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INFLUENCE OF PEA AND LUPIN OLIGOSACCHARIDES ON CAECAL SHORT-CHAIN FATTY ACIDS PRODUCTION AND NITROGEN EXCRETION PATTERNS IN RATS

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The impact of casein diet supplementation with oligosaccharides extracted from lupin and pea seeds on caecal short-chain fatty acids production and nitrogen balance was investigated in Wistar rats. The experimental diets were supplemented with 8% extract from lupin or pea seeds and contained 3.9 or 4.9% of α-galactosides, respectively. The control diet was supplemented with 5% of cellulose. The addition of lupin and pea oligosaccharides increased SCFA production in the caecum, compared to the cellulose control group. The pea extract stimulated acetate and propionate production, whereas the lupin preparation caused the highest caecal butyric acid pool. Amounts of SCFA produced per gram of ingested pea and lupin oligosaccharides were similar. The administration of lupin and pea preparations was accompanied by an increase in ammonia and protein contents in caecal digesta. Feeding oligosaccharides caused higher nitrogen excretion in faeces but had no considerable effect on nitrogen losses in urea nor N retention as compared to the control group.

INTRODUCTION

The diets containing oligosaccharides are known to induce significant changes in the metabolism of the gut [Bielecka *et al.*, 2002]. The oligosaccharides not digested in the upper parts of the gastrointestinal tract (GI) reach the large bowel where they induce changes in the metabolism of microflora, *e.g.* they stimulate the production of short-chain fatty acids [Topping & Clifton, 2001]. Changes in the intestinal microflora may affect the normal digestive process, altering the absorption of nutrients, *e.g.* inducing changes in general nitrogen metabolism [Younes *et al.*, 1995a, b]. The most extensively studied oligosaccharides are fructooligosaccharides whose dietary effects are well known [Cummings *et al.*, 2001]; yet information concerning the *in vivo* effects of oligosaccharides of grain legumes is insufficient. Preparations of α-galactosyl derivatives of sucrose are widely produced by extraction from plant sources, particularly soybean, also lupin and pea [Gulewicz *et al.*, 2000]. α-Galactosides are known, in part, to be responsible for flatulence problems [Saini & Gladstones, 1986] and a lower nutritional value of a diet [Zduńczyk *et al.*, 1998]. However, in recent years, these carbohydrates have been an object of interest due to their potential beneficial influence on the large intestine's ecosystem [Guillon & Champ, 2002]. The purpose of this study was to evaluate the potential influence of α-galactosides obtained from pea and lupin seeds on caecal fermentation (especially its main product – SCFA) and nitrogen utilization in rats.

MATERIAL AND METHODS

α-Galactosides were extracted from *L. angustifolius* L. cv. Mirela and *Pisum sativum* L. cv. Opal seeds as described by Gulewicz *et al.* [2000]. The composition of the preparations was determined with the HPLC method and chromatographic peaks were identified on the basis of comparison with retention times of a mixture of standards. After purification, the dry carbohydrate fractions contained sucrose and α-galactosides in the amount of 24.5% and 61.0% in a pea preparation, and 35.0% and 48.5% in a lupin preparation, respectively. The composition of the oligosaccharide fraction in both preparations is presented in Table 1.

The experiment was conducted on Wistar rats aged 40–45 days and weighing 107±3 g at the beginning of the test. The control group was fed with the casein diet containing 5% of cellulose, and experimental groups were fed with the diets in which cellulose and part of corn starch were replaced with lupin or pea extract. The diets contained

TABLE 1. The composition of α-galactosides fraction in legume preparation (%).

α-Galactoside	Lupin preparation	Pea preparation
Raffinose	6.4	12.7
Stachyose	67.4	67.2
Verbascose	26.2	20.1
Total	100	100

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135 g/kg crude protein (casein supplemented with DL-methionine) and standard amount of a mineral mix (according to AIN-93G Mineral Mix) and vitamin mixtures (according to AIN-93G Vitamin Mix). The content of energy from dietary components (12% protein, 48% carbohydrates, 40% fat) was similar to that of the Western-type diet [Dabai *et al.*, 1996].

The composition of experimental and control diets is presented in Table 2.

TABLE 2. Composition of diets (%).

	Diet		
	Control	Lupin extract	Pea extract
Component			
Casein	14.8	14.8	14.8
DL-methionine	0.2	0.2	0.2
Soybean oil	10	10	10
Mineral-mix[1]	3	3	3
Vitamin-mix[2]	2	2	2
Corn starch	65	62	62
Cellulose	5	-	-
Legume extract	-	8	8
Ingredients from legume seeds			
Protein	-	0.67	0.60
Ash	-	0.23	0.20
Sucrose	-	2.80	1.96
Total α-galactosides	-	3.89	4.87
Others	-	0.41	0.37

[1]AIN-93G-MX according to Reeves [1997], per kg mix: 357 g calcium carbonate anhydrous (40.04% Ca), 196 g potassium phosphate monobasic (22.76% P, 28.73% K), 70.78 g potassium citrate, tripotassium monohydrate (36.16% K), 74 g sodium chloride (39.34% Na, 60.66% Cl), 46.6 g potassium sulfate (44.87% K, 18.39% S), 24 g magnesium oxide (60.32% Mg), 6.06 g ferric citrate (16.5% Fe), 1.65 g zinc carbonate (52.14% Zn), 1.45 g sodium meta-silicate × $9H_2O$ (9.88% Si), 0.63 g manganous carbonate (47.79% Mn), 0.3 g cupric carbonate (57.47% Cu), 0.275 g chromium potassium sulfate × $12H_2O$ (10.42% Cr), 81.5 mg boric acid (17.5% B), 63.5 mg sodium fluoride (45.24% F), 31.8 mg nickel carbonate (45% Ni), 17.4 mg lithium chloride (16.38% Li), 10.25 mg sodium selenate anhydrous (41.79% Se), 10 mg potassium iodate (59.3% I), 7.95 mg ammonium paramolybdate × $4H_2O$ (54.34% Mo), 6.6 mg ammonium vanadate (43.55% V), 221.026 g powdered sucrose.

[2]AIN-93G-VM according to Reeves [1997], g/kg mix: 3.0 nicotinic acid, 1.6 Ca pantothenate, 0.7 pyridoxine-HCl, 0.6 thiamin-HCl, 0.6 riboflavin, 0.2 folic acid, 0.02 biotin, 2.5 vitamin B-12 (cyanocobalamin, 0.1% in mannitol), 15.0 vitamin E (all-rac-α-tocopheryl acetate, 500 IU/g), 0.8 vitamin A (all-trans-retinyl palmitate, 500000 IU/g), 0.25 vitamin D-3 (cholecalciferol, 400000 IU/g), 0.075 vitamin K-1 (phylloquinone), 974.655 powdered sucrose.

Experimental and control groups consisted of eight male rats housed individually in metabolic cages. The selection of the animals and their maintenance over the two-week experiment followed common regulations. The environment was controlled with a 12-h light-dark cycle, a temperature of 21±1°C, relative humidity of 50±5% and 20 air changes/h. Animals had free access to water and diets.

During the first part of experimental feeding, digestibility and balance of nitrogen were determined. The initial 5-day preliminary period was aimed at adapting the gut microflora to the carbohydrates, followed by a 5-day experimental period when faeces and urine were collected once daily. Nitrogen in faeces and urine was determined for each rat according to Kjedhal's method. Apparent nitrogen digestibility, the biological value of dietary protein and nitrogen retention were used as criteria of nutritional quality of diets.

At the termination of the experiment, the rats were anaesthetised using sodium pentobarbitone according to the recommendation for euthanasia of experimental animals [Close *et al.*, 1997]. After laparotomy, the caecum with contents was removed and weighed. Samples of fresh digesta were used for immediate analysis of protein, ammonia and short-chain fatty acids. In fresh caecal digesta ammonia extracted and trapped in a solution of boric acid in the Conway's dishes was determined by direct titration with sulphuric acid [Hofirek & Haas, 2001]. The caecal digesta was centrifuged at 10000 × g for 5 min, and protein content of the supernatant was determined by the method of Lowry *et al.* [1951] using BSA (bovine serum albumin) as a standard.

The caecal digesta was analysed for SCFA concentration with the gas chromatography (Shimadzu GC-14A with a glass column 2.5 m × 2.6 mm, containing 10% SP-1200/1% H_3PO_4 on 80/100 Chromosorb W AW, column temperature 110°C, detector FID temperature 180°C, injector temperature 195°C). The caecal digesta was weighed (sample of *ca.* 0.2 g), mixed with 0.2 mL of formic acid, diluted with deionised water and centrifuged at 10 000×g for 5 min. Supernatant was decanted for injection into the gas chromatograph. Caecal SCFA pool size was calculated as the product of SCFA concentration in digesta and caecal digesta mass. The protein and ammonia pools were calculated in the same manner.

The results are presented as mean values and (pooled) standard errors of the mean (SEM). The results were analysed using the Shapiro-Wilk normality test and one-way ANOVA. Significant differences between the groups were determined with Duncan's multiple range test and considered significant at $p=0.05$.

RESULTS

Different composition of diets did not affect diet intake, whereas feed and protein utilization were significantly improved by oligosaccharide supplementation (Table 3). The weight of caecal digesta was significantly higher in the lupin preparation group than in the pea extract and the control groups. Compared to the control rats fed a cellulose-

TABLE 3. Daily diet intake, daily body weight gain and indices of feed and protein utilization.

	Control	Lupin extract	Pea extract	SEM[1]
Daily diet intake (g)	17.51	17.83	17.63	0.35
Daily body weight gain (g)	3.90[b]	4.34[a]	4.35[a]	0.02
Feed conversion (g/g)	4.49[a]	4.11[b]	4.05[b]	0.02
Protein efficiency ratio (g/g)	1.70[b]	1.77[a]	1.78[a]	0.01

[1]SEM – standard errors of the means (standard deviation for all rats divided by square root of rat number, n=24); a, b – values within each row with the same superscript are not different at $p \leq 0.05$

-containing diet, the dietary α-galactoside preparations were characterised with higher pools of ammonia and Lowry's protein, especially in the case of the lupin seeds extract (Table 4). Diet supplementation with the pea preparation significantly increased the total SCFA and individual acids, except for butyrate content in the caecum of animals, compared to the cellulose-fed rats. In the case of the lupin seed extract, those differences were significant for propionate, butyrate and iso-acids. The pea preparation caused significantly higher caecal contents of acetate, propionate, valerate, iso-valerate as well as significantly lower ones of butyrate, iso-butyrate, compared to the lupin preparation. Both extracts caused comparable production of short-chain fatty acids per gram of consumed oligosaccharides. When C2:C3:C4 profile is considered, the fermentation of the pea preparation produced a higher proportion of acetate than the lupin diet and a lower proportion of butyrate compared to the control and lupin groups.

TABLE 4. Caecal parameters in rats fed with experimental diets.

	Control	Lupin extract	Pea extract	SEM
Caecal digesta (g/100 g BW)	1.11^{b}	1.38^{a}	1.20^{b}	0.04
Ammonia (mg/100g BW)	0.266^{c}	0.704^{a}	0.552^{b}	0.04
Lowry's protein (mg/100g BW)	0.144^{c}	0.331^{a}	0.228^{b}	0.03
Total SCFA (μmol/100 g BW)	125.0^{b}	136.0^{ab}	155.0^{a}	5.22
Acetate (μmol/100 g BW)	73.55^{b}	74.53^{b}	91.34^{a}	3.88
Propionate (μmol/100 g BW)	24.41^{c}	30.24^{b}	34.60^{a}	1.12
Iso-butyrate (μmol/100 g BW)	2.90^{c}	4.66^{a}	3.88^{b}	0.11
Butyrate (μmol/100 g BW)	17.03^{b}	18.74^{a}	15.14^{c}	0.89
Iso-valerate (μmol/100 g BW)	2.99^{c}	3.67^{b}	4.82^{a}	0.15
Valerate (μmol/100 g BW)	4.10^{b}	4.15^{b}	5.21^{a}	0.18
Total SCFA (μmol/g NDC[1])	142.0^{b}	197.1^{a}	180.2^{a}	6.04
C_2 :C_3:C_4 profile[2]:				
C_2	59	55	59	0.85
C_3	20	22	22	0.42
C_4	14	14	10	0.25

[1]non-digestible carbohydrates; [2] μmol/100 μmol total SCFA; a, b, c – values within each row with the same superscript are not different at $p \leq 0.05$

TABLE 5. Nitrogen balance in rats.

	Control	Lupin extract	Pea extract	SEM
N intake (mg/5 days)	1839^{b}	1974^{a}	1960^{a}	15.59
N excretion (mg/5 days)				
in faeces	201.8^{c}	258.8^{a}	228.7^{b}	1.98
in urine	756	807	798	5.94
N apparent digestibility[1] (%)	89.0^{a}	86.9^{c}	88.3^{b}	0.26
N retention[2] (%)	47.9	46.0	47.6	0.89
BV[3] (%)	66.0	65.2	65.2	1.24

[1]Apparent digestibility: [N intake – N faecal / N intake]×100; [2]Retention: [N intake – N faecal – N urinary / N intake] × 100; [3]Biological value: [N intake – (N faecal – N metabolic) – (N urinary – N endogenous)] × 100 / [N intake – (N faecal – N metabolic)];
a, b, c – values within each row with the same superscript are not different at $p \leq 0.05$

The nitrogen balance for rats fed casein diets supplemented with cellulose or α-galactosides preparations obtained from lupin and pea seeds is presented in Table 5. In rats, nitrogen loss was significantly affected when lupin and pea oligosaccharide mixtures were added to the diet. The rats given diets supplemented with α-galactosides excreted more nitrogen in faeces than those fed with cellulose. Furthermore, the faecal nitrogen output in rats fed with the lupin preparation was significantly greater compared to the pea group and consequently the lowest nitrogen digestibility was recorded in the lupin extract group. The effect of experimental treatments on urinary nitrogen excretions was not significant. As a result, nitrogen retention was not influenced by the diet.

DISCUSSION

In our study, the choice of legume seed oligosaccharides was motivated by their increasing occurrence in a diet according to the current dietary guidelines recommending an increased fibre intake. The control diet was supplemented with cellulose, which is more resistant towards the microbial flora than oligosaccharides from raffinose family which are more fermentable types of fibre. Feeding rats for 14 days with both legume seed preparations did not influence the feed intake, but improved its conversion, and as a consequence the daily body weight gain in rats fed with the oligosaccharide-supplemented diet was higher than in the control group. In our earlier study, a lower level (2.92%) of white lupin oligosaccharides in a diet did not affect the feed intake nor the final body weight of rats [Zduńczyk *et al.*, 1998]. Some authors observed a compensatory increase in the intake of a diet, the nutritive value of which decreased upon the addition of dietary fibre [Lopez-Guisa *et al.*, 1988]. Such effects were not noticed in our study, when a diet was supplemented with 5% cellulose or fructans with different degree of polymerization (oligofructose, inulin) and compared with a non-DF diet [Juśkiewicz *et al.*, 2005].

The main end-products formed during the fermentation of non-digestible carbohydrates are short-chain fatty acids (SCFA), mainly acetic, propionic and butyric acid [Topping & Clifton, 2001]. Some researchers have suggested that values for the caecal SCFA pool appear to be a more accurate reflection of caecal fermentation than SCFA concentrations when feeding rats with oligosaccharides of various fermentabilities [Berggren *et al.*, 1993]. Other researchers have also found that different amounts of the same oligosaccharide entering to the diet for rats correlated better with caecal pool than with caecal concentration of SCFA [Remesy *et al.*, 1992]. In our earlier studies [Juśkiewicz *et al.*, 2005; Zduńczyk *et al.*, 2004], we found that 4–5% dietary inulin did not increase whereas 8% dietary inulin significantly decreased the caecal concentration of total short-chain fatty acids, when compared to a sucrose diet. On the other hand, there were considerably greater SCFA pools caused by dietary inulin. Therefore, the calculated SCFA pool produced in the caecum provides more precise information on the effect of the preparations applied on the intensity of fermentation in the gastrointestinal tract.

In the present study, the dietary addition of both

oligosaccharide preparations increased caecal short-chain fatty acids production, however to a different extent. The pea extract caused greater acetate and propionate pools, whereas the lupin preparation resulted in a higher content of caecal butyrate. On the other hand, both lupin and pea preparations gave similar amount of total SCFA per gram of α-galactosides consumed. When C_2:C_3:C_4 profile is considered, lupin extract fermentation produced a higher proportion of butyrate and lower proportion of acetate compared to the pea group. In our recent study on rats [Juśkiewicz *et al.*, 2005], the supplementation of a diet with long-chain inulin or short-chain oligofructose did not change butyrate proportion, but caused a higher proportion of propionate and a lower proportion of acetate, when compared to the cellulose-containing diet. The butyrate is recently the most interesting acid among SCFAs. Butyric acid appears to be essential in the maintenance of a healthy caecum and colon, it is the preferred energy substrate for the mucosa cells and has been suggested to protect against hindgut disease, *e.g.* ulcerative colitis and cancer [Lupton, 2000]. Compared to the control rats fed a diet with cellulose, both legume preparations caused the enhancement of caecal protein and ammonia pool, and that process was stronger for the lupin extract which, however, contained less α-galactosides than the pea preparation. This indicated some differences between physiological responses of GI to diet supplementation with lupin and pea preparations. A higher Lowry's protein pool in rats fed the lupin seed extract was a consequence of a greater bulk effect and an increased bacterial density in the caecum. One of the side effects of the proliferation of bacteria, which is a prerequisite for SCFA production, is the conversion of nitrogen as bacterial protein. The main source of this nitrogen is ammonia derived from urea [Topping, 1996]. That process leads not only to lowering blood urea concentration but also to a temporary raising of intracaecal ammonia concentration, and consequently to an increase in ammonia pool observed in rats fed with oligosaccharides in our study. Moreover, the legume α-galactosides are probably responsible for the inhibition of protein digestion in the upper part of the gastrointestinal tract of monogastric animals [Gdala *et al.*, 1997]. As a result, a part of dietary protein could escape digestion in the small intestine and was metabolised by microflora, thus increasing ammonia content of the caecum.

The studies on rats conducted by Pastuszewska *et al.* [2000] showed that nitrogen excretion patterns are strongly influenced by different types of dietary carbohydrates. The microbial degradation of complex carbohydrates that occurs in the large intestine during bacterial fermentation could lead to considerable changes in the caecal/colonic N metabolism. The faecal N excreted is derived from incomplete digestion of bacterial, dietary protein, secreted digestive enzymes, and sloughed mucosal cells [Eggum, 1992]. In our study, the addition of α-galactosides to a basal diet for 10 days increased the flow of substrate to the caecum, and to a certain extent stimulated bacterial growth and increased N incorporation into the bacterial cell walls. Compared to cellulose, which is a poorly fermentable carbohydrate, the higher fermentable α-galactosides led to an increase in bacterial growth resulting in a nitrogen enrichment of faecal matter. In an experiment with rats [Younes *et al.*, 1995b], it was recorded that more fermentable oligosaccharides reduced N excretion in urine, which was linked to the fact that, under such conditions, blood urea constitutes the most readily available source of N for bacterial protein synthesis in the caecum [Viallard, 1984]. There are still disagreements between researchers as far as the nutritional effect of different carbohydrates is concerned. In our experiment, it was demonstrated that α-galactosides obtained from lupin and pea seeds were not able to change the N retention index when compared to cellulose. A similar effect, but with rats receiving resistant starch for 10 days as well as 1 month, was recorded in an experiment of Brunsgaard *et al.* [1995], where it was concluded that resistant starch in the diet increased faecal nitrogen excretion without affecting urinary nitrogen excretion. The same effects were observed in experiments with humans [Cummings, 1996]. On the other hand, Younes *et al.* [1995a] observed a shift of nitrogen excretion from the urine to the faeces following 17-day RS intake.

CONCLUSIONS

The α-galactoside preparations obtained from lupin and pea seeds, and incorporated into the casein diet at a dose of 3.9 and 4.9%, respectively, did not affect diet intake, whereas feed and protein utilization were significantly improved by experimental treatments. It was in contradiction with usual changes associated with legume intake. Both preparations increased caecal SCFAs content, however the pea extract containing more α-galactosides resulted in a greater production of short-chain fatty acids. On the other hand, the lupin preparation caused higher butyrate pool than the pea extract. Compared to cellulose, the preparations containing α-galactosides increased N output in faeces, without changes in N urinary patterns. The observations made strengthen the case for wider utilization of the non-digestible carbohydrate fraction from legume seeds.

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WPŁYW OLIGOSACHARYDÓW GROCHU I ŁUBINU NA PRODUKCJĘ LOTNYCH KWASÓW TŁUSZCZOWYCH W JELICIE ŚLEPYM ORAZ DROGI WYDALANIA AZOTU U SZCZURÓW

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W doświadczeniu na szczurach rasy Wistar zbadano wpływ dodatku do diet kazeinowych oligosacharydów ekstrahowanych z nasion łubinu i grochu na produkcję lotnych kwasów tłuszczowych (LKT) w jelicie ślepym oraz drogi wydalania azotu. Dodatek ekstraktu do diet wynosił 8%, z czego dodatek α-galaktozydów z nasion łubinu i grochu wyniósł, odpowiednio 3.9 i 4.9%. Dieta kontrolna zawierała 5% celulozy (tab. 2). Dodatek do diety oligosacharydów łubinu i grochu spowodował wzrost produkcji LKT w jelicie ślepym w porównaniu do grupy kontrolnej (tab. 4). Ekstrakt z nasion grochu stymulował produkcję kwasów octowego i propionowego, natomiast ekstrakt łubinowy kwasu masłowego. Ilość lotnych kwasów tłuszczowych produkowanych w przeliczeniu na 1 gram spożytych oligosacharydów była podobna dla grochu i dla łubinu. Podawaniu w diecie preparatów z nasion łubinu i grochu towarzyszył wzrost ilości amoniaku i białka w treści jelitowej (tab. 4). Dodatek oligosacharydów do diety spowodował większe wydalanie azotu z kałem, natomiast nie miał istotnego wpływu na straty azotu w moczu oraz retencję azotu w organizmie, w porównaniu do grupy kontrolnej (tab. 5).