{a**}$ | 0.014 | 0.003 |
| HDL cholesterol, mmol · l$^{-1}$ | 1.18$^{a}$ | 0.93$^{b*}$ | 1.12$^{ab**}$ | 0.021 | 0.010 |
| Profile HDL, % | 64.32 | 64.07 | 63.96 | 0.988 | 0.960 |

[1] see Table 1; $^{ab}$ − means with different superscripts within a row are significantly different at $P < 0.05$ (ANOVA);*,** − data significantly different between lupine groups at $P < 0.05$ (Student's t-test); HDL – high-density lipoprotein cholesterol; profile HDL – % HDL fraction in total cholesterol; results are expressed as mean ± standard error

and HDL cholesterol concentrations were lower in RL than in SBM rats. The HDL profile did not differ among dietary groups.

## Discussion

**Chemical composition and microbial status of raw and fermented lupine meal**. The chemical composition of narrow-leafed lupine seeds used in this study was comparable with the values given by Wasilewko and Buraczewska (1999), Kasprowicz-Potocka et al. (2015) and Stanek et al. (2015).

Fermentation is a natural process in which microorganisms utilize and convert nutrients present in the biomass and reduce contents of compounds with anti-nutritional activity. The fermentation process influenced the change in the content of protein and amino acids, but strongly reduced true protein content, what is in agreement with observation of Kasprowicz-Potocka et al. (2015). Lupine protein is known as easily fermented by bacteria and protozoa in the rumen. Yeasts are also capable of utilizing a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous components of the cell, such as amino acids (and consequently peptides and proteins). They possess a whole repertoire of genes encoding enzymes to the biosynthesis of all amino acids. From the other side the content of true protein should be higher in the case of biomass growth (Dobrzański et al., 2006). Recorded decrease in the true protein may be due to the poor fermentation and low biomass production resulted from media composition. Also, partial denaturation of the protein may occurred during the drying of fermented lupine seeds. The drying process can decrease protein dispersibility and solubility, or decrease biological value and protein utilization (Buraczewska et al., 1998). Changes in the content of basic nutrients (crude ash, crude fat and crude fibre) may be the results of increase or reduction in (N-free extract) (NFE) or protein fractions (Feng et al., 2007; Kasprowicz-Potocka et al., 2015). Similar observations were noted by Hassan et al. (2008) and Yabaya et al. (2009). According to these authors, the increase in nutrients content (crude protein and fibre) was mainly due to the activity of yeast during fermentation.

In the present study we hypothesized that lactic acid bacteria (LAB) or yeast would use some structural sugars and would reduce fibre fraction in the product. Lignified and insoluble carbohydrate structures present in crude fibre, acid detergent fibre (ADF) and neutral detergent fibre (NDF) are generally hardly fermented in comparison to their soluble counterparts (NFE or oligosaccharides) (Bach Knudsen and Jørgensen, 2001). Moreover, the increase in the NDF and also ADF content could be due to the technological processes conditions, which is usually associated with an increase in NDF-bound protein and its lower availability (Buraczewska et al., 1998; Hassan et al., 2008). On the other hand, the total use of simple sugars (NFE) was relatively low, which could be connected with the activity of microorganisms used, which need optimal pH and temperature to act or prior hydrolysis of material (Dobrzański et al., 2006).

During fermentation yeast produces different types of hydrolases, which degrade the oligosaccharides into simple sugars (Egounlety and Aworh, 2003). The easily degraded sugars like RFO could be partially or fully decomposed during fermentation,

which was confirmed by results of own research. Vidal-Valverde et al. (1993) by fermenting lentils received a total reduction of these compounds. Also, lupine fermentation conducted by Fritsch et al. (2015) using *Bifidobacterium lactis* significantly reduced the RFO content.

Fermentation using bacteria (Fritsch et al., 2015) or yeast (Kasprowicz-Potocka et al., 2015) caused also the decrease in phytate content by about 20%, and it was confirmed in our study. The observed changes indicate that fermentation increases the activity of native phytase, which can disintegrate insoluble organic complexes with minerals.

Only the concentration of alkaloids in the fermented seeds was similar to that in the raw seeds. This is in line with observations of Kasprowicz-Potocka et al. (2015) who found that lupine alkaloids are resistant to both fermentations with the yeast *Saccharomyces cerevisiae,* as well as *Candida utilis*. Jiménez-Martínez et al. (2007) found 91% reduction of alkaloid content after fermentation of lupine seeds by *Rhizopsus oligosporus*, and Santana et al. (2002) discovered some bacteria species, which reduced about 85% of lupanine in the aqueous lupine media. Bacteria and yeast used in the actual research were not suitable for lupine alkaloids degradation.

**Evaluation of microbiological quality of feed is an important element in assessing the quality of feed.** The obtained fermentation product was characterized by a more favourable microbiological status in relation to raw lupine seeds. A significant increase in the number of lactic acid bacteria was observed, correlated with a low pH of the product, and a slight reduction of *Enterobacteriaceae* bacteria that are not resistant to an acidic environment. A similar effect of yeast fermentation was observed by Kasprowicz-Potocka et al. (2015). The obtained results also correspond with the results of Mbata et al. (2009), in which natural fermentation of maize and nuts resulted in lowering the pH value to 3.5, which contributed to the elimination of *Escherichia coli* bacteria, while increasing the number of lactic acid bacteria and yeasts. This is also confirmed by studies of Canibe and Jensen (2012).

**Feed intake, body weight and protein utilization and digestibility.** Replacement of SBM in the mixture with raw lupine seeds or fermentation product did not affect the animal feed intake. However unprocessed seeds present in the diet caused a reduction of body weight gain and body weight gain per g of protein intake by approximately 10%. It could be expected that fermentation of seeds improved palatability of feed, but this effect was no observed in this research. Stanek et al. (2015) found, that diets containing the seeds of three narrow-leafed lupine cultivars reduced feed intake and significantly limited the growth rate of rats compared to the control group (SBM in diet). Also, studies of Zdunczyk et al. (1998) showed a reduction of body weight gain per g of protein intake in the groups receiving raw lupine seeds in the mixture. Similar relationships were found by Sobotka et al. (2013) and Kasprowicz-Potocka et al. (2015). These authors observed that the replacement of SBM with protein of raw narrow-leafed and yellow lupine seeds significantly reduced the growth rate of rats but did not affect feed intake. Also, Bartkiene et al. (2013), using products fermented by *Pediococcus acidilactici* (soyabean, linseed and white and yellow lupine) in diets for rats, found better weight gain and feed utilization in relation to the control group. Fermented seeds were free of oligosaccharides and had a reduced content of phytate, which can impact positively feed utilization and digestibility of protein. It is in agreement with Gdala and Buraczewska (1997) and Zdunczyk et al. (1998) who found, that RFOs can reduce protein digestibility in the small intestine of animals.

**Gastrointestinal parameters.** The results of present studies indicate the diversity of processes in the gastrointestinal tract of rats depending on the composition of the diet. Diets with lupine seeds (raw or fermented) did not affect the parameters of the small intestines, however, they significantly affected the caecum and colon environment. The lower acidity of the caecum digesta in animals from SBM and FL group compared to animals from RL group may suggest the formation of optimum conditions for the development of beneficial microflora and limiting the development of harmful bacteria (Sobotka et al., 2013). It could be also the results of the favourable microbial status of fermented lupine, accompanied with lower pH.

From the other side in rats receiving raw lupine seeds the increase of the mass of the caecum and colon digesta was observed which may be due to the increased mass of bacteria in the colon resulted from the presence of RFOs. RFOs are utilized by microflora as a growth medium in the distal parts of the digestive tract and decomposed into the gases. It was confirmed by Juśkiewicz et al. (2006) and Sobotka et al. (2013). The increase in both the mass of the intestinal tissue and the mass of the digesta is connected with the higher mass of undigested portions of the diet reaching the colon, and a higher share of fermentable non-starch polysaccharides, particularly RFO sin diet with raw seeds. The presence of these

substances in lupine seeds could result in higher metabolic activity of microorganisms thus increase in the energy demand, which in turn would limit the amount of energy available to rats (Bakker et al., 1998). In the case of fermented products in the diet with reduced RFOs content the activity of bacteria in the caecum and colon was lower. It's partially confirmed by microbial activity in this part of the gastrointestinal tract measured by enzymes activity (α-galactosidase). Similarly to own studies, Zdunczyk et al. (1998) found that the amount of ammonia produced during the bacterial degradation of lupine seeds in rats is lower than other feed components. This fact is confirmed by the lower activity of β-glucoronidase in the groups receiving lupine. The obtained results corresponds with the results presented by Bartkiene et al. (2013) and Kasprowicz-Potocka et al. (2015), who noted similar trends for diets involving fermented and raw lupine seeds.

The activity of microorganisms in the caecum is correlated with their production of SCFA. In these studies significant differences in the pool and profile of SCFA were found. Similarly, lower concentration of acetate and valerate in groups fed with lupines was observed by Juśkiewicz et al. (2006) and Kasprowicz-Potocka et al. (2015) in comparison to control group. In animals receiving raw lupine seeds, higher production of SCFA, acetate and butyrate calculated as μmol per 100 g of body mass in relation to the fermented seeds was found. It is consistent with previous observations. In own studies, similarly as in Sobotka et al. (2013), the increase of butyrate concentration was accompanied by an increase of caecum tissue mass in rats receiving raw lupine seeds in the diet. Kasprowicz-Potocka et al. (2015) also observed a higher pool of butyrate in the group receiving raw lupine seeds. Butyrate is one of the major nutrients and an energy source for the epithelial cells lining the colon and it regulates growth and differentiation of cells (Guarner and Malagelada, 2003). In contrast, acetates are the major fermentation product of *Bifidobacterium* and affect the formation of the caecum environment and enter the peripheral circulation, where they are metabolized in the tissues. The reduced concentration of pooled SCFA may mean that the microbial activity in the digesta from animals fed with fermented lupines is partially inhibited; thereby the introduced biomass constitutes a protein source in the initial sections of the gastrointestinal tract, and does not show probiotic effect in its subsequent sections.

The analysis of the lipids profile in blood serum did not reveal any significant disorders in rats. Replacement of SBM with lupines in the diet (raw or fermented) resulted only in a decrease of TAG concentrations in blood serum. Similarly as in own studies, a decrease of TAG in the blood and liver of animals receiving lupine in the diet was also reported by Spielmann et al. (2007). This indicates a beneficial impact of the lupine protein on the metabolism of TAG and shows the effect of preventing the development of cardiovascular diseases. In addition, in the group fed a diet involving raw lupine seeds, a significant decrease in total cholesterol and HDL cholesterol concentrations was found. Also other studies confirm hypocholesterolaemic advantages of lupine (Osman et al., 2011). The inhibition of cholesterol synthesis and cholesterol reduction in the blood could be affected by production of SCFA in digesta (Wong et al., 2006). However, fermentation of lupine seeds caused a significant increase in the concentration of total cholesterol in the blood serum of rats. The obtained results corresponds with the study of Chango et al. (1998) and Kasprowicz-Potocka et al. (2015).

## Conclusions

Fermentation caused total reduction of raffinose family oligosaccharides and partial reduction of phytate, but the nutritional value of lupine seeds generally was not significantly improved. An increase in the content of structural carbohydrates and similar level of alkaloids as in raw seeds was found. Fermentation of lupine seeds increased apparent total tract digestibility of protein in rats. Raw and fermented lupine seeds had an impact on gastrointestinal processes and microflora, but no improvements were observed.

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