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Effect of blue lupin seed or pea seed as a substitute for GM soybean meal in diets of fattening pigs on intestinal health

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This study aimed to determine the composition of the fecal microflora of pigs fed with feed mixtures containing pulses and meals. Two experiments were performed with growing pigs. In their feed mixtures, GM soybean meal was replaced with either peas or blue lupine, and the modified mixtures were further fortified with rapeseed meal. Three-breed piglets were used in the study: ♀ (landrace × yorkshire) × ♂ duroc, (100 pigs in total, sex ratio: barrows : gilts - 1:1). Each experiment was performed with 50 animals divided into 5 groups, each of 10 pigs (control group - C and experimental groups - E1, E2, E3, E4). Population numbers of bacteria from the *Enterobacteriaceae* and *Lactobacillus* families, as well as from the genera *Clostridium spp.*, *Shigella spp.*, and *Salmonella spp.* were determined with the deep inoculation method (*Clostridium*) and the surface inoculation method (the other microorganisms tested) in serially-diluted samples of feces collected from the rectum of slaughtered fatteners. The number of bacteria was presented per feces dry matter, and the final result was expressed as colony forming units (cfu) per 1 g of feces. Analyses conducted in the experiment demonstrated an increased count of *Lactobacillus spp.* bacteria, a decreased count of *Enterobacteriaceae* bacteria, a stable population number of *Clostridium spp.* bacteria, and no *Salmonella* in the fatteners, administered feed mixtures with legume seeds, compared to the control animals. *Shigella* bacilli were confirmed in fecal samples taken from three experimental

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groups in Experiment II, while they were absent in the feces of animals from Experiment I. In both experiments, the correct ratio of villi and crypts was found, ensuring adequate intestinal absorption surface. The study results suggest that pulses, including legume grains like blue lupine, can positively affect the enteric microflora, promote the proliferation of bacteria beneficial for the host and promote the proper development of villi and crypts.

KEYWORDS: pigs / nutrition / meals / seeds of pulses / enteric microflora / villi:crypts

A properly functioning gastrointestinal tract (GIT) ensures regular absorption of nutrients and electrolytes and provides a barrier to pathogens and toxins. The sterile intestines of piglets are colonized by microflora immediately after birth, with these microorganisms deriving from the mother and the external environment. The initial colonization by *E. coli* and *Streptococcus spp*. creates the anaerobic environment for the successive microbes, i.e. *Bacteroides spp., Bifidobacterium spp., Clostridium spp.*, and *Lactobacillus spp.* [Konstantinov *et al.* 2006, Petri *et al.* 2010]. The microbiome composition fixes around the 3rd-4th week of piglets life. The natural microflora of the GIT includes both beneficial microorganisms, like lactic acid bacteria; conditionally-pathogenic ones, like *Escherichia coli*, and bacteria from the *Clostridium, Salmonella*, or *Streptococcus* genera. The gross weight of bacteria in adult animals' GIT points to their significant contribution to digestive processes. Exposure to various microorganisms affects its various colonization rates, determining its development and functions [Shirkey *et al.* 2006, Willing and Van Kessel 2007]. The populations of life-long observed bacterial species from the *Lactobacillus* and *Streptococcus* genera change with age, diet, and rearing environment [Wang *et al.* 2013]. The quantitative and qualitative changes in gut microflora are due to feed/diet [Arnal and Lallès 2016], feed additives [Rekiel and Gajewska 2006], stress, and infections [Lee *et al*. 2016]. The composition and counts of bacteria depend on the breed, age, and physiological condition of animals, and interactions between endogenous microorganisms colonizing their alimentary tract, exposure to pathogenic microorganisms, and additional supplementation with, e.g. probiotics or EMs [Giang *et al.* 2011, Mishra *et al.* 2014, Dowarah *et al.* 2016, Balasubramanian *et al.* 2018, Reszka *et al.* 2020]. The microorganisms affect the physiological condition of the gastrointestinal tract. A correlation has been found in pigs between their microbiota and their health and production parameters, including e.g., quality of meat and adipose tissue [Gardiner *et al.* 2020]. Form and composition of feed for monogastric animals influence the bacterial population and other parameters the height of the villi and the depth of the crypts in the intestinal epithelium. Gut health has broad implications for the systemic health of animals, and the correlation between animal performance and gut health is well known [Nguyen *et al.* 2021].

The phytotherapeutic effect of legumes has been known for centuries in Asia and the Mediterranean region. They have been used for preventive and therapeutic purposes, but also nutritional purposes as diet components [Guarrera 2005]. Today, they have become the raw material for the production of nutraceuticals used in the prophylaxis of diet-related diseases in humans [Duranti 2006]. They provide nutrients

to humans and animals and have also been scientifically proven to exhibit features and properties of pharmaceuticals [Bryant *et al.* 2022]. In their experiment with model animals, Bartkiene *et al.* [2013] attempted to determine the effect of either non-processed or LAB-fermented flours (soybean, flaxseed, lupine - yellow and white lupine) on rat intestinal epithelium, assuming their protective role against bacterial pathogens. They demonstrated a beneficial effect of the diet applied on the enzymatic activity as well as on the growth and body weight of animals. In turn, high counts of lactic acid bacteria, *Bifidobacterii*, and enterococci were detected in the small intestine, cecum, and colon of rats fed a diet with fermented yellow lupine seeds. The results of investigations bring some practical implications, especially to growing pigs that often suffer from the disturbed coli/lacto ratio.

The intake of legume seeds with a feed ration accelerates digesta passage in the gastrointestinal tract, reducing the intensity of fermentation processes essential for the health status, body homeostasis, and animal welfare [Probert 1995].

The gut microbiological ecosystem is critical in ensuring the proper nutritional, physiological, and immune functions of a pig body. The qualitative and quantitative composition and functions of a normal microbiological ecosystem have not been explicitly defined yet. Therefore, they cannot be fully exploited as a tool to maximize animal health and performance [Fouhse *et al.* 2016].

Feed materials used in feed mixtures, including their contents and quality, and their potential to cover nutritional demands for energy and dietary components affect their potential applications (Písaříková *et al.* 2008, Jezierny *et al.* 2010, Mierlita and Popovici 2013) which may be related to the development of the intestinal epithelium and the quantitative and qualitative composition of the gut microbiota. In view of the above, the aim of the study was to determine the composition of the faecal microflora and the ratio of crypt to villi in three sections of the small intestine of fattening pigs that were given compound feed containing pulses (seeds) and meals as protein sources.

Material and methods

According to the Polish law and the EU Directive (no. 2010/63/EU), the experiment did not require approval from the Local Ethical Committee as it was done by local farmers on a small scale (in the production conditions).

Two experiments were performed with growing pigs. In their feed mixtures, GM soybean meal was replaced with locally produced feed materials: peas or blue lupine, and the modified mixtures were additionally fortified with rapeseed meal.

Animals

Three-breed piglets (♀ [landrace × yorkshire] × ♂ duroc) were used in the study. A total of 100 pigs were included, with an equal sex ratio (barrows : gilts - 1:1). Each experiment involved 50 animals divided into 5 groups, each containing 10 pigs (control group - C and experimental groups - E1, E2, E3, E4). The experiment I was begun at body weight of ca. 26.5 kg, and the experiment II - at 33.5 kg b.w. The

animals were weighed every two weeks over the fattening period, and a slaughter day was fixed when their average body weight exceeded 120 kg. All animals were slaughtered on the same day. The animals were under veterinary supervision over the experimental period.

Feeding

GM soybean meal was a protein component of feed mixtures for control groups, whereas peas of Hubal variety (Experiment I) and blue lupine of Regent variety (Experiment II) served as protein sources in feed mixtures for experimental groups. Percentage contribution of peas, blue lupine, and GM soybean meal in the feed mixtures is presented in Table 1, whereas the composition of feed mixtures used in the study is provided in Tables 2 and 3.

Table 1. Scheme of experiment

Material	Groups[1]				
	C	E1	E2	E3	E4
Experiment I - percentage of feed material in mixture					
Pea seeds – Ist and IInd period of fattening	-	5.0	10.0	15.0	17.5
Rapeseed meal:					
Ist period of fattening	-	2.5	2.5	2.5	7.8
IInd period of fattening	-	2.5	2.5	2.5	6.0
GM soybean meal:					
Ist period of fattening	13.0	9.7	8.3	6.4	2.0
IInd period of fattening	10.5	6.6	4.8	3.0	-
Experiment II - percentage of feed material in mixture					
Blue lupine seeds in Ist and IInd periods of fattening	-	5.0	10.0	15.0	17.5
Rapeseed meal:					
Ist period of fattening	-	2.5	2.5	2.5	6.0
IInd period of fattening	-	2.5	2.5	2.5	5.6
GM soybean meal:					
Ist period of fattening	15.0	10.5	8.0	5.5	2.0
IInd period of fattening	12.8	8.2	5.6	3.1	-

[1] C – control group, E1-E4 – experimental groups.

The feed mixtures used in Experiment I were isoenergetic (fattening period I – 13.21 MJ EM/kg; fattening period II – 13.17 MJ EM/kg) and isoprotein (fattening period I – 16.41%; fattening period II – 15.30%), likewise these used in Experiment II (fattening period I – 13.21 MJ EM/kg; fattening period II – 13.19 MJ EM/kg), (fattening period I – 16.62%; fattening period II – 15.36%) – Grela and Skomiał [2015]. The pigs were fed *ad libitum* and had free access to water.

Microbiological analysis of feces

Fecal samples for testing were collected from 5 animals from each group. Population numbers of bacteria from *Enterobacteriace* and *Lactobacillus* families

Table 2. Feed material in the feed mixture in the I[st] and II[nd] period of fattening (%)

Raw feed materials	Groups[1]				
	C	E1	E2	E3	E4
I fattening stage					
barley	35.0	30.0	25.0	15.0	5.0
triticale	24.0	19.2	20.3	22.1	28.3
wheat	20.0	25.0	25.0	30.0	30.0
oats	5.0	5.0	5.0	5.0	5.0
soybean meal GM	13.0	9.7	8.3	6.4	2.0
rapeseed meal	-	2.5	2.5	2.5	7.8
pea seeds	-	5.0	10.0	15.0	17.5
soybean oil	-	0.6	0.9	1.0	1.4
premix*	3.0	3.0	3.0	3.0	3.0
Analyzed nutritional value (%)					
dry matter	87.6	87.4	86.9	87.3	87.5
crude protein	16.4	16.5	16.3	16.5	16.3
ether extract	2.6	2.7	2.7	2.7	2.8
crude fiber	3.9	3.9	4.0	4.1	4.1
crude ash	3.9	4.0	3.9	4.1	3.9
Calculated nutritional value (%)					
metabolic energy (MJ/kg)	13.22	13.21	13.21	13.21	13.21
lysine	1.05	1.06	1.09	1.10	1.10
methionine + cysteine	0.64	0.64	0.64	0.63	0.67
threonine	0.70	0.71	0.71	0.71	0.72
tryptophan	0.20	0.19	0.19	0.19	0.19
calcium	0.81	0.82	0.82	0.82	0.86
phosphorus	0.54	0.55	0.55	0.54	0.58
sodium	0.17	0.19	0.21	0.23	0.24
II fattening stage					
barley	35.0	25.0	15.0	10.0	8.2
triticale	32.0	26.5	30.0	30.0	30.0
wheat	10.0	21.6	24.7	30.0	30.0
oats	10.0	10.0	10.0	6.9	5.0
soybean meal GM	10.5	6.6	4.8	3.0	-
rapeseed meal	-	2.5	2.5	2.5	6.0
pea seeds	-	5.0	10.0	15.0	17.5
soybean oil	-	0.3	0.5	0.4	0.8
premix *	2.5	2.5	2.5	2.5	2.5
Analyzed nutritional value (%)					
dry matter	86.8	86.4	87.0	86.7	86.1
crude protein	15.3	15.4	15.5	15.2	15.1
ether extract	2.5	2.6	2.6	2.6	2.7
crude fiber	4.1	4.2	3.9	4.2	3.9
crude ash	4.5	4.7	4.5	4.7	4.6
Calculated nutritional value (%)					
metabolic energy (MJ/kg)	13.17	13.16	13.17	13.17	13.16
lysine	0.94	0.94	0.95	0.96	0.97
methionine + cysteine	0.60	0.61	0.61	0.60	0.61
threonine	0.64	0.64	0.64	0.64	0.65
tryptophan	0.18	0.18	0.18	0.17	0.17
calcium	0.68	0.70	0.69	0.69	0.71
phosphorus	0.51	0.51	0.51	0.50	0.52
sodium	0.15	0.17	0.19	0.21	0.21

[1] C – control group, E1-E4 - experimental groups.
*Premix composition: lysine – 12.10%; methionine – 2.65%; threonine – 5.05%; tryptophan – 0.25%; calcium – 20.50%; phosphorus – 1.80%; sodium – 5.00%; iron – 4000 mg; manganese – 2400 mg; zinc – 2600 mg; copper – 800 mg; iodine – 55.0 mg; selenium – 13.50 mg; vitamin A – 260,000 IU; vitamin D3 – 69,000 IU; vitamin E – 4 700 mg; vitamin K3 – 68 mg; vitamin B1 – 68 mg; vitamin B2 – 170 mg; vitamin B6 – 105 mg; vitamin B12 – 830 mcg; vitamin C – 1000 mg; folic acid – 27.00 mg; pantothenic acid – 410 mg; niacinamide B3 – 690 mcg; biotin – 3450 mg; choline chloride – 10,000 mg; aroma, antioxidant: 1b (E320-BHA, E321-BHT, E324 – ethoxyquin) 550 mg/kg; enzymes: 4a E-1 640 6 – phytase (EC 3.1.3.2.6 n-5000 FTU/g) 17 500 FTU/kg, (E1600 endo 1,4-beta-xylanase, EC 3.2.1.8–22,000 VU/g; 425,000 VU/kg, endo 1,3 beta-glucanase EC 3.2.1.6–30,000 VU/g, 57,000 VU/kg); raw material composition: calcium carbonate, monocalcium phosphate, (monophosphate) sodium chloride 1.8.1.9, herbal mix 10 g/kg.

Table 3. Feed material in the feed mixture in the Ist and IInd period of fattening (%)

Raw feed materials	Groups[1]				
	C	E1	E2	E3	E4
I fattening stage					
barley	36.6	12.0	11.0	5.4	2.3
triticale	15.0	30.0	30.0	33.0	35.0
wheat	25.0	33.7	35.0	35.0	33.0
oats	5.0	3.0	–	–	–
soybean meal GM	15.0	10.5	8.0	5.5	2.0
rapeseed meal	–	2.5	2.5	2.5	6.0
blue lupin seeds	–	5.0	10.0	15.0	17.5
soybean oil	0.4	0.3	0.5	0.6	1.2
premix *	3.0	3.0	3.0	3.0	3.0
Analyzed nutritional value (%)					
dry matter	87.7	87.4	87.3	87.1	87.4
crude protein	16.3	16.4	16.3	16.2	16.4
ether extract	2.6	2.6	2.7	2.7	2.8
crude fiber	3.9	3.9	4.2	4.2	4.1
crude ash	3.9	4.1	4.1	4.1	3.9
Calculated nutritional value (%)					
metabolic energy (MJ/kg)	13.23	13.19	13.20	13.21	13.20
lysine	1.09	1.06	1.04	1.03	1.05
methionine + cysteine	0.66	0.65	0.64	0.63	0.64
threonine	0.72	0.71	0.70	0.70	0.71
tryptophan	0.21	0.20	0.19	0.19	0.18
calcium	0.87	0.89	0.89	0.89	0.91
phosphorus	0.53	0.52	0.51	0.50	0.51
sodium	0.17	0.18	0.18	0.18	0.18
II fattening stage					
barley	36.7	12.0	11.4	8.8	5.8
triticale	20.0	30.0	30.0	33.0	35.0
wheat	20.0	32.3	35.0	35.0	33.0
oats	8.0	7.5	3.0	–	–
soybean meal GM	12.8	8.2	5.6	3.1	–
rapeseed meal	–	2.5	2.5	2.5	5.6
blue lupin seeds	–	5.0	10.0	15.0	17.5
soybean oil	–	–	–	0.1	0.6
premix *	2.5	2.5	2.5	2.5	2.5
Analyzed nutritional value (%)					
dry matter	87.6	87.9	87.4	87.5	88.0
crude protein	15.4	15.6	15.3	15.2	15.3
ether extract	2.2	2.1	2.3	2.2	2.3
crude fiber	3.9	3.8	4.2	4.0	4.1
crude ash	4.0	3.9	4.0	4.1	4.3
Calculated nutritional value (%)					
metabolic energy (MJ/kg)	13.20	13.19	13.19	13.19	13.20
lysine	0.98	0.95	0.96	0.96	0.96
methionine + cysteine	0.63	0.63	0.61	0.60	0.60
threonine	0.66	0.65	0.65	0.64	0.65
tryptophan	0.19	0.19	0.18	0.17	0.17
calcium	0.74	0.75	0.76	0.76	0.78
phosphorus	0.50	0.49	0.48	0.47	0.48
sodium	0.14	0.15	0.15	0.15	0.16

[1] C – control group, E1-E4 - experimental groups. *Premix composition under Table 2.

and these of bacteria from *Clostridium*, *Shigella spp.,* and *Salmonella spp.* genera were determined with the deep inoculation method (*Clostridium*) and the surface inoculation method (the other microorganisms tested) in serially-diluted samples of feces collected from the rectum of slaughtered fatteners. To this end, 10-g samples of feces were weighed, disintegrated, transferred to conical flasks, and poured

with 90 cm^3 of a sterile physiological saline solution. A stock solution of 10^{-1} was obtained after thorough sample mixing. Then, 10 cm^3 of the previous dilution was supplemented with 80 cm^3 of a sterile physiological saline solution and a dilution of 10^{-2} was prepared. From the resulting solution, 10 cm^3 of the solution was taken, 80 cm^3 of a sterile physiological saline solution was added and dilutions of 10^{-3} were prepared. This was repeated until a dilution of 10^{-6} was obtained. After deep inoculation of *Clostridium*, 1 mL of a suspension of each of the earlier prepared serial dilutions was spread onto Petri dishes and poured with agar cooled to a temperature of ca. 50-55°C. The agar and the bacterial culture were gently and thoroughly mixed and left to solidify [Kunicki-Goldfinger 1998]. In the surface inoculation method, 0.1 mL of a suspension of each of the earlier prepared serial dilutions was spread onto the surface of the solidified agar medium using a plate spreader. Thus prepared cultures were incubated at a temperature of 37.7°C, for 48 hours.

The following culture media were used in analyses: Salmonella Shigella LAB-AGAR, TSC LAB-AGAR Base and Endo, and MRS LAB-AGAR (BIOMAXIMA, Warsaw, Poland).

The numbers of bacteria tested are presented per feces dry matter. They were counted on plates with 30 to 300 grown colonies. In the surface inoculation method, the number of bacteria in 1 mL of the analyzed agar was computed according to the following formula:

$$\boldsymbol{x = \frac{(n1 + n2)}{2} \times d \times 10}$$

whereas in the deep inoculation method, according to the following formula where:

$$\boldsymbol{x = \frac{(n1 + n2)}{2} \times d}$$

n1, n2 – the number of colonies counted on Petri dishes in replications for one selected serial dilution;

d – dilution factor of the serial dilution used for computations.

The final result is expressed as colony forming units (cfu) per 1 g of feces.

Histology

Immediately after slaughtering the fatteners, samples were taken for histological analysis, about 3 cm each from three segments of the small intestine: duodenum, jejunum and ileum. A total of 150 samples were collected in two experiments (75 samples in each experiment - 5 groups × 5 fattening pigs × 3 intestinal segments). Individual small intestinal segments were washed with 0.9% saline solution and then fixed in 4% $CaCO_3$ buffered formalin solution. The fixed specimens were dehydrated, cleaned and soaked in paraffin in a tissue processor (Thermo Scientific, Waltham, USA), and then embedded in paraffin blocks using embedding equipment

(Medite, Germany). The blocks were divided into 10 μm-thick sections using a rotary microtome (Thermo Scientific, Waltham, USA), and the sections were successively transferred to slides coated with egg albumin and glycerol mixture. The slides were deparaffinized and hydrated, and then stained with hematoxylin and eosin (H-E) for further analysis. A Carl Zeiss microscope (Jena, Germany) was used to calculate the ratio of villi and crypts in each intestinal segment.

Statistical analysis

Statistical analysis of the results was performed using the IBM SPSS Statistics 21 package. The tables present the means and standard deviations (SD). The normality of the distribution of variables in the groups was tested by the Shapiro-Wilk test. For parameters with a normal distribution, ANOVA was used to compare the groups. In the absence of normal distribution, the Kruskal-Wallis test was used**.**

Results and discussion

The results of analyses presented in Table 4 were published in our earlier works: Sońta *et al*. [2020ab]. The production results of the fatteners in the experiment were favorable. The body weight gains of fatteners exceeded 1000 g/day, whereas the feed conversion ratio ranged from 2.35 to 2.59 kg/kg.

Table 4. Fattening results of experiment ($\overline{x} \pm$SD)

Specification	Groups[1]					*p*-value
	C	E1	E2	E3	E4	
	Experiment I					
Initial body weight (kg)	26.4±1.1	27.1±0.8	26.9±0.9	26.5±0.8	26.5±1.0	0.336
Final body weight (kg)	123.4±9.6	123.0±8.7	124.8±7.5	122.1±7.3	116.5±9.7	0.255
Average daily body weight gain (g)	1104±119	1090±97	1113±87	1086±80	1022±108	0.294
Feed conversion/kg of body weight gain (kg/kg)	2.52	2.60	2.58	2.57	2.63	-
	Experiment II					
Initial body weight (kg)	33.5 ± 1.4	33.1 ± 1.5	33.9 ± 1.5	33.7 ± 1.8	33.4 ± 1.5	0.810
Final body weight (kg)	125.5 ± 8.7	123.5 ± 5.8	125.1 ± 4.9	126.6 ± 7.7	121.1 ± 7.4	0.452
Average daily body weight gain (g)	1260 ± 118	1238 ± 82	1249 ± 70	1272 ± 107	1201 ± 92	0.529
Feed conversion/kg of body weight gain (kg/kg)	2.35	2.54	2.48	2.53	2.59	-

[1] C – control group, E1-E4 – experimental groups.

The importance of soybean in the feeding of monogastric animals, including GM soybean meal commonly administered to pigs and poultry, has been addressed in ample research, including e.g., Choct *et al.* [2010]. The usability of legume seeds in livestock feeding has also been presented in many works [Sońtaand Rekiel 2017]. According to Hanczakowska and Świątkiewicz [2015] and Martín-Pedrosa *et al.* [2016], the best pulse-based feedstuffs for pigs include yellow lupine followed by peas, blue lupine, faba bean, and chickpeas, whereas the least recommended ones include grass pea, vetch, white lupine, and raw bean seeds.

The production results obtained by fattening pigs in both experiments indicate the high genetic potential of the pigs used in the experiments and the correct balance of feed mixtures used in feeding growing animals. The optimization of the above-mentioned genetic and environmental factors proved beneficial for the development of the microbiome and intestinal epithelium presented in the following section, and thus the efficient functioning of the animals' digestive tract.

The feed mixtures used in both experiments contained GM soybean meal and either peas (Experiment I) or blue lupine (Experiment II), and were fortified with rapeseed meal. Compared to soybean meal, the rapeseed meal is rich in sulfur amino acids – methionine and cysteine; therefore, it was additionally used in the feed mixtures in the present study. Its synergy with other feed mixture components boosts fattening results. Dietary fiber of the feed mixtures could help optimize the composition of gut microbiota of the fatteners from both experiments, which is in line with previous findings [Montagne *et al*. 2003, Rekiel *et al*. 2005, Mateos *et al*. 2006, Jha *et al.* 2019].

Experiment I

The highest numbers of *Enterobacteriace* family bacteria were determined in the fecal samples from groups C and E3, and the lowest one in the sample from group E1 (Tab. 5). In turn, the highest count of *Lactobacillus spp.* genus bacteria was found in the fecal sample from group E1, and the lowest one in that from group C. In the case of *Clostridium spp.* bacteria, the highest counts were found in the fecal samples from the control pigs and the lowest ones in these from E2 and E3 fatteners. Neither *Salmonella* nor *Shigella* were found in the analyzed samples from this experiment.

In group C and in groups E1 and E3, the villi to crypt ratio was identical in the three sections of the small intestine (Tab. 6). In contrast, in group E2 in the duodenum and E4 in the duodenum and jejunum, the ratio of villi to crypts narrowed.

Table 5. The number of bacteria in feces, cfu*

Specification	Groups[1]				
	C	E1	E2	E3	E4
Enterobacteriace	$78 \cdot 10^6$	$31 \cdot 10^6$	$35 \cdot 10^6$	$78 \cdot 10^6$	$67 \cdot 10^6$
Lactobacillus spp.	$150 \cdot 10^5$	$260 \cdot 10^5$	$212 \cdot 10^5$	$240 \cdot 10^5$	$230 \cdot 10^5$
Clostridia spp.	$4 \cdot 10^2$	$3 \cdot 10^2$	$1 \cdot 10^2$	$1 \cdot 10^2$	$3 \cdot 10^2$
Salmonella	lack	lack	lack	lack	lack
Shigella	lack	lack	lack	lack	lack

cfu* - colony forming units, [1]C – control group, E1-E4 – experimental groups.

Table 6. Ratio of the number of villi to crypts in sections of the intestine

Specification	Groups[1]				
	C	E1	E2	E3	E4
Duodenum	4/3	4/3	3/3	4/3	3/3
Jejunum	4/3	4/3	4/3	4/3	3/3
Ileum	4/3	4/3	4/3	4/3	4/3

[1] C – control group, E1-E4 – experimental groups

Experiment II

The highest number of *Enterobacteriace* family bacteria was determined in the fecal samples from group C. In the samples from experimental groups, their count was the same but lower than in group C (Tab. 7). The lowest count of *Lactobacillus spp.* genus bacteria was determined in the sample from group C, and the highest one in the sample from group E1. A colony of *Salmonella* was detected in the samples of feces from group E4 fatteners. *Shigella* was detected in the fecal samples from groups C, E1, and E2, with the highest cfu number noted in E1 sample.

Table 7. The number of bacteria in feces, cfu*

Specification	Groups[1]				
	C	E1	E2	E3	E4
Enterobacteriace	$4 \cdot 10^6$	$2 \cdot 10^6$	$2 \cdot 10^6$	$2 \cdot 10^6$	$2 \cdot 10^6$
Lactobacillus spp.	$0.8 \cdot 10^7$	$100 \cdot 10^7$	$79 \cdot 10^7$	$74 \cdot 10^7$	$84 \cdot 10^7$
Clostridia spp.	$6.7 \cdot 10^6$	$1.8 \cdot 10^6$	$4.8 \cdot 10^6$	$8.5 \cdot 10^6$	$0.6 \cdot 10^6$
Salmonella	lack	lack	lack	lack	$1 \cdot 10^2$
Shigella	$1 \cdot 10^2$	$11 \cdot 10^3$	$1 \cdot 10^2$	lack	lack

*Colony forming units, [1] C – control group, E1-E4 – experimental groups.

Table 8. Ratio of the number of villi to crypts in sections of the intestine

Specification	Groups[1]				
	C	E1	E2	E3	E4
Duodenum	5/5	7/6	6/5	6/5	6/5
Jejunum	6/5	6/5	5/4	5/4	6/5
Ileum	5/5	5/5	5/5	5/4	5/5

[1] C – control group, E1-E4 – experimental groups.

The effect of the use of blue lupine seeds on the villi to crypt ratio in the three intestinal sections varied (Tab. 8). The highest number of villi and crypts and the highest ratio were found in the duodenum in group E1, and in the jejunum in groups C, E1 and E4. In the ileum, the ratio of villi to crypts was comparable in group C once in groups E1, E2, E4, slightly lower in E3.

The present study demonstrated an increased count of *Lactobacillus spp.* bacteria in fecal samples, which is consistent with the results reported by Tuśnio *et al*. [2017], who showed increased population numbers of *Lactobacillus spp.* and *Clostridium spp.* bacteria in the colon of piglets administered feed mixtures with pea seeds compared to the control animals whose feed mixture contained only soybean meal as the sole source of protein. Legumes are sufficient sources of dietary fiber that may be fermented in the colon by microflora [Tuśnio *et al*. 2017]. The *Lactobacillus* genus bacteria are deemed beneficial enteric bacteria, whereas *Clostridia* are found undesirable due to their adverse effect on animal health. Increased population numbers of such microorganisms as *Lactobacillus* contribute to enhanced mucin production, which improves the intestinal barrier [Che *et al*. 2014]. Most of the *Enterobacteriace*

family bacteria colonizing the gastrointestinal tract of pigs are harmless species. However, some of them are considered pathogenic and responsible for intestinal diseases. Therefore, decreased counts of these bacteria obtained in the present study (Experiment I and Experiment II) should be acknowledged as beneficial. Legume seeds, including mainly seeds of different lupine varieties, have a high content of dietary fiber, which may decrease their nutritional value mainly due to its non-starch polysaccharides (NSP) but positively influence functions of the gastrointestinal tract [Hanczakowska and Księżak 2012]. The non-starch polysaccharides protect plant cell walls by impairing their enzymatic digestion [Mierlita and Popovici 2013]. However, as Zduńczyk *et al*. [2014a] claimed, their increased inclusion level in turkey diet may stimulate the proliferation and activity of the gut microbiome. The presence of NSP in feed materials may reduce the energy value and digestibility of feed mixtures and nutrient availability [Choct *et al.* 2010, Mierlita and Popovici 2013]. The NSP content in lupine and pea seeds reaches 442-547 and 180 g/kg d.m., respectively [Górecka 2008, Jeroch and Lipiec 2018]. A high content of water-soluble NSP in the diet correlates with digesta viscosity in the small intestine. It may positively affect digesta passage in the intestines, proliferation of desirable microorganisms, and health status of the gastrointestinal tract [Zduńczyk *et al.* 2014a]. However, the results of experiments conducted by Langhout [1999], Persia *et al.* [2002], and Lan [2004] into the effect of NSP on the enteric bacteria populations are inexplicit. While investigating NSP effects, Langhout (1999) demonstrated a significant increase in the number of pathogenic bacteria in the intestines at the expense of beneficial microflora. In turn, the results reported by Persia *et al.* [2002] and Lan [2004] indicate that NSP had a positive effect on the intestines, and thus on the health and welfare of animals by inhibiting the proliferation of enteric pathogens. Zduńczyk *et al.* [2014b], who fed laying hens with a feed mixture containing 20% of blue lupine, demonstrated an increased total bacteria count in the cecum and increased population numbers of *Lactobacillus, Enterococcus*, and *Bifidobacterium spp.* bacteria as well as decreased counts of *E. coli* and bacterial populations from *Bacteroides*, *Prevotella*, and *Porphyromonas* genera compared to the hens fed a diet with soybean meal. According to the authors cited above, the lupine seed oligosaccharides could promote the competitive exclusion of potential pathogens in the intestines. Those authors also claimed that lactic acid bacteria (LAB) could reduce pathogens' number and virulence. The LAB adhesion in the mucosa stimulates the host's immune response. Another experiment carried out by Zduńczyk *et al.* [2016] demonstrated decreased counts of *E. coli* and *Clostridiaceae* in the cecum of turkeys receiving a feed mixture with yellow lupine seeds. Those authors claimed the bacteriostatic effect of short-chain fatty acids (SCFA) observed in the cecum to be among the factors responsible for beneficial changes in enteric microflora. A diet is one of the strongest determinants of the intestinal microbiome composition. Once provided with complex carbohydrates, microorganisms produce SCFA, including, e.g., butyrate or lactic acid, which exert positive effects on the body by, i.a., alleviating inflammatory responses. Diets poor in complex carbohydrates,

like dietary fiber, modify intestinal microbiome diversity, including that of protective bacteria from the genus *Bifidobacterium.*

Maintaining the coli/lacto balance in pig intestines is not an easy task because feed mixtures for growing animals contain mineral additives (with calcium-phosphorus compounds) and protein feed materials exhibiting high affinity to acids. Their use in feed mixtures contributes to digesta pH change from acidic to slightly acidic, neutral or even slightly alkaline (pH 6-8), promoting pathogenic flora proliferation. The present study results confirm that the animals from both experiments were healthy, with no gastrointestinal disorders, and that their intestinal microflora proved safe for their health. This was confirmed by the absence of *Salmonella* in fecal samples of the fatteners. The high count of *Salmonella* bacilli triggers poorer growth performance [Farzan and Friendship 2010] and indicates a strong correlation between the composition of intestinal microbiota, host health, and animal performance. The results obtained in the present study confirm the proper course of fattening [Sońta *et al.* 2020 ab] and are indicative of the completely healthy and efficiently functioning gastrointestinal tracts of the fatteners. Populations of microorganisms colonizing the gastrointestinal tract could contribute to the effective feed utilization by fatteners. A previous study [Vigors *et al.* 2016] demonstrated that the populations of microorganisms vary among animals, affecting their better or worse production results. In their study, the more productive animals were those with higher counts of *Lactobacillus spp*., and - as the authors clamed - the increased pig performance was due to the suppressed innate immune response.

The feed consumed by the animals stimulates morphological development, such as enhancing the height of the villi and the depth of the crypts, which and as a result, growing pigs can achieve very good growth rates, which ensures faster slaughter weights. A study by Chávez *et al.* [2016], Tzora *et al.* [2017] and Itza-Ortiz *et al.* [2018] confirmed that animals with higher body weights and higher daily gains had greater height and area of intestinal villi than those with lower weight gains. Nutrients are absorbed in the small intestine, whose surface area is increased through folds and functional structures such as villi and microvilli [Vázquez and Hernán 2012]. According to Itza-Ortiz *et al.* [2019], the height of the villi is important in the absorption process. Also, the higher the number of villi and properly formed crypts with the possibility of their permanent reconstruction, the potentially better the absorption of nutrients. In our study, in both completed experiments, the pigs grew rapidly, which was due to the optimal ratio of villi to crypts, providing the appropriate absorptive surface of the intestines for healthy animals. The large proportion of pea seeds (group E4 - experiment I) may have reduced proliferation at the level of the crypts, which is consistent with the growth rate of pigs in this group - it is lower in relation not only to group C but also to groups E1, E2 and E3.

The production, microbiological, and histological results confirm the validity of using leguminous seeds, such as peas and blue lupines, in pig fattening diets.

Conclusions

The study results enable concluding that pulses, including legume grains like peas and blue lupine, can positively affect the enteric microflora and promote the proliferation of bacteria beneficial to the host organism. At the same time, with a certain ratio of intestinal villi to crypts, they promote good absorption of nutrients, which is confirmed by the high growth rate and very good feed utilization of the pigs included in the experiment.

Disclosures

The authors declare no conflicts of interest.

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