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EFFECTS OF PARTIAL REPLACEMENT OF SOYBEAN MEAL WITH RAPESEED MEAL, NARROW-LEAVED LUPIN, DDGS, AND PROBIOTIC SUPPLEMENTATION, ON PERFORMANCE AND GUT MICROBIOTA ACTIVITY AND DIVERSITY IN BROILERS*

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Abstract

This study examines the impact of soybean meal (SBM) substitutes, including solvent-extracted 00 rapeseed meal (RSM), narrow-leaved lupin (LUPIN), and distillers dried grains with solubles (DDGS) (each used at a ratio of 250 g/kg^{-1} in the diet), as well as administered probiotic (*L. casei, L. plantarum*, *Rhodopseudomonas palustris*, *S. cerevisiae*), on gut microbiota activity, diversity and performance. The experimental treatments were arranged in a 4 × 2 factorial design, with the factors being protein source in the diets (SBM only, RSM, LUPIN or DDGS) given from 8 to 35 days of age, and with or without a probiotic preparation administered in drinking water during the entire rearing period. The performance declined in birds fed with SBM substitutes (P≤0.01). The RSM diet decreased concentration of short chain fatty acids (SCFAs) (P<0.01) in ileal and caecal digesta as well as decreased bacterial enzymes activity in the caeca. The LUPIN diet increased viscosity and decreased SCFAs concentration in ileum, while the DDGS diet increased butyrate concentration in caeca. SBM substitutes and probiotic were involved in changing the *Clostridiales* and *Lactobacillales* diversity in the ileal and caecal digesta. Probiotic administration did not affect performance, but it did alleviate some negative effects of SBM substitutes on microbiota activity and diversity.

Key words*:* narrow-leaved lupin, rapeseed meal, DDGS, probiotic, gut microbiota, broiler chickens

Solvent-extracted rapeseed meal (RSM), byproducts of maize-to-ethanol fermentation (distillers dried grains with solubles, DDGS) and low-alkaloid lupins can be

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used as alternative protein sources to replace soybean meal (SBM) in poultry diets. The carbohydrate fractions of these alternative protein sources differ in chemical composition (mainly nonstarch polysaccharides and oligosaccharides, and resistant starch in DDGS) and in physicochemical properties (solubility, viscosity). They are not digested with the aid of enzymes secreted in the upper part of the gastrointestinal tract (GIT), and instead they are fermented and serve as substrates for growth of microbiota in the distal parts of the broiler gut (Bjerrum et al., 2006; Sergeant et al., 2014). The mechanisms regulating the equilibrium of broilers' intestinal microbiota after ingestion of different plant protein feeds are still poorly understood (Pan and Yu, 2014). Rubio et al. (1998) reported that broilers fed diets with *Lupinus angustifolius* had increased *Lactobacilli* counts but unaltered *E. coli* counts in the ileum and caecum compared with broilers fed a SBM-based diet. Loar II et al. (2012) showed that high levels of maize-DDGS decreased *E. coli* counts but had no effect on *L. monocytogenes* counts in the ileum and caecum. The function of ileal and caecal microbial fermentation products in energy utilization, mineral absorption, epithelial growth stimulation and inhibition of pathogens is a subject of extensive research (Józefiak et al., 2004; Pan and Yu, 2014; Konieczka et al., 2018; Yadav and Jha, 2019). The metabolic capacity of the microbiota is extremely diverse and can positively or negatively affect the health of the host. The main end products of bacterial fermentation are lactate, short chain fatty acids (SCFAs; acetate, propionate, butyrate, isobutyrate, valerate, isovalerate), and gases (H_2, CO_2) (Jørgensen et al., 1996; Józefiak et al., 2004; Sergeant et al., 2014). In addition to being an important energy source for the intestinal epithelium, SCFAs stimulate enterocyte growth and proliferation, and regulate mucin production and intestinal immune responses (Van der Wielen et al., 2000; Pan and Yu, 2014). Within the commensal intestinal microbiota, species with the potential to improve poultry performance are particularly important, as they are also involved in cross-talk between the microbiota, gut epithelium and immune system, providing resistance to enteric pathogens. These species can be enriched in the broiler gastrointestinal tract by providing specific probiotic microorganisms (Yadav and Jha, 2019; Pan and Yu, 2014). No significant differences have been found between effects of various methods of probiotic delivery – via feed, drinking water, litter or oral gavage – on the broiler gut microbiota (Olnood et al., 2015). Probiotic species contribute to an increase in the activity of many bacterial glycolytic enzymes, such as α-galactosidase, which hydrolyses dietary α-galactosides (RFO and other oligosaccharides); β-galactosidase, which contributes to the hydrolysis of β-galactosides; and α- and β-glucosidase, which contribute to the hydrolysis of nonstarch polysaccharides (cellulose, β-glucans). The enhanced activity of some bacterial enzymes, particularly β-glucosidase and β-glucuronidase, may be detrimental to the birds (Jin et al., 2000; Pool-Zobel et al., 2002). In the literature, information on the interactions between SBM substitutes and probiotic relating to the composition and activity of the intestinal microbiota is limited (Pan and Yu, 2014; Yadav and Jha, 2019). Moreover, most of the data on the activity of different probiotics are obtained using diets not containing coccidiostats. The ionophore coccidiostats used in commercial poultry production reduce the counts and activity of some microbiota members, including *Clostridium perfringens* and *Lactobacillus* (Bjerrum,

2006; Czerwiński et al., 2012), but they are not added to the diets fed in the final 3–5 days before slaughter.

The aim of this study was to examine the effect of partially replacing SBM in broiler diets (with RSM, narrow-leaved lupin or DDGS) on the activity and diversity of the ileal and caecal microbiota and growth performance parameters; further we aimed to assess the effects of free access to drinking water with or without a multimicrobial probiotic preparation.

Material and methods

All procedures involving animals were approved by the III Local Animal Care and Use Committee in Warsaw, Warsaw University of Life Sciences, Poland (approval no. 21/2012) and were performed in accordance with the principles of the European Union and Polish Law on Animal Protection.

Materials

Narrow-leaved lupin seeds (*L. angustifolius* cv. Kadryl, from harvest year 2012) and other components of diets were of commercial grade. Liquid multimicrobial (MM) probiotic preparation (EM Probiotic™) contained *Lactobacillus casei* and *Lactobacillus plantarum,* both at 5×10^6 CFU/ml, *Saccharomyces cerevisiae* at 5×10^3 CFU/ml and *Rhodopseudomonas palustris* according to producer declaration (Greenland Technology EM, Janowiec, Poland); the recommended dose was 3 ml MM probiotic per L of drinking water. The water containing the MM probiotic was prepared daily and provided *ad libitum*.

Birds and housing

A total of 192 one-day-old Ross 308 broiler females were placed in battery cages and randomly allocated to one of two treatments. All birds received the same starter diet, but half the birds were given tap water and the other half were given water containing MM probiotic. On day 8, birds were deprived of feed for 4 h, then individually weighed and transferred to the battery of individual wire-mesh cages (length 36 cm × width 36 cm × height 52 cm); each bird was treated as a replication. The chickens drinking water without and with MM probiotic were randomly allocated to 4 dietary treatments (19 birds each, all with a body weight close to the group average of 159 g). Throughout the growth period, trial birds were kept at a room temperature of 22°C and on a light/dark cycle of 18 h/6 h. Birds had free access to feed and drinking water (without or with MM probiotic) supplied in troughs placed outside each cage.

Experimental design and diets

The experimental design comprised two factors: type of main protein sources in the diet and absence or presence of MM probiotic administration. Four isoprotein diets were prepared (Table 1). Diets were formulated to provide nutrients according to

the requirements and to contain the same amount of lysine, sulfur amino acids, threonine and crude fat. All diets were supplemented with Avizyme 1505X preparation, providing, per kg diet: endo-1.4-β-xylanase (EC 3.2.1.8), 2760 U; subtilisin (EC 3.4.21.62), 4800 U; and α-amylase (EC 3.2.1.1), 480 U, according to the producer's declaration (Danisco Animal Nutrition). The diets were cold pelleted with a CL-2 CPM laboratory pellet mill (California Pellet Mill Co., Crawfordsville, IN, USA).

Table 1. Ingredient composition and calculated chemical composition of experimental diets, g/kg air-dry matter

Item	Grower				Finisher			
	SBM	RSM	LUPIN	DDGS	SBM	RSM	LUPIN	DDGS
Ingredients								
wheat	374.6	302.2	260.0	271.9	427.5	355.0	313.6	324.7
maize	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
soybean meal	348.2	181.7	221.2	227.2	299.9	133.3	172.1	178.8
rapeseed meal	–	250.0	–	–	–	250.0	–	–
narrow-leaved lupin	–	–	250.0	–	–	–	250.0	–
DDGS	–	–	–	250.0	–	–	–	250.0
rapeseed oil	40.8	34.5	31.7	13.7	40.6	34.3	31.8	13.6
NaCl	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
$NaHCO_3$	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
limestone	11.1	9.0	10.3	12.6	13.0	10.7	12.1	14.4
monocalcium phosphate	13.1	12.0	13.5	10.5	11.1	10.2	11.6	8.7
L-lys HCl (78%)	1.5	1.8	2.2	1.4	0.5	0.6	1.0	2.8
DL-met (99%)	1.8	0.5	2.2	3.8	1.3	–	1.7	0.9
L-thr (99%)	0.6	–	0.6	0.6	0.2	–	0.2	0.2
vitamin-mineral mix[1,2]	5.3[1]	5.3[1]	5.3[1]	5.3[1]	2.9[2]	2.9[2]	2.9[2]	2.9[2]
Calculated[3]								
ME (MJ/kg)	12.66	11.65	11.33	11.74	12.80	11.83	11.54	11.87
crude protein (N × 6.25)	215	215	215	215	200	200	200	200
lysine (total)	12.0	12.0	12.0	12.0	10.0	10.0	10.0	10.0
Met + Cys (total)	8.6	8.6	8.6	8.6	7.7	7.7	7.7	7.7
threonine (total)	8.0	8.0	8.0	8.0	7.0	7.0	7.0	7.0
crude fat	60.6	60.6	60.6	60.6	60.6	60.6	60.6	60.6

[1,2] Provided per kg of complete diet: [1]vit. A 13500 IU; vit. D_3 3600 IU; (mg) vit. E 80; vit. B_1 3.25; vit. B_2 7.5; biotin 0.2; vit. B_6 5; vit. B_{12} 0.0325; vit. K_3 3; niacin 45; folic acid 1.5; Ca pantothenate 15; choline chloride 447.6; Mn 100; Zn 75; Se 0.275; Co 0.4; Cu 17.5; Fe 67.5; I 1; Ca 0.95 g; Avizyme 1505X 0.3 g and salinomycin 60 mg.

[2]Vit. A 8250 IU; vit. D_3 2750 IU; (mg) vit. E 67.5; vit. B_1 0.5; vit. B_2 4; biotin 0.1; vit. B_6 2; vit. B_{12} 0.01; vit. K_3 1; niacin 25; folic acid 1; Ca pantothenate 7; choline chloride 250; Mn 45; Zn 80; Se 0.15; Cu 20; Fe 30; I 1; Ca 0.47 g; Avizyme 1505X 0.3 g.

[3]Calculated on the basis of chemical analysis and the tabular data of feed materials (Smulikowska and Rutkowski, 2005).

Experimental and analytical procedures

Each diet was fed to 2 groups of birds. One group drank tap water, the other group was provided with the MM probiotic preparation on every second day. Birds were fed grower diets with coccidiostat from day 8 to day 28 and a finisher diet without coccidiostat (Table 1) from day 29 until the end of the experiment. Feed intake and body weight were measured at weekly intervals after 4 h of feed deprivation; the growth performance was calculated for the period 8–35 days. After completion of the growth experiment, the birds were sacrificed and the ileum and caecum were removed from each. The digesta from each segment were gently squeezed into sterile tubes and pooled by segment (two birds per sample) to provide sufficient material for analyses (n=8).

Samples of approximately 1 g of digesta were packed into sterile Eppendorf tubes and kept frozen at –20°C for Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis and microbial enzymes activity measurements. Approximately 3 g of ileal digesta samples were immediately centrifuged (10 000 × g, 10 min, 4°C), and the viscosity of the supernatant (0.5 ml aliquot) was measured using a Brookfield LVDV II+ digital cone/plate viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA). The viscometer was maintained at 40°C by connecting the sample cup to a water bath. The viscosity units were mPa·s (mPa·s = Cp= 0.01 × dyne·s/cm^2). The digesta samples for SCFA determination were converted to their respective sodium salts by adjusting the pH to 8.2 with a 1 M NaOH solution and stored at –20°C. SCFA analyses were performed according to the procedure described by Barszcz et al. (2011), using isocaproic acid as an internal standard on an HP 5890 Series II gas chromatograph (Hewlett Packard, Waldbronn, Germany) with a flame-ionization detector (FID) and Supelco Nukol fused silica capillary column (30 m × 0.25 mm internal diameter, film 0.25 mm).

The glycolytic activities of bacterial enzymes (α- and β-glucosidase, α- and β-galactosidase, and β-glucuronidase) in the ileal and caecal digesta were measured in terms of the rate of p- or o-nitrophenol release from respective nitrophenylglucosides, according to a previously described technique (Konieczka and Smulikowska 2018). Enzyme activity was expressed as μmol of p- or o-nitrophenol formed per min (U) per g of sample.

Feed materials were analyzed for their chemical composition according to AOAC (2005). The glucosinolate content in the RSM was analyzed according to the ISO 9167-1 (1992) method using an HPLC 1050 series apparatus (Hewlett Packard, San Fernando, CA).

Microbial community analysis

Terminal restriction fragment length polymorphism (T-RFLP) analyses were performed according to a previously described technique (Czerwiński et al., 2010), but the fluorescently labeled terminal restriction fragments (T-RFs) were separated in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Data were analyzed using GeneMapper version 3.5 software and an internal size standard LIZ 1200 (Applied Biosystems, USA). The peaks of the T-RF length polymorphism electropherograms were compared to *in silico* digests of bacterial 16S rRNA gene sequences deposited

in GenBank and/or Ribosomal Database Project (RDP) using the MiCA platform (http://mica.ibest.uidaho.edu/). As a result, the most abundant T-RF fragments were identified.

Statistical analysis

All data had been explored earlier in order to reject any possible outliers (outliers were defined as observations whose distance to the location estimate exceeded three times standard deviation). Differences between treatments were examined with two–way analysis of variance and least significance difference tests with the use of STATGRAPHICS® Centurion XVI software package version 16.1.03 (Statistical Graphic Corporation, 1982–2010). The model included the main effects of protein source in the diet and the absence or the presence of MM probiotic administration. Differences were considered significant at $P<0.05$.

Results

Broilers were fed with the experimental diets from day 8 and growth performance was measured from day 8 to day 35 (Table 3). There was no mortality during that period. The feed intake was similar in all of the treatment groups, but body weight gain (BWG) was lower and feed conversion ratio (FCR) was worse in birds fed with LUPIN, RSM and DDGS diets compared with birds fed SBM ($P\leq0.01$). MM probiotic administered with water did not affect growth performance, and there were no interactions among dietary treatments.

Table 2. Composition of plant protein feeds (g/kg as-fed basis)

Item	DM	Crude protein (CP)	Crude ash (CA)	Crude fat (CF)	Crude fibre	NFE[1]
Soybean meal	882	443	64	14	39	322
Rapeseed meal[2]	894	330	76	39	128	321
Narrow-leaved lupin[3]	904	281	34	49	163	377
DDGS	891	264	49	121	71	386

[1]Nitrogen-free extractives = DM – CP – CA – CF – Crude fibre; [2]Contains 2.7 μmol total glucosinolates/g; [3]cv Kadryl.

The activities of all five bacterial enzymes in the ileal contents (Table 4) were significantly higher in birds fed the RSM diet compared with those of birds fed the other diets ($P<0.001$). The MM probiotic administration significantly reduced α-glucosidase, α-galactosidase and β-glucuronidase activities in the birds fed the RSM diet and enhanced β-galactosidase activity in the group fed the SBM diet (Dt × Pro; $P<0.05$).

The activity of α-glucosidase in the caecal contents (Table 4) was not affected by dietary treatments. The activities of the four remaining enzymes were lowest in birds fed with the RSM diet. The LUPIN and DDGS diets did not affect β-glucuronidase activity but lowered β-glucosidase activity (P<0.001), while β-galactosidase activity was higher in birds fed the LUPIN diet and lower in birds fed the DDGS diet than in birds fed the SBM diet (P<0.001). MM probiotic administration significantly lowered α-glucosidase and β-glucuronidase activities in all groups (P<0.05 and P<0.01), enhanced β-glucosidase activity in the group fed the LUPIN diet and lowered β-galactosidase activity in birds fed the SBM and LUPIN diets (Dt × Pro; P<0.05 and P<0.001, respectively).

Table 3. Main effects of the dietary treatments on the growth performance (8–35 days of age)

Dietary treatments	BWG (kg)	Feed intake (kg)	FCR (kg feed/kg BWG)
Diet (Dt)[1]			
SBM	2.13 B	3.09	1.45 A
RSM	1.95 A	3.08	1.58 C
LUPIN	2.00 A	3.11	1.55 BC
DDGS	1.97 A	3.04	1.54 B
MM probiotic (Pro)[2]			
–	2.03	3.11	1.53
+	2.00	3.06	1.53
SEM	0.013	0.020	0.007
Probability (P-value)			
Dt	0.001	0.680	0.001
Pro	0.118	0.241	0.671
Dt × Pro	0.432	0.683	0.337

[1]As in Table 1; [2] *Lactobacillus casei* and *Lactobacillus plantarum*, 5×10^6 U/ml*; Saccharomyces cerevisiae*, 5×10^3 U/ml and *Rhodopseudomonas palustris*, provided in drinking water (see Material and Methods);

A, B – within columns, for each main effect, means with different letters are significantly different at P<0.01.

The total SCFA concentration in the ileal digesta averaged 5.55 μmol/g in birds fed the RSM and LUPIN diets, but it was, on average, 26% higher in SBM- and DDGS-fed chicks (P<0.001) (Table 5). The proportions of the major SCFA components acetate and propionate were similar in birds fed with SBM, RSM and DDGS diets, but in birds fed the LUPIN diet, the ratio of acetate was lower and that of propionate was higher (P<0.001). MM probiotic administration increased the ratio of isobutyrate (Dt × Pro; P<0.05). The viscosity of the ileal digesta averaged 1.84 mPas·s in birds fed with SBM, RSM and DDGS but was 4.06 mPas·s in birds fed the LUPIN diet (P<0.001). The viscosity of the ileal digesta decreased by 27% (P<0.05) in the group fed the LUPIN diet after MM probiotic administration (Dt × Pro; P<0.05)

Table 4. Main effects of the dietary treatments on the bacterial enzyme activities (U/g)[1] in ileal and caecal digesta

Dietary treatments	Glucosidase		Galactosidase		β-glucuronidase
	α-	β-	α-	β-	
Ileum					
Diet (Dt)[2]					
SBM	1.36 B	1.80 A	1.71 A	2.53 A	2.46 B
RSM	3.14 C	5.20 B	4.82 B	5.10 B	5.15 C
LUPIN	1.25 B	1.88 A	1.76 A	2.08 A	2.29 AB
DDGS	1.03 A	1.90 A	1.69 A	2.38 A	2.11 A
Probiotic (Pro)[3]					
–	1.69	2.83 b	2.55	2.95	3.02
+	1.70	2.56 a	2.44	3.09	2.98
SEM	0.116	0.198	0.180	0.190	0.170
Probability (P-value)					
Dt	0.001	0.001	0.001	0.001	0.001
Pro	0.906	0.030	0.234	0.400	0.707
Dt × Pro	0.013[4]	0.079	0.016[4]	0.017[5]	0.015[4]
Caecum					
Diet (Dt)[2]					
SBM	0.65	13.97 D	1.75 B	6.06 C	4.09 bc
RSM	0.63	2.35 A	0.69 A	1.63 A	3.38 a
LUPIN	0.72	9.72 C	1.09 A	9.61 D	4.24 c
DDGS	0.49	7.54 B	1.81 B	3.78 B	3.56 ab
MM probiotic (Pro)[3]					
–	0.77 b	8.39	1.38	6.27 B	4.19 B
+	0.48 a	8.39	1.29	4.27 A	3.44 A
SEM	0.060	0.607	0.094	0.487	0.132
Probability (P-value)					
Dt	0.526	0.001	0.001	0.001	0.036
Pro	0.019	0.999	0.576	0.001	0.003
Dt × Pro	0.803	0.033[6]	0.264	0.001[7]	0.709

[1] U = μmol of *p-(o-)*nitrophenol formed per min per g of sample; [2]As in Table 1; [3]As in Table 3; [4] MM probiotic significantly lowered activity only in birds fed the RSM diet; [5]MM probiotic significantly enhanced activity only in birds fed the SBM diet; [6] MM probiotic significantly enhanced activity only in birds fed the LUPIN diet; [7]MM probiotic significantly lowered activity in birds fed the SBM and LUPIN diets; a, b, A, B – within columns, for each main effect, means with different letters are significantly different at: a, b – P<0.05; A, B – P<0.01.

The total SCFA concentration in the caecal digesta was lowest in birds fed with RSM, followed by those fed with LUPIN, SBM and DDGS diets (P<0.001, Table 6). The total SCFA concentration increased significantly due to MM probiotic administration in SBM- and LUPIN-fed chicks, but it did not change in birds fed RSM and DDGS diets (Dt × Pro; P<0.05). The major SCFA components were acetic, butyric and propionic acids, followed by putrefactive SCFAs (valeric, isobutyric and isovaleric acids). The acetate ratio was lower (P<0.05) but the butyrate ratio was higher (P<0.001) in birds fed the DDGS diet, compared to the birds of the remaining groups. The MM probiotic administered with water did not significantly affect the SCFA ratios.

Table 5. Main effects of the dietary treatments on the total short-chain fatty acid (SCFA) concentration and viscosity of the ileal digesta

Dietary treatments	Total SCFA (µmol/g)	SCFA (% of total)			Viscosity mPa·s
		C2	C3	C4i	
Diet (Dt)[1]					
SBM	7.01 B	96.1 B	2.36 A	1.90 AB	1.79 A
RSM	5.42 A	95.8 B	2.73 AB	2.58 C	1.83 A
LUPIN	5.68 A	94.1 A	3.61 B	2.24 BC	4.06 B
DDGS	6.98 B	96.4 B	2.36 A	1.84 A	1.89 A
MM probiotic (Pro)[2]					
–	6.22	95.4	2.76	2.15	2.50
+	6.33	95.8	2.77	2.13	2.30
SEM	0.186	0.206	0.156	0.075	0.163
Probability (P-value)					
Dt	0.001	0.001	0.012	0.001	0.001
Pro	0.715	0.259	0.983	0.849	0.351
Dt × Pro	0.062	0.274	0.823	0.043[3]	0.046[4]

[1, 2]As in Tables 1 and 3; [3]Probiotic significantly increased the C4i ratio in birds fed the LUPIN diet; [4]MM probiotic lowered viscosity only in birds fed the LUPIN diet; C2 – acetate, C3 – propionate, C4i – isobutyrate; A, B – within columns, for each main effect, means with different letters are significantly different at P<0.01.

Table 6. Main effects of the dietary treatments on the total short-chain fatty acid (SCFA) concentration in caecal digesta

Dietary treatments	Total SCFA (µmol/g)	SCFA (% of total)					
		C2	C3	C4	C4i	C5	C5i
Diet (Dt)[1]							
SBM	69.4 C	75.9 b	4.89	16.5 A	0.79	1.21 AB	0.73
RSM	49.4 A	75.7 b	4. 82	16.5 A	0.90	1.46 C	0.60
LUPIN	61.4 B	76.2 b	4.30	17.3 A	0.70	0.98 A	0. 53
DDGS	74.5 C	72.6 a	4.98	20.2 B	0.82	1.36 BC	0.76
MM probiotic (Pro)[2]							
–	61.5	75.4	4.65	17.2	0.84	1.22	0.68
+	65.9	74.8	4.84	18.1	0.77	1.28	0.63
SEM	1.80	0.500	0.149	0.456	0.030	0.046	0.038
Probability (P-value)							
Dt	0.001	0.027	0.437	0.004	0.136	0.001	0.133
Pro	0.074	0.536	0.536	0.308	0.263	0.462	0.589
Dt × Pro	0.031[3]	0.474	0.327	0.110	0.698	0.258	0.605

[1,2]As in Tables 1 and 3; [3]MM probiotic significantly increased total SCFA in birds fed the SBM and LUPIN diets; C2 – acetate, C3 – propionate, C4 – butyrate, C4i – isobutyrate, C5 – valerate, C5i – isovalerate.

a, b, A, B – within columns, for each main effect, means with different letters are significantly different at: a, b – P<0.05, A, B – P<0.01.

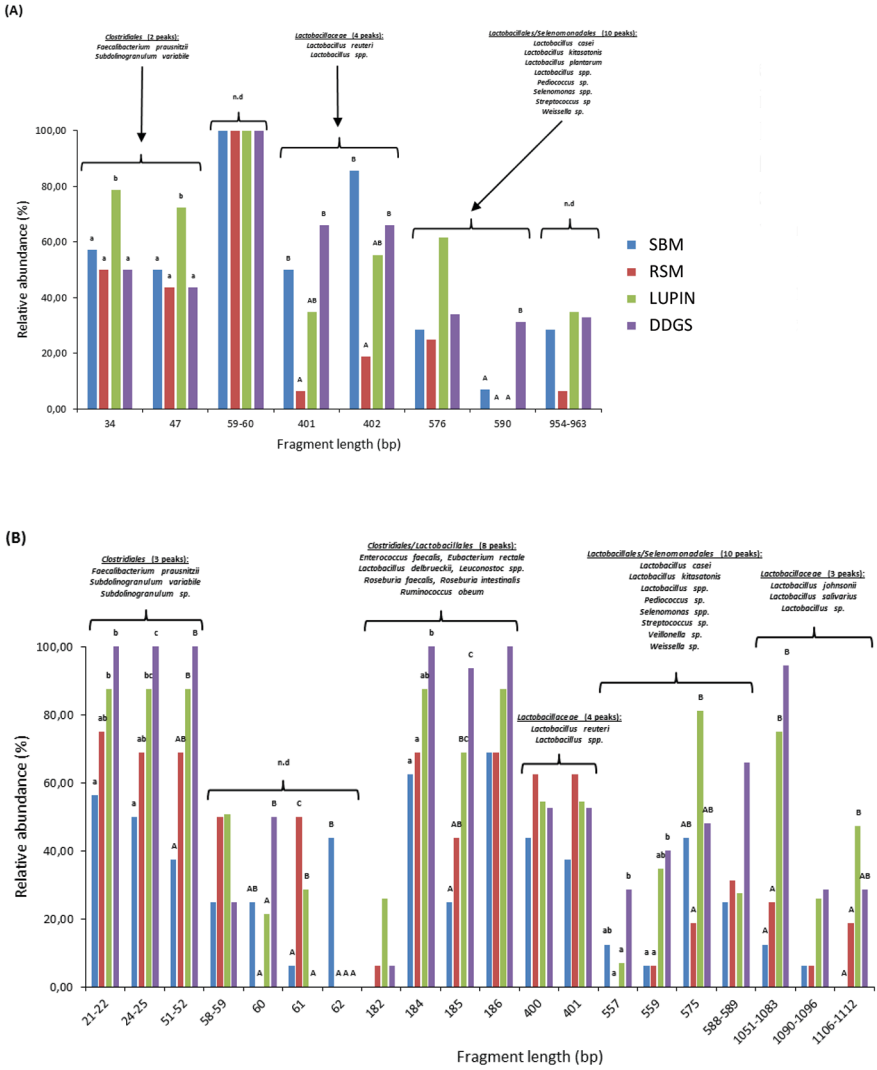

Figure 1. Effects of plant protein feeds on data as measured by T-RFLP analysis using a universal primer set and *Hha*I digest (see the Material and methods). Each bar represents the average value of 16 samples taken from broilers fed soybean meal (SBM), rapeseed meal (RSM), narrow-leaved lupin seeds (LUPIN) or DDGS diets (with and without MM probiotic). Some of the observed fragment lengths could not be identified from the available databases – these are indicated by n.d. The top (A) and bottom (B) panels show the results from ileal and caecal digesta samples, respectively

The T-RFLP fingerprints revealed a higher abundance ($P<0.05$) of several bacterial populations, including the *Clostridiales* order in the ileum and the *Clostridiales* and *Lactobacillales* orders in the caecum, in broilers fed the LUPIN diet in comparison with those fed the SBM diet. In broilers fed the DDGS diet, the abundances of the *Clostridiales*, *Lactobacillales* and *Selenomonadales* orders did not differ in the

ileum but were higher in the caecum (P<0.05) compared with SBM- and RSM-fed birds. The RSM diet reduced the abundance of some peaks representing members of the *Lactobacillales* order in the ileum, but it had no effect on the microbiota composition in the caecum in comparison with birds fed the SBM diet (Figure 1). MM probiotic administration increased the abundance (P<0.01) of bacterial populations, including the *Clostridiales* and *Lactobacillales* orders in the ileum, but it reduced the abundance (P<0.01) of some peaks representing members of the *Lactobacillales* and *Selenomonadales* orders in the caecum in comparison with nonsupplemented birds (Figure 2).

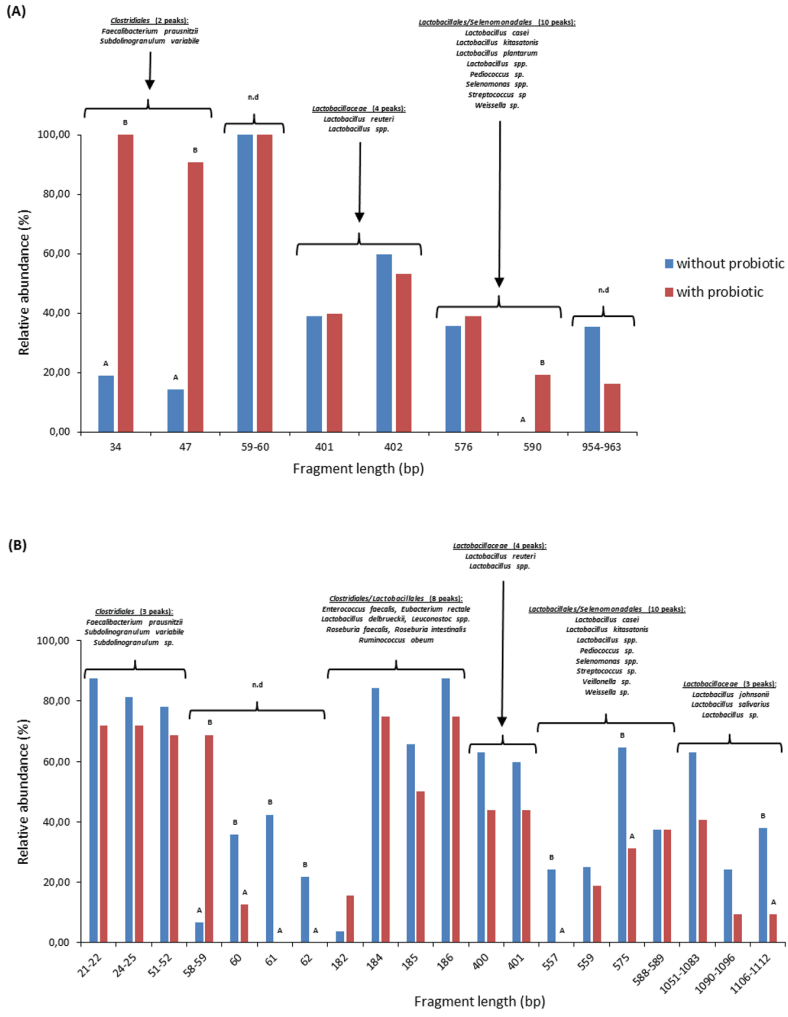

Figure 2. Effects of MM probiotic supplementation on data from T-RFLP analysis using a universal primer set and *Hha*I digest (see the Material and methods). Each bar represents the average value of 32 samples taken from broilers receiving water without or with MM probiotic. Some of the observed fragment lengths could not be identified from the available databases – these are indicated by n.d. The top (A) and bottom (B) panels show the results of ileal and caecal digesta samples of broilers, respectively

Combining the results of the fingerprint data obtained by *Hha*I and *Msp*I digests, or those from additional restriction endonuclease digests, enabled a more precise, yet still tentative, identification of the fragments. In particular, for all of the dietary treatments, the most abundant peaks obtained by use of the universal primers for both digests (for *Msp*I digests, data not shown) perfectly matched the *in silico* digests of several members of the *Clostridiales* order in the ileum (*Faecalibacterium prausnitzii*, *Subdolinogranulum variabile*) and caecum (*Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia faecalis*, *Roseburia intestinalis*, *Ruminococcus obeum*, *Subdolinogranulum variabile*). We also found that some abundant peaks perfectly matched the *in silico* digests of several members of the *Lactobacillales* order in the ileum (*L. casei*, *L. johnsonii*, *L. kitasatonis*, *L. plantarum*, *L. reuteri*, *L. salivarius*, *Pediococcus sp.*, *Streptococcus sp.*, *Weissella sp.*) and caecum (*Enterococcus faecalis*, *L. casei*, *L. delbrueckii*, *L. johnsonii*, *L. kitasatonis*, *L. reuteri*, *L. salivarius*, *Leuconostoc sp.*, *Pediococcus sp*., *Streptococcus sp*., *Weissella sp.*), as well as the *Selenomonadales* order in the ileum (*Selenomonas sp.*) and caecum (*Selenomonas sp*., *Veillonella sp.*). Combining the results of the fingerprints obtained by the *Hha*I and *Msp*I digests, we also found that MM probiotic administration increased the abundance ($P<0.01$) in the ileum and lowered the abundance ($P<0.01$) in the caecum of some peaks that probably represented *L. casei* (Figure 2), but *L. plantarum* was not detected in the caecum in any treatment group (data not shown).

The T-RFLP fingerprints obtained by a primer set that specifically targeted members of the genera *Lactobacillus*, *Enterococcus*, and *Leuconostoc* for *Hha*I and *Mse*I digests provided fingerprint patterns that revealed more differences between the *Lactobacillales* composition of the ileal and caecal populations (data not shown). Several fragments potentially representing the *Lactobacillaceae* family were detected in the ileum (*L. casei*, *L. crispatus*, *L. curvatus*, *L. gallinarum*, *L. johnsonii*, *L. kitasatonis*, *L. panis*, *L. reuteri*, *L. sakei, L. salivarius*, *Pediococcus pentosaceus*) and caecum (*L. casei*, *L. crispatus*, *L. curvatus*, *L. delbrueckii*, *L. johnsonii*, *L. kitasatonis*, *L. reuteri*, *L. sakei*, *L. salivarius*, *Pediococcus pentosaceus*). Several fragments potentially representing the *Enterococcaceae* family were found in the ileum (*E. durans*) and caecum (*E. avium*, *E. casseliflavus*, *E. devriesei*, *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. gilvus*, *E. hermanniensis*, *E. hirae*), in addition to fragments potentially representing the *Leuconostocaceae* family that were found in the ileum (*Weissella cibaria*) and caecum (*Leuconostoc gasicomitatum*, *Weissella cibaria*).

Discussion

This report demonstrated that diets containing RSM, narrow-leaved lupin or DDGS were well utilized by chickens, as the growth performances in the experimental groups were within the limits expected for broiler Ross 308 females of a similar age (BW 2.006 kg, FCR 1.558 kg/kg; Aviagen, 2014). The experimental diets were all formulated to contain the same level of crude fat to avoid a possible interaction between fat level and probiotic activity (Sharifi et al., 2012). Therefore, the

metabolizable energy (AME) values of diets containing alternative protein sources were lower than the AME value of the SBM diet. Lower AME values did not depress feed intake but did result in a lower body weight gain and a lower feed conversion ratio for experimental birds compared with SBM-fed birds. Administration of multi-microbial probiotic in drinking water failed to improve the growth performance and feed efficiency; similar results were reported by Olnood et al. (2015). However, in other studies, improved BWG and FCR were observed in broilers supplemented with *Lactobacillus acidophilus* probiotic (De Cesare et al., 2017) and in broilers provided two multistrain *Lactobacillus* probiotics (Timmerman et al., 2006). The latter authors suggested that when productivity rates of broilers increased, the effects of probiotics on performance decreased, which may be the case in the present study.

The SBM substitutes used in the present study differ with respect to the composition of their carbohydrate fractions, including the values of soluble NSP, starch, polyphenols and α-galactosides (Pustjens et al., 2013; Pedersen et al., 2014; Zduńczyk et al., 2015). In chickens, resistant starch and protein that escape digestion and absorption in the upper part of the gut, as well as soluble NSP and α-galactosides, are responsive to enzymes from resident and exogenous intestinal microbes. The end products of intestinal microbial degradation are an important energy source for the intestinal epithelium, and they also regulate mucin production and intestinal immune responses, which play a key role in the development of microbiota and in the prevention of pathogen colonization (Józefiak et al., 2004; Rehman et al. 2007; Van der Wielen et al., 2000). *In vitro* experiments (MacFarlane and MacFarlane, 2003) have shown that starch and pectin are degraded rapidly by intestinal microbiota and that their main product is acetate. Xylans and arabinogalactans are slowly degraded and give rise to acetate and propionate. Butyrate is produced in substantial amounts only from the degradation of starch, while isobutyrate and isovalerate are formed during the catabolism of branched-chain amino acids.

We found that in RSM- and LUPIN-fed birds, the SCFAs concentrations in ileal digesta were approximately 20% lower than the SCFAs concentrations in DDGS- and SBM-fed chickens, but the values had a negligible effect on performance. As was previously mentioned, in chickens, lactate and SCFAs are primarily utilized as a source of energy for the intestinal epithelium, but the yield of energy for the host is limited. Jørgensen et al. (1996) determined the yield of energy from the NSP fermentation in broilers to be only 3–4% in relation to the metabolizable energy intake. In the current study, MM probiotic administration reduced viscosity of ileal contents in LUPIN-fed birds; this may be considered a positive effect, as high viscosity has an adverse effect on fermentation processes (Rehman et al., 2007) and negatively correlates with the metabolizable energy value of lupin diets (Konieczka and Smulikowska, 2018). The MM probiotic administration also reduced the activity of α-glucosidase, α-galactosidase and β-glucuronidase in birds fed the RSM diet. Lowering the activity of β-glucuronidase may be a particularly important effect, as high activity of β-glucuronidase is indicative of increased *E. coli* and *Clostridium* populations (Beaud et al., 2005). The microbial β-glucuronidase may also release noxious metabolites from nontoxic glycosides (Jin et al., 2000) and may be involved in the formation of toxic aglycones (Pool-Zobel et al., 2002).

In the caecum, digesta are fermented longer and the concentration of SCFAs is higher than it is in the ileum (Van der Wielen et al., 2000; Józefiak et al., 2004; Rehman et al., 2007); our study confirmed this finding. The SCFAs produced in the caecum have an important role in bird health and contribute to epithelial development; in particular, butyrate is the preferred energy source for enterocytes. It has been shown that SCFAs, mainly butyrate, have bacteriostatic effects on some enteric bacteria and may reduce *Enterobacteriaceae* (including *Salmonella)*, but they do not affect beneficial bacteria such as *Lactobacillus* spp. (Rinttilä and Apajalahti, 2013; Van der Wielen et al., 2000). We found that the activity of microbial β-glucosidase decreased after partial substitution of SBM by all alternative protein sources, and that the activity of β-galactosidase was lower in diets with RSM and DDGS. However, it was only in birds fed the RSM diet that the SCFAs concentration dropped to a value 30% lower than that in birds fed with the SBM diet. RSM is rich in polyphenolic compounds (Zduńczyk et al., 2015), which most likely impedes the fermentable substrate flow to the caecum; that impediment may negatively influence the proliferation or the metabolic activity of the gut microbiota (MacFarlane and MacFarlane, 2003). We found that in birds fed the DDGS diet, the ratio of butyrate in the caecum was higher than that in the caecum of SBM-fed birds; butyrate can originate from the fermentation of resistant starch, which is present in DDGS in substantial amounts (Loar II et al., 2012).

MM probiotic increased caecal SCFAs concentrations in birds fed SBM and LUPIN diets, and it reduced the α-glucosidase and β-glucuronidase activities in all groups. Decreased activity of β-glucuronidase may be indicative of decreased *E. coli* and *Clostridium* populations. Previously, De Caesare et al. (2017) reported that *Lactobacillus acidophilus* probiotic enhanced β-glucosidase activity in broilers fed a maize-SBM-based diet.

Bjerrum et al. (2006) observed that microbial diversity and abundance were more evident in the caecum than the ileum of chickens, a finding which matches the present results. The experimental evidence concerning effects of substitution of SBM by the alternative protein sources used in the present experiment is scarce. Previously, Rubio et al. (1998) reported that *Lactobacilli* (*L. fermentum*, *L. acidophilus.*, *L. salivarius*, *L. brevis*) counts increased in the ileum and the caecum of chickens fed a diet with narrow-leaved lupin. Zduńczyk et al. (2014) found that hens fed a diet with narrow-leaved lupin had in their caeca increased counts of *Lactobacillus*, *Enterococcus* and *Bifidobacterium* sp. and decreased counts of *E. coli* and bacteria of the genera *Bacteroides*, *Prevotella* and *Porphyromonas.* We found that substitution of SBM by narrow-leaved lupin and DDGS increased the abundance of several bacterial populations, including *Clostridiales* and *Lactobacillales*, in the ileum and caecum, compared with the bacterial populations in broilers fed the SBM-based diet. The increased ratio of butyrate found in the caecum of birds fed with DDGS indicates that this alternative protein source may have a positive effect on the microbial ecology of the chicken gut.

There is an extensive search for probiotic products that can help establish and maintain the balance of the gut microbiota in commercial broilers. Gao et al. (2017) reported that probiotic feeding accelerated gut microbiota maturation, stimulated

many intestinal *Lactobacillus* sp. and led to an altered bacterial correlation network. De Cesare et al. (2017) reported that *Lactobacillus acidophilus* probiotic positively affected the microbial species producing butyric acid. Olnood et al. (2015) showed that *L. johnsonii* probiotic administration reduced the number of *Enterobacteria* and *C. perfringens* in the ileum and caecum. In the present study, the MM probiotic administration increased the abundance of *Clostridiales* and *Lactobacillales* in the ileum and lowered the abundance of several bacterial populations of *Lactobacillales* and *Selenomonadales* orders in the caecum. The present study also found that MM probiotic administration increased the abundance of some peaks probably representing *L. casei* in the ileum.

It can be concluded that the use of SBM substitutes in broiler diets is unlikely to cause adverse changes in the microbiota composition, but it will modulate the activities of the ileal and caecal microbiota in different ways. With DDGS inclusion, the changes are negligible. RSM enhances the activity of glycolytic bacterial enzymes in the ileum and lowers that activity in the caecum, and narrow-leaved lupin increases the viscosity of the digesta and the activity of some bacterial enzymes. Multimicrobial probiotic administration may partly alleviate the negative effects of RSM and narrow-leaved lupin but has no positive effect on growth performance.

References

AOAC (2005). Official Methods of Analysis (18th ed.). Association of Official Analytical Chemists, Arlington, VA

Aviagen (2014). Ross 308 broiler: performance objectives. Accessed August 2017. http://en.aviagen.com/assets/Tech.Center/Ross.Broiler/Ross-308-Broiler-PO-214-EN.pdf.

Barszcz M., Taciak M., Skomiał J. (2011). A dose-response effects of tannic acid and protein on growth performance, caecal fermentation, colon morphology, and β-glucuronidase activity of rats. J. Anim. Feed Sci., 20: 613–625.

Beaud D., Tailliez P., Anba-Mondoloni J. (2005). Genetic characterization of the beta-glucuronidase enzyme from a human intestinal bacterium, *Ruminococcus gnavus*. Microbiology, 151: 2323–2330.

Bjerrum L., Engberg R.M., Leser T.D., Jensen B.B., Finster K., Pedersen K. (2006). Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poultry Sci., 85: 1151–1164.

Czerwiński J., Højberg O., Smulikowska S., Engberg R.M., Mieczkowska A. (2010). Influence of dietary peas and organic acids and probiotic supplementation on performance and caecal microbial ecology of broiler chickens. Brit. Poult. Sci., 51: 258–269.

Czerwiński J., Højberg O., Smulikowska S., Engberg R.M., Mieczkowska A. (2012). Effects of sodium butyrate and salinomycin upon intestinal microbiota, mucosal morphology and performance of broiler chickens. Arch. Anim. Nutr., 66: 102–116.

De Cesare A., Sirri F., Manfreda G., Moniaci P., Giardini A., Zampiga M., Meluzzi A. (2017). Effect of dietary supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) on caecum microbioma and productive performance in broiler chickens. PloS One, 12: e0176309.

Gao P., Ma C.H., Sun Z., Wang L., Huang S., Su X., Xu J., Zhang H. (2017). Feed-additive probiotics accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. Microbiome, 5: 91.

ISO 9167-1 (1992). Rapeseeds – Determination of glucosinolates content. Part 1. Method using gradient elution high performance liquid chromatography. Geneva: ISO.

Jin L.Z., Ho Y.W., Abdullah N., Jalaludin S. (2000). Digestive and bacterial enzyme activities in broilers fed diets supplemented with *Lactobacillus* cultures. Poultry Sci., 79: 886–891.

Jørgensen H., Zhao X-Q., Bach Knudsen K.E., Eggum B. (1996). The influence of dietary fibre source and level on the development of the gastrointestinal tract, digestibility and energy metabolism in broiler chickens. Brit. J. Nutr., 75: 379–395.

Józefiak D., Rutkowski A., Martin S.A. (2004). Carbohydrate fermentation in the avian ceca: a review. Anim. Feed Sci. Tech., 113: 1–15.

Konieczka P., Smulikowska S. (2018). Viscosity negatively affects the nutritional value of blue lupin seeds for broilers. Animal, 12: 1144–1153.

Konieczka P., Nowicka K., Madar M., Taciak M., Smulikowska S. (2018). Effects of pea extrusion and enzyme and probiotic supplementation on performance, microbiota activity and biofilm formation in the broiler gastrointestinal tract. Br. Poult. Sci., 59: 654–662.

Loar II R.E., Donaldson J.R., Corzo A. (2012). Effects of feeding distillers dried grains with solubles to broilers from 0 to 42 days posthatch on broiler performance, carcass characteristics, and selected intestinal characteristics. J. Appl. Poult. Res., 21: 48–62.

MacFarlane S., MacFarlane G.T. (2003). Regulation of short-chain fatty acid production. Proc. Nutr. Soc., 62: 67–72.

Olnood Ch.G., Beski S.S.M., Iji P.A., Choct M. (2015). Delivery routes for probiotics: effects on broiler performance, intestinal morphology and gut microflora. Anim. Nutr., 1: 192–202.

Pan D., Yu Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes, 5: 108–119.

Pedersen M.B., Dalsgaard S., Knudsen K.E.B., Yu S., Lærke H.N. (2014). Compositional profile and variation of distillers dried grains with solubles from various origins with focus on non-starch polysaccharides. Anim. Feed Sci. Tech., 197: 130–141.

Pool-Zobel B., Van Loo J., Rowland I., Roberfroid M.B. (2002). Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer. Brit. J. Nutr., 87: S273–SS281.

Pustjens A.M., Schols H.A., Kabel M.A., Gruppen H. (2013). Characterisation of cell wall polysaccharides from rapeseed (*Brassica napus*) meal. Carbohydr Polym., 98: 1650–1656.

Rehman H.U., Vahjen W., Awad W.A., Zentek J. (2007). Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. Arch. Anim. Nutr., 61: 319–335.

Rinttilä T., Apajalahti J. (2013). Intestinal microbiota and metabolites – Implications for broiler chicken health and performance. J. Appl. Poult. Res., 22: 647–658.

Rubio L.A., Brenes A., Setién I., de la Asunción G., Durán N., Cutuli M.T. (1998). Lactobacilli counts in crop, ileum and caecum of growing broiler chickens fed on practical diets containing whole or dehulled sweet lupin (*Lupinus angustifolius*) seed meal. Brit. Poult. Sci., 39: 354–359.

Sergeant M.J., Constantinidou C., Cogan T.A., Bedford M.R., Penn C.W., Pallen M.J. (2014). Extensive microbial and functional diversity within the chicken cecal microbiome. PLoS One 9: e91941; doi:10.1371/journal.pone.0091941.

Sharifi S.D., Dibamehr A., Lotfollahian H., Baurhoo B. (2012). Effects of flavomycin and probiotic supplementation to diets containing different sources of fat on growth performance, intestinal morphology, apparent metabolizable energy, and fat digestibility in broiler chickens. Poultry Sci., 91: 918–927.

Smulikowska S., Rutkowski A. (Eds) (2005). Recommended Allowances and Nutritive Value of Feedstuffs – Poultry Feeding Standards (in Polish). 4th Edition. The Kielanowski Institute of Animal Physiology and Nutrition, PAS, Jabłonna (Poland).

Statistical Graphic Corporation 1982–2010. STATGRAPHICS® Centurion XVI version 16.1.03. Statistical Graphic System, Statistical Graphic Corporation .

Timmerman H.M., Veldman A., van den Elsen E., Rombouts F.M., Beynen A.C. (2006). Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. Poultry Sci., 85: 1383–1388.

Van der Wielen P.W., Biesterveld S., Notermans S., Hofstra H., Urlings B.A., van Knapen F. (2000). Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl. Environ. Microbiol., 66: 2536–2540.

Yadav S., Jha R. (2019). Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J. Anim. Sci. Biotech., 10: 2; https://doi.org/10.1186/s40104-018-0310-9.

Zduńczyk Z., Jankowski J., Juśkiewicz J., Mikulski D., Słominski B.A. (2013). Effect of different dietary levels of low-glucosinolate rapeseed (canola) meal and non-starch polysaccharide-degrading enzymes on growth performance and gut physiology of growing turkeys. Can. J. Anim. Sci., 93: 353–362.

Zduńczyk Z., Jankowski J., Rutkowski A., Sosnowska E., Drażbo A., Zduńczyk P., Juśkiewicz J. (2014). The composition and enzymatic activity of gut microbiota in laying hens fed diets supplemented with blue lupine seeds. Anim. Feed Sci. Tech., 191: 57–66.

Zduńczyk Z., Jankowski J., Kaczmarek S., Juśkiewicz J. (2015). Determinants and effects of postileal fermentation in broilers and turkeys. Part 2: cereal fibre and SBM substitutes. World Poultry Sci. J., 71: 49–57.

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