Effect of inulin and a probiotic supplement in the diet of pigs on selected traits of the gastrointestinal microbiome

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Summary

The addition of inulin and/or a probiotic to feed mixtures for pigs can lead to changes in microflora composition and production of short-chain organic acids (SCFA) in the final segment of the digestive tract. The aim of the study was to determine the effect of an inulin and/or a probiotic supplement on Enterobacteriaceae taxa, the susceptibility of the bacteria to six antibiotics, and SCFA content in the cecum and colon, as well as changes in the histological structure of the intestines. The experiment was conducted on 80 fattening pigs with an initial body weight of 30.0 ± 0.5 kg, divided into 4 groups: control (I-C) and three experimental ones, fed the diets either with a probiotic (group II-P), inulin (III-I) or a both additives (group IV-PI). The animals were given ad libitum access to grower (30-70 kg) and finisher (71-115 kg) feed mixtures. During slaughter, from 8 pigs of each group, samples of contents and tissue from the cecum and colon were collected for laboratory analysis. The results of the study indicate differences in the expression of antibiotic resistance in the Enterobacteriaceae isolates depending on the type of dietary supplement. The inulin supplement caused changes in SCFA concentration, mainly an increase in the concentration of propionic and butyric acid, and also increased the thickness of the muscular layer.

The synbiotic (mixture of prebiotics and probiotics) was confirmed to have a beneficial effect on the gastrointestinal microbiome, which may be of significance in preventing the spread of Enterobacteriaceae infections in pigs.

Keywords: inulin, probiotic, pigs, microbiota

Optimization of pig diets, apart from balancing of nutrients, increasingly involves the use of feed supplements, including organic acids, eubiotics, probiotics and prebiotics, mainly oligosaccharides. The use of inulin and probiotic bacterial strains in the diet of animals and humans has been highly successful (19, 25, 26, 29). By modifying the microflora composition in the large intestine, pro- and prebiotics lower the pH in the intestinal contents and increase the production of short-chain fatty acids (SCFAs), which can result in morphological changes in the digestive tract and increased nutrient absorption (16).

The stability of the intestinal microbiome in pigs depends on many environmental factors, feed components, genetic predispositions and body condition. Colonization of the intestine of healthy animals by potentially pathogenic bacterial strains can be limited

by competition with indigenous intestinal bacteria, mainly lactic acid bacteria, leading to "colonization resistance" to bacteria ingested with food. Interaction and competition between useful bacteria, particularly of the genus *Lactobacillus*, and pathogenic taxa of the family Enterobacteriaceae play a key role in the functioning of the digestive tract in pigs (21). Potentially pathogenic bacteria in the gastrointestinal tract of animals in favorable conditions can multiply and lead to the emergence and spread of factors of resistance to certain antibiotics (1, 3, 5, 6, 14).

The aim of the study was to determine the effect of an inulin and/or probiotic supplement on Enterobacteriaceae flora, the susceptibility of bacterial strains to six antibiotics, and the content of volatile fatty acids in the contents of the cecum and colon, as well as changes in the histological structure of the intestines.

Material and methods

The study was approved by the Second Local Ethics Committee. The experiment was carried out on 80 cross-breed growers (Polish Landrace × Polish Large White) × (Duroc) initially weighing 30.0 ± 0.5 kg, which were assigned to 4 treatment groups: I – C (control), II – P, receiving a probiotic, III – I, receiving inulin, and IV – PI, receiving a probiotic and inulin (Tab. 1). The experimental factor was the application of a probiotic supplement containing *Lactococcus lactis*, *Carnobacterium divergens*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae*, and/or inulin HPX from chicory roots (Orafti; DP ≥ 23). The diets were isocaloric and isoprotein. The animals were housed in pens, with 4 animals in each pen. The fatteners were fed *ad libitum* with complete diets, i.e. grower (30-70 kg) and finisher (71-115 kg). The feed mixtures comprised grain meal (wheat and barley), soybean meal, soybean oil, and mineral feeds (monocalcium phosphate and fodder chalk). The diets were balanced for metabolizable energy, protein, amino acids, minerals, and vitamins (11). The animals had free access to drinkers and feeders (*ad libitum* feeding system). Air temperature, relative humidity, and cooling conditions were the same for all the treatment groups.

The pigs were slaughtered at about 115 kg BW. The slaughter was performed by electrical stunning in accordance with technology currently employed in the meat industry. Digesta samples from the cecum and distal colon as well as specimens of the jejunum were taken immediately after slaughter and frozen for chemical, microbial and histological evaluation.

Concentration of SCFAs in the cecum and distal colon of eight pigs from each group was determined according to the method of Ziotecki and Kwiatkowska (34). The following modifications were introduced: digesta samples (5 g) were collected into pre-weighed and pre-tared 50 ml test tubes, mixed with 8 ml ultrapure water and pH-measured using the Testo 205 portable pH meter. SCFAs were converted to their respective sodium salts by adjusting pH to approx. 8.2 with 1 M NaOH. The samples were stored at -20°C and thawed at room temperature prior to analysis. Subsequently, the samples were centrifuged (10 min, 4,000 rot/min) and the supernatant liquid was collected and dispensed in a volume of about 1.2 ml using Eppendorf pipettes, and refrozen (-20°C) for further analysis. The supernatant was transferred into chromatographic vials and mixed with isocaproic acid (internal standard: IS) at a ratio of 30 ml of IS to 200 ml of supernatant. The samples were analyzed in duplicate using a HP 5890 Series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with a flame ionization detector (FID) and a Supelco Nukol fused silica capillary column (30 m × 0.25 mm). Helium was used as a carrier gas at a 103 ml/min flow rate. The oven was initially kept at 100°C for 2 min., then heated at $10^{\circ}\text{C}/\text{min}$. to 140°C , and this temperature was maintained for 20 min. The injector temperature was maintained at 220°C and the detector at 250°C . The total run time was approximately 27 min. The concentration of each VFA was estimated in relation to IS using a mixture of VFA standard solutions.

Tab. 1. Experimental design (inulin or maize in g kg⁻¹ of mixtures)

Feed and supplement	Feeding groups			
	I – C	II – P	III – I	IV – PI
Maize	40.0	40.0	20.0	20.0
Maize + probiotic*	–	+ P	–	–
Inulin**	–	–	20.0	–
Inulin** + probiotic	–	–	–	20.0 + P

Explanations: * probiotic – *Lactococcus lactis* IBB500 > 10^9 cfu/g, *Carnobacterium divergens* S1 > 10^9 cfu/g, *Lactobacillus casei* 0915 > 10^9 cfu/g, *Lactobacillus plantarum* 0862 > 10^9 cfu/g, *Saccharomyces cerevisiae* 0141 > 10^7 cfu/g; ** inulin with degree of polymerisation – DP ≥ 23

The small intestine specimens were placed in Bouin's solution (a 30 : 15 : 1 mixture of picric acid, formalin and glacial acetic acid). Then they were dehydrated, paraffin-embedded and sectioned at 5 μm using a microtome. Two slides were prepared from each sample; each slide contained a minimum of 3 sections stained with haematoxylin and eosin. Villus height, crypt depth and muscularis externa thickness (15 measurements per slide) were determined using a light microscope with an Olympus BX51 camera and Olympus image analysis software.

The results obtained for the SCFAs and histology values were analyzed statistically using Statistica software (2003). The mean, SEM and p-value were calculated. One-way ANOVA was performed and significance of differences between the mean values was determined with Tukey's test at $P \leq 0.05$.

The material for microbiological analysis was collected with sterile cotton swabs from the contents of the cecum and colon of five pigs from each group. The bacteria were multiplied in laboratory conditions using LB enrichment broth (bioMérieux). Following 24 h incubation the material was transferred to SS medium (bioMérieux). Following growth on selective medium bacterial colonies were selected for further analysis. Biochemical analysis was carried out on the entire collection of isolated strains. Each strain was tested using an API 20E commercial test kit (bioMérieux). Susceptibility to chemotherapeutics was determined by the disc diffusion method on Mueller-Hinton agar (bioMérieux). The following antibiotics were used to determine the drug susceptibility of the isolates: ampicillin (AM at a concentration of 10 $\mu\text{g}/\text{ml}$), streptomycin (S 10 IU), tetracycline (TE 30 IU), nitrofurantoin (FM 300 IU), chloramphenicol (C 30 $\mu\text{g}/\text{ml}$), and sulfonamides with trimethoprim (SXT 1.25 + 23.75 $\mu\text{g}/\text{ml}$). Following incubation the results were interpreted according to the disc manufacturer's instructions. CFUs were determined according to the method described by Hong et al. (13).

Results and discussion

The concentration of short-chain organic acids in the contents of the cecum is presented in Table 2. No significant differences ($P \leq 0.05$) between groups were noted in the content of acetic or isovaleric acid. Supplementation with inulin (group III) or inulin and the probiotic (group IV) led to an increase ($P \leq 0.05$)

Tab. 2. Content of volatile fatty acids in $\mu\text{M/g}$ of the cecum (means $\pm$ SD, $n = 8$)

SCFA ($\mu\text{M/g}$ of the cecum)	Feeding groups			
	I	II	III	IV
Acetic acid	48.32 $\pm$ 3.22 ^a	49.23 $\pm$ 3.56 ^a	49.68 $\pm$ 2.97 ^a	50.66 $\pm$ 3.16 ^a
Propionic acid	14.91 $\pm$ 1.06 ^b	15.16 $\pm$ 1.11 ^b	16.75 $\pm$ 1.09 ^a	17.38 $\pm$ 1.21 ^a
Isobutyric acid	0.63 $\pm$ 0.09 ^a	0.57 $\pm$ 0.08 ^{ab}	0.55 $\pm$ 0.09 ^{ab}	0.48 $\pm$ 0.07 ^b
Butyric acid	9.56 $\pm$ 0.11 ^c	10.23 $\pm$ 0.91 ^{bc}	11.42 $\pm$ 0.89 ^{ab}	12.43 $\pm$ 0.86 ^a
Isovaleric acid	0.61 $\pm$ 0.07 ^a	0.58 $\pm$ 0.07 ^a	0.55 $\pm$ 0.08 ^a	0.54 $\pm$ 0.06 ^a
Valeric acid	0.73 $\pm$ 0.07 ^b	0.74 $\pm$ 0.06 ^b	0.95 $\pm$ 0.08 ^a	1.02 $\pm$ 0.08 ^a
Total SCFA	74.73 $\pm$ 6.24 ^c	76.42 $\pm$ 5.98 ^{bc}	79.67 $\pm$ 6.12 ^{ab}	82.37 $\pm$ 7.22 ^a

Explanations: a, b, c – values with different letters in the same row differ significantly at $P \leq 0.05$

Tab. 3. Content of volatile chain fatty acids in $\mu\text{M/g}$ of the final segment of the colon (means $\pm$ SD, $n = 8$)

SCFA ($\mu\text{M/g}$ of the colon)	Feeding groups			
	I	II	III	IV
Acetic acid	43.52 $\pm$ 3.45 ^b	44.25 $\pm$ 3.32 ^{ab}	45.82 $\pm$ 2.99 ^a	46.24 $\pm$ 3.64 ^a
Propionic acid	16.93 $\pm$ 1.07 ^b	18.13 $\pm$ 1.12 ^a	17.53 $\pm$ 0.99 ^{ab}	18.35 $\pm$ 1.13 ^a
Isobutyric acid	1.22 $\pm$ 0.13 ^a	1.17 $\pm$ 0.11 ^a	1.13 $\pm$ 0.17 ^a	1.11 $\pm$ 0.11 ^a
Butyric acid	13.46 $\pm$ 0.89 ^c	14.24 $\pm$ 0.97 ^b	15.36 $\pm$ 1.07 ^a	15.83 $\pm$ 1.12 ^a
Isovaleric acid	1.64 $\pm$ 0.17 ^a	1.66 $\pm$ 0.14 ^a	1.68 $\pm$ 0.15 ^a	1.69 $\pm$ 0.14 ^a
Valeric acid	1.36 $\pm$ 0.11 ^c	1.43 $\pm$ 0.12 ^{bc}	1.64 $\pm$ 0.14 ^{ab}	1.85 $\pm$ 0.15 ^a
Total SCFA	78.02 $\pm$ 6.34 ^c	80.76 $\pm$ 7.22 ^{bc}	83.05 $\pm$ 6.76 ^{ab}	84.95 $\pm$ 7.34 ^a

Explanations: as in Tab. 2

in the concentration of propionic, butyric and valeric acid and a decrease in the content of isobutyric acid with respect to the control.

Differentiated relationships were found for the content of selected fatty acids in the contents of the large intestine (Tab. 3). The addition of the synbiotic (group IV) increased the concentration of acetic, propionic, butyric and valeric acids, but caused no significant changes in the content of isobutyric and isovaleric acid. The inulin supplement (group III) increased the concentration of propionic acid in the colon contents. The probiotic supplement (group II) had a stronger effect on the concentration of SCFA in the colon than in the cecum.

The results of the microscopic measurements of fragments of the jejunum (villus height, crypt depth and muscular layer thickness) are presented in Table 4. No significant changes were found in villus height and crypt depth or in the ratio of villus height to crypt depth. The pigs in group III (inulin supplement) and IV (inulin + probiotic) had the thickest muscular layer.

In the contents of the cecum and colon 12 *Enterobacteriaceae* taxa were identified, whose numbers depended on the segment of

the digestive tract and on the supplements applied (Tab. 5). The most frequently identified taxa were *Citrobacter freundii* and species of the genera *Proteus* and *Providencia*. *Enterobacter cloacae*, *Salmonella arizonae* and *Pantoea* were identified in only a few cases.

Analysis of the pool of *Enterobacteriaceae* obtained from the animals in all the groups and from both sampling sites showed increased drug susceptibility: in the strains isolated from cecum increased sensitivity to antibiotics (for the control group) was recorded for two specifics (AM, S) and group III for four (C, TE, S, AM), while in the colon in group IV to four chemotherapeutics (C, TE, FM and AM) (Tab. 6).

The titre of *Enterobacteriaceae* bacteria (log cfu/g) isolated from the intestinal contents of the pigs of groups II and IV, both from the cecum and the colon (group II: 3.7 $\pm$ 0.63 and 3.9 $\pm$ 0.42; group IV: 3.5 $\pm$ 0.33 and 3.7 $\pm$ 0.46, respectively), was significantly different than in the other groups of animals in both sampling sites, where in general the results were similar – group I: 5.4 $\pm$ 0.52 and 5.8 $\pm$ 0.48; group III: 5.5 $\pm$ 0.64 and 5.7 $\pm$ 0.55 log cfu/g, respectively.

Since antibiotic growth promoters were withdrawn from use in 2006, the search has continued for other feed additives aimed at improving animal health, modifying intestinal bacterial flora and achieving good production results (ADG, FCR). The most effective are considered to be pro-, pre- and eubiotics (2, 16) and organic acids. These additives can substantially restrict the development of pathogenic microflora.

Research by some authors (18, 24, 28) indicates that adding fructans to feed mixtures for pigs increases production of short-chain organic acids in the digestive tract. In the present study, the highest total content of selected SCFAs in the cecum was noted in groups IV (82.4 $\mu\text{M/g}$) and III (79.8 $\mu\text{M/g}$), which received inulin in their feed (group III) or inulin with a probiotic (group IV), with the highest concentrations noted for propionic and butyric acid, and the lowest for isobutyric acid. In the contents of the colon, supplementation with inulin or

Tab. 4. Effects of dietary treatments on villus height, crypt depth, and villus height to crypt depth ratio in the jejunum of fatteners (means $\pm$ SD, $n = 8$)

Item	Feeding groups			
	I	II	III	IV
Villus height, μm	335.3 $\pm$ 24.34 ^a	339.5 $\pm$ 22.65 ^a	343.3 $\pm$ 23.65 ^a	343.9 $\pm$ 23.43 ^a
Crypt depth, μm	311.4 $\pm$ 24.67 ^a	312.3 $\pm$ 23.66 ^a	315.2 $\pm$ 23.13 ^a	316.1 $\pm$ 22.39 ^a
Villus height/Crypt depth	1.08 $\pm$ 0.09 ^a	1.09 $\pm$ 0.08 ^a	1.09 $\pm$ 0.09 ^a	1.09 $\pm$ 0.10 ^a
Muscularis externa, μm	513.4 $\pm$ 33.98 ^b	505.6 $\pm$ 29.87 ^b	539.3 $\pm$ 32.65 ^a	534.6 $\pm$ 31.45 ^a

Explanations: as in Tab. 2

Tab. 5. *Enterobacteriaceae* taxa isolated from the cecum and colon of pigs

Taxa	Cecum				Colon			
	I	II	III	IV	I	II	III	IV
<i>Citrobacter braakii</i>	+	-	++	+	+	-	++	+
<i>Citrobacter freundii</i>	++	++	+++	+	++	+++	+++	+
<i>Escherichia coli</i>	+	+	-	-	-	-	-	-
<i>Enterobacter cloacae</i>	+	-	-	-	-	-	-	-
<i>Pantoea spp.</i>	+	-	-	-	+	-	-	-
<i>Proteus mirabilis</i>	+	++	+	-	+	+	-	-
<i>Proteus penneri</i>	+	-	-	+	-	-	-	+
<i>Proteus vulgaris</i>	+	-	++	-	+	-	+	-
<i>Providencia alcalifaciens</i>	+	-	-	-	-	-	-	-
<i>Providencia rettgeri</i>	-	-	-	-	+	-	-	-
<i>Providencia stuartii</i>	-	-	-	-	+	-	-	-
<i>Salmonella enteritidis</i>	-	-	-	-	+	-	-	-

Explanations: - no isolates; + a few isolates; ++ numerous isolates; +++ very numerous isolates

inulin and a probiotic was also found to have a positive effect on the concentration of propionic, acetic, butyric and valeric acid. A pronounced effect of inulin supplementation (15 g/kg feed) on the concentration of SCFAs in the cecum and the large intestine was also found in a study by Pierce et al. (24). The authors observed a significant increase in the content of propionic acid in the cecum and a decrease in the concentration of isobutyric and isovaleric acid.

No significant changes, however, were noted in crypt depth or in the ratio of villus height to crypt depth. A study on piglets by Walsh et al. (31), using two polysaccharides (fucoidan and laminarin), also showed no significant differences between the study groups in villus height or crypt depth in the jejunum or in the ratio between them. The lack of significant differences in crypt depth in the jejunum of fattening pigs may be due to the fact that the digestive system of the pigs reaches maturity at the age of 4-5 months. This situation may also be dictated by the lower content of bifidobacteria in the early segments of the digestive tract in comparison with, for example, the large intestine, where inulin could be fully exploited.

The pathogenic potential of many *Enterobacteriaceae* taxa is well known and can be diagnosed in some diseases (10, 22). Among these, of particular epidemiological importance for pigs are bacteria of the genera *Escherichia* (20), *Salmonella* (12), *Enterobacter* (30), *Proteus* (8), *Citrobacter* (9, 23) and *Providencia* (7, 32). Strains belonging to these genera were isolated from the cecum and colon of the four experimental groups whose diet was supplemented with a pre- and/or probiotic.

The largest numbers of taxa were identified in group I, in the samples from both the cecum (9) and the colon (8) of the pigs. The lowest taxonomic variation was noted in both sampling sites in the group IV animals, whose diet was supplemented with both the pre- and probiotic.

Tab. 6. Susceptibility of *Enterobacteriaceae* strains (n = 40) to chemotherapeutic agents (%) isolated from the cecum and colon of pigs – as phenotypic characteristic

Groups		Chemotherapeutics					
		C	TE	SXT	S	FM	AM
		Cecum					
I	S	65 ^b	62 ^b	100 ^a	33 ^c	85 ^{ab}	10 ^d
	I	35 ^b	23 ^c	0	30 ^b	15 ^c	50 ^a
	R	0	15 ^b	0	37 ^a	0	40 ^a
II	S	10 ^d	25 ^{cd}	83 ^a	55 ^c	70 ^b	70 ^b
	I	80 ^a	55 ^c	0	0	22 ^{cd}	30 ^{cd}
	R	10 ^c	20 ^b	17 ^b	45 ^a	8 ^c	0
III	S	80 ^{ab}	80 ^{ab}	90 ^a	55 ^b	90 ^a	65 ^b
	I	10 ^c	5 ^d	0	12 ^c	5 ^d	35 ^a
	R	10 ^{bc}	15 ^b	10 ^{bc}	33 ^a	5 ^c	0
IV	S	15 ^d	47 ^c	100 ^a	40 ^c	43 ^c	80 ^{ab}
	I	85 ^a	13 ^d	0	43 ^{bc}	57 ^b	20 ^d
	R	0	40 ^a	0	17 ^c	0	0
		Colon					
I	S	10 ^d	0	100 ^a	20 ^{cd}	65 ^{bc}	30 ^c
	I	55 ^{ab}	55 ^{ab}	0	25 ^c	35 ^c	60 ^a
	R	35 ^b	45 ^{ab}	0	55 ^a	0	10 ^d
II	S	15 ^d	25 ^{cd}	100 ^a	40 ^c	85 ^{ab}	20 ^{cd}
	I	15 ^d	60 ^{ab}	0	23 ^{cd}	15 ^d	70 ^a
	R	70 ^a	15 ^d	0	37 ^c	0	10 ^d
III	S	30 ^{cd}	70 ^b	100 ^a	5 ^d	85 ^{ab}	35 ^{cd}
	I	60 ^a	27 ^c	0	35 ^{bc}	15 ^{cd}	55 ^a
	R	10 ^{cd}	3 ^d	0	60 ^a	0	10 ^{cd}
IV	S	60 ^b	60 ^b	100 ^a	20 ^{cd}	85 ^{ab}	75 ^{ab}
	I	40 ^a	30 ^b	0	40 ^a	15 ^{cd}	25 ^b
	R	0	20 ^a	0	20 ^a	0	0

Explanations: S – sensitive, I – intermediate, R – resistant; C – chloramphenicol (30), TE – tetracycline (30), SXT – trimethoprim + sulfamethox. (1.25 + 23.75), S – streptomycin (10), FM – nitrofurantoin (300), AM – ampicillin (10); numbers in parentheses indicate the content of the chemotherapeutic in the disc; a, b, c, d – values with different letters in the same row differ significantly at $P \leq 0.05$.

Despite the use of all available and permissible means to reduce the population of pathogenic bacteria, all isolates having pathogenic potential and exhibiting antibiotic resistance have clinical significance. The antibiotics that proved most effective in restricting the growth of the pool of isolates tested were sulfonamides, nitrofurantoin and ampicillin (Tab. 6). It is also worth noting the effectiveness of both streptomycin and chloramphenicol in limiting the growth of the strains analyzed.

A number of factors influencing the colonization of the pig intestine by pathogenic bacteria depend on dietary supplements. Fermenting feed components, in both liquid and solid form, reduce pH by activating naturally occurring phytases and improve intestinal

health by reducing the abundance of *Enterobacteriaceae* throughout the intestine (4, 15, 17, 33). Fermenting carbohydrates contained in food can affect bacterial ecosystems, both qualitatively and quantitatively, depending on the source of the carbohydrates. Bacteria stimulate the secretion of mucus in the intestines and the regeneration of intestinal epithelial cells (21). The addition of organic acids to fodder reduces the number of enterobacteria in the intestine of healthy animals (13). The immunostimulatory and anti-inflammatory properties of conjugated linoleic acid and omega-3 fatty acids are also exploited in dietary supplementation (27). The analyses presented here also suggest a potential practical application of pre- and probiotics as dietary supplements for effective regulation of the *Enterobacteriaceae* population colonizing the digestive tract of pigs. Every factor ensuring microecological balance in the digestive tract of living organisms, in this case pre- and probiotics, is of epidemiological significance.

Diet supplementation with inulin or inulin with a probiotic can significantly influence the microbiome of the digestive tract, including *Enterobacteriaceae*, as well as the concentration of SCFAs, particularly propionic and butyric acid. Monitoring of the antibiotic resistance of *Enterobacteriaceae* colonizing the intestine of animals while taking into account the effect of pre- and probiotics added to their feed can be an important element limiting the emergence and spread of antibiotic-resistant strains. The dietary supplements analyzed increase the potential competitiveness of useful physiological microflora present in the intestine of pigs with respect to potentially pathogenic *Enterobacteriaceae* taxa.

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