ASSESSMENT OF GUT MICROBIOTA AND SELECTED BLOOD PARAMETERS IN WEANED PIGLETS FOLLOWING SUPPLEMENTATION WITH A PROBIOTIC AND VITAMIN C

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Abstract

The aim of the study was to assess the gut microbiota and selected haematological and biochemical blood parameters of weaned piglets following dietary supplementation with a probiotic and vitamin C. The piglets were divided into a control group (group C) and an experimental group (group E), with 30 piglets in each group. All animals received the same feed *ad libitum*. The animals in the control group (group C) received feed with no added probiotic and vitamin C. The piglets in the experimental group (group E) were given a supplement containing a probiotic (*Bacillus cereus* 1×10^9 CFU/kg) in the amount of 1.5 g/piglet/day and vitamin C in the amount of 300 mg/piglet/day. The supplement was administered for 28 days. The total numbers of bacteria of the family *Enterobacteriaceae* and of the genus *Lactobacillus* were determined in faeces. The erythrocyte count, haemoglobin level, haematocrit, leukocyte count, and percentages of neutrophils and lymphocytes were determined in the blood. The biochemical analysis concerned the concentration of triacylglycerols, total cholesterol, and LDL and HDL cholesterol. In group E a significant decrease ($P < 0.01$) in the total number of *Enterobacteriaceae* bacteria was observed in the faeces of the piglets, accompanied by an increase in the number of lactobacilli relative to group C. *E. coli* was found to predominate over other microorganisms. *Salmonella choleraesuis* bacteria were present in the faeces of both groups before administration of the supplement, but were not found after its use. The supplement with probiotic and vitamin C caused a significant increase in the erythrocyte, haemoglobin and haematocrit levels in the blood of the piglets and a significant decrease in the concentration of triacylglycerols, total cholesterol and LDL cholesterol.

Key words: blood, gut microbiota, piglets, probiotic, vitamin C

Weaning of piglets and the switch to solid food are highly stressful for these young animals. The greatest problem during this period is diarrhoea of complex aetiology, in which besides the infectious agents (enterotoxigenic strains of *Escherichia coli*,

rotaviruses and coronaviruses), environmental and nutritional factors play the most important role. The simultaneous change in diet and housing conditions leads to a quantitative and qualitative imbalance of the gut microbiota, and even to the multiplication of pathogenic bacteria. To prevent gastrointestinal disorders in young pigs, the use of probiotics, to which numerous health benefits are ascribed, is becoming increasingly common.

Probiotics exert a beneficial effect on the intestinal microbiota. They are used to protect the intestinal wall against colonization by pathogenic bacteria (e.g. *Salmonella* spp. and *E. coli*) and to increase the number of normal gut microflora (Asml et al., 2015; Corcionivoschi et al., 2010; Shengfa and Nyachoti, 2017). Furthermore, the levorotatory lactic acid in probiotic products reduces the absorption of toxic substances such as ammonia into the blood (Mojka, 2014). Among the mechanisms of action of probiotic bacteria, substances they produce that prevent the development of pathogenic microbes play an important role. These include organic acids, hydrogen peroxide and bacteriocins (Śliżewska et al., 2006). Administration of probiotics containing strains of the genus *Bacillus* or *Lactobacillus* to weaned piglets reduces mortality and the incidence of conditions with diarrhoeal symptoms (Asml et al., 2015; Dalmini et al., 2017; Giang et al., 2012). In addition, probiotic substances form a barrier against pathogenic microbes by adhering to the intestinal epithelium, thus increasing the body's resistance. Probiotics also stimulate the immune system by increasing immunoglobulin production, enhancing macrophage and lymphocyte activity, and stimulating production of γ -interferon (Śliżewska et al., 2006).

In livestock farming, strains of probiotic bacteria, including lactic acid bacteria of the genus *Lactobacillus*, bacteria of the genera *Bifidobacterium* and *Enterococcus*, yeast *Saccharomyces* spp., and spore-forming bacteria, including *Bacillus* spp., can be enriched with elements (mainly Fe), colostral immunoglobulins, and vitamins (Janik et al., 2006). Research conducted by Kunavue and Lien (2012) and by Liu et al. (2018) has also shown that the addition of organic acids, such as fulvic acid, or xylooligosaccharides may enhance their health-promoting activity. Lee et al. (2014) have demonstrated that the addition of natural acidifiers derived from the fermentation of citrus fruit juices can also stimulate the growth of probiotic strains.

In our study, a probiotic was used in combination with ascorbic acid (vitamin C) as a feed additive. We posited that this combination would have a beneficial effect on the gut microbiota of weaned piglets and on their haematological and biochemical parameters.

Vitamin C, among other vitamins, plays an important role in animal nutrition, as it contributes to an acidic pH in the gastrointestinal tract, which inhibits pathogenic bacteria while favouring the occurrence of beneficial microbes. The use of this vitamin should mainly be recommended in pigs, which only synthesize it to a small degree as compared to other livestock animals (Lechowski, 2009). The antioxidant effect of vitamin C is particularly important, as it reacts with free radicals to transform them into less toxic compounds (Janda et al., 2015; Sroka et al., 2005). Ascorbic acid also acts on the immune system by stimulating the formation of certain immunoglobulins, especially IgM (Lauridsen and Jensen, 2005). It is important to administer vitamin C to piglets, especially in stress situations, which include weaning. It is also

a potential source of protection against environmental oxidative stress and transport stress in pigs (Adenkola et al., 2009; Carr and Maggini, 2017).

The aim of the study was to assess the gut microbiota and selected haematological and biochemical parameters of the blood following supplementation of piglet feed with a probiotic and vitamin C.

Material and methods

Animals used in the experiment

The research on animals was carried out with Ethics Committee approval (no. 22/2012).

The research was carried out on a pig farm in the Lublin Voivodeship, Poland. The pigs were kept in a closed cycle in a deep litter system and the herd consisted of 500 animals. The study was conducted on F1 Duroc × Pietrain and Pietrain × Duroc crossbreds (sow × boar). The piglets were under veterinary care throughout the rearing period and showed no disease symptoms. Sixty piglets were used in the study.

The piglets were weaned from their mothers at the age of 40 days, and the experiment was begun when they were 47 days old. The animals were of the same age and had similar body weight (16.00–16.90 kg). All piglets were fed complete compound feed produced on-farm, containing wheat (39.75%), spring barley (39.75%), rapeseed oil (0.5%) and the commercial supplement mix ‘Protamino piggi’ from Sano (20%). The feed did not contain either vitamin C or a probiotic. All piglets were fed the same feed *ad libitum* and had unrestricted access to water.

The piglets were divided into a control group (group C) and an experimental group (group E), with 30 piglets in each group. The piglets were kept in two pens – group E in one and group C in other. The ratio of males to females was 1:1 in each group.

The animals in the control group (group C) received feed with no added probiotic and vitamin C. The piglets in the experimental group (group E) were given a supplement containing a probiotic (*Bacillus cereus* 1×10^9 CFU/kg) (Lohmann Animal Health, Grodzisk Mazowiecki, Poland) in the amount of 1.5 g/piglet/day and vitamin C (JFARM, Ostrołęka, Poland) in the amount of 300 mg/piglet/day in a small portion of feed, before receiving their regular feed ration. The supplement was administered for 28 days.

Faeces and blood were collected from the animals from both groups at three different times: 1) day 0, i.e. seven days after weaning, before administration of the probiotic and vitamin C; 2) day 28, i.e. after 4 weeks of administration of the supplement; 3) day 35, i.e. seven days after supplementation was discontinued.

Bacteriological testing of faeces

Faeces for bacteriological cultures were collected from the rectum of 20 animals from the experimental group and 20 from the control group. Quantitative and qualitative bacteriological tests of the faeces were performed according to Polish

Standard PN-EN ISO 7218:2008. The total number of bacteria of the *Enterobacteriaceae* family was determined by plating diluted samples on VRBG agar (BTL, Łódź, Poland) according to Polish Standard PN-ISO 21528-2:2005. To prepare dilutions of the samples, 1 g of faeces was placed in 90 ml of Ringer's solution and homogenized. A series of dilutions of the homogenate was made, from 10^{-2} to 10^{-7} , by transferring 1 ml of material to a tube containing 9 ml of Ringer's solution. After plating onto nutrient agar, the Petri dishes were incubated at 37°C for 24 hours. To isolate pure bacterial cultures, a culture was grown on MacConkey agar (BTL, Łódź, Poland) and the colonies were counted following incubation. The number of *Lactobacillus* bacteria was determined by plating samples on MRS agar (BTL, Łódź, Poland) and incubating them at 37°C in aerostats (Oxoid, Basingstoke, United Kingdom) for 24–48 hours, after which the bacterial colonies were counted (PN-EN 15787:2009). The number of microbes is presented as decimal logarithms per g fresh weight of faeces in colony-forming units (log CFU/g). Bacteria of the family *Enterobacteriaceae* were identified to species by the microtube method using API 20 E tests (bio Merieux, Warsaw, Poland).

Haematological and biochemical testing of blood

Blood was taken from the anterior vena cava of 60 animals (30 piglets from each group). The blood samples (4.9 ml) were collected into S-Monovette tubes (Sarstedt AG & Co, Numbrecht, Germany) and tubes with K2 EDTA (Profilab Sc. Warsaw, Poland). A scil Vet abc Plus haematology analyser (HORIBA Medical, Montpellier, France) was used to determine selected haematological parameters, i.e.: erythrocyte count, haemoglobin concentration, haematocrit, leukocyte count, and percentages of neutrophils and lymphocytes.

The concentrations of triacylglycerols, total cholesterol, and HDL and LDL cholesterol were determined using a BS-130 automatic biochemistry analyser (Mindray, Shanghai, China).

Statistical analysis

The results of the tests are presented in tables. The levels of the analysed parameters in each group were compared by one-way analysis of variance and Student's t-test, using SPSS 12.0 PL statistics software.

Results

In the faeces of piglets receiving the probiotic and vitamin C (group E), there was a significant reduction ($P < 0.01$) in the number of *Enterobacteriaceae* bacteria between faeces sampled on days 0 and 28 (by 1.28 log units), and a significant increase ($P < 0.01$) between days 28 and 35 (by 1.07 log units) (Table 1). In the faeces of the piglets in the control group (group C), there was a significant decrease ($P < 0.01$) in the bacterial count between days 0 and 28 of the study (by 0.95 log units) and a significant increase ($P < 0.01$) between days 28 and 35 (by 1.57 units). At 35 days

the number of these bacteria was also 0.62 units higher ($P<0.01$) than the initial level (day 0).

Table 1. Mean total number of bacteria (log CFU/g) in the faeces of piglets receiving the probiotic with vitamin C

Taxonomy	Group	Sampling day			Total	SEM
		0 days	28 days	35 days		
Family	C	7.43 B	6.48A*	8.05 C**	7.01	0.120
<i>Enterobacteriaceae</i> (E)	E	7.24 B	5.96 A*	7.03 B**	7.14	
Genus <i>Lactobacillus</i>	C	8.53 B	8.70 B**	7.68 A**	8.35**	0.120
(L)	E	8.93 a	9.42 b**	8.98 a**	9.11**	

A, B, C – values with different letters in rows differ significantly at $P\leq 0.01$.

a, b – values with different letters in rows differ significantly at $P\leq 0.05$.

** – values in columns differ significantly at $P\leq 0.01$.

* – values in columns differ significantly at $P\leq 0.05$.

C – control group.

E – experimental group.

The feed supplement given to the piglets caused a significant ($P<0.05$) decrease in the total number of bacteria of the family *Enterobacteriaceae* (by 0.52 log units) in the faeces of piglets from the experimental group as compared to the control after 28 days of administration. The number of these bacteria decreased significantly ($P<0.01$), by 1.02 log units, seven days after supplementation was discontinued.

There was a significant ($P<0.01$) increase in the total number of *Lactobacillus* bacteria relative to the control group immediately after supplementation, by 0.72 log units, and a significant ($P<0.01$) increase of 1.3 log units seven days after it was completed (Table 1). In the experimental group, a significant increase in the number of these bacteria ($P<0.05$) was found between faeces sampled at 0 and 28 days, by 0.49 log units, while a significant ($P<0.05$) decrease of 0.44 log units was demonstrated between faeces sampled at 28 and 35 days. In the control group, a significant reduction ($P<0.01$) in the number of *Lactobacillus* bacteria was noted between days 0 and 35 and between days 28 and 35 of the study, by 0.85 and 1.02 units, respectively.

In the qualitative tests of piglet faeces, *E. coli* bacteria were predominant over other microorganisms (Figures 1–3). In faeces sampled at 28 days, a slight decrease in the percentage of these microorganisms was noted in the experimental group (group E). However, seven days after the supplement was discontinued, this percentage had increased (Figures 2–3). Before the start of administration of the probiotic with vitamin C (group E), *Salmonella choleraesuis* was found in the faeces of piglets in both groups (Figure 1). However, these bacteria were not detected on days 28 and 35. The remaining microorganisms isolated were part of the physiological gut microbiota and accounted for only a small percentage.

The probiotic and vitamin C supplementation resulted in a statistically significant increase ($P<0.01$) in the erythrocyte count and haemoglobin level and a significant increase ($P<0.05$) in the haematocrit in the blood of piglets sampled at 28 days, as compared with the control (Table 2).

Figure 1. Identified bacteria of the family *Enterobacteriaceae* (in %) in the faeces of piglets seven days after weaning, before administration of the supplement (day 0)

Figure 2. Identified bacteria of the family *Enterobacteriaceae* (in %) in the faeces of piglets after four weeks of administration of the supplement (day 28)

Figure 3. Identified bacteria of the family *Enterobacteriaceae* (in %) in the faeces of piglets seven days after supplementation was discontinued (day 35)

Table 2. Haematological parameters of the blood of piglets

Parameter	Group	Sampling day			Total	SEM
		0 days	28 days	35 days		
Erythrocytes ($10^{12}/L$)	C	6.68	6.59**	7.07	6.80*	0.120
	E	6.98	7.67**	7.80	7.48*	
Haemoglobin (Mmol/L)	C	7.10	6.97**	7.07*	7.05**	0.119
	E	7.45	8.07**	8.15*	7.89**	
Haematocrit (L/L)	C	0.36	0.37*	0.39	0.37**	0.045
	E	0.38	0.42*	0.43	0.42**	
Leukocytes ($10^9/L$)	C	22.77	18.80	20.81	20.79	1.013
	E	24.86	23.31	20.62	22.93	
Neutrophils (%)	C	28.13	27.84	25.87	27.28	0.813
	E	26.96	30.73	29.35	29.01	
Lymphocytes (%)	C	65.02	63.18	61.80	63.24	1.434
	E	69.75	69.50	64.74	68.26	

** – values in columns differ significantly at $P \leq 0.01$.

* – values in columns differ significantly at $P \leq 0.05$.

C and E – as in Table 1.

Administration of the supplement caused no significant changes in leukocyte parameters ($P > 0.05$).

The use of the probiotic and vitamin C in the diet significantly reduced ($P < 0.05$) the concentration of triacylglycerols, total cholesterol and LDL cholesterol in the blood relative to the control group immediately after supplementation was discontinued (Table 3).

Table 3. Mean values of biochemical parameters in the blood of piglets

Parameter	Group	Sampling day			Total	SEM
		0 days	28 days	35 days		
TG (Mmol/L)	C	0.45	0.42*	0.46	0.44*	0.017
	E	0.43	0.31*	0.41	0.38*	
CHOL (Mmol/L)	C	2.58	2.82*	2.55	2.65*	0.061
	E	2.62 B	2.06 A*	2.24	2.31*	
CHOL _{HDL} (Mmol/L)	C	0.98	1.09*	0.97	1.01	0.031
	E	1.04 b	0.84 a*	0.93	0.94	
CHOL _{LDL} (Mmol/L)	C	1.40	1.54*	1.37	1.44	0.061
	E	1.43 b	1.08 a*	1.12 a	1.21	

A, B, C – values with different letters in rows differ significantly at $P \leq 0.01$.

a, b – values with different letters in rows differ significantly at $P \leq 0.05$.

* – values in columns differ significantly at $P < 0.05$.

TG – triacylglycerols.

CHOL – total cholesterol.

CHOL_{LDL} – LDL cholesterol.

CHOL_{HDL} – HDL cholesterol.

C and E – as in Table 1.

Discussion

We have confirmed that the inclusion of a probiotic (*Bacillus cereus*) combined with vitamin C in the diet may have positive effects on some indicators of intestinal health, such as a significant decrease in the number of bacteria of the family *Enterobacteriaceae* and a significant increase in the number of *Lactobacillus* bacteria in the faeces of piglets.

A beneficial effect was probably exerted by the action of the probiotic enhanced by vitamin C as an acidifier. Administration of vitamin C to sows for three and six weeks has also resulted in a significant ($P < 0.05$) decrease in the number of these bacteria in their faeces (Trawińska et al., 2012). Similar results have been obtained by Jadamus et al. (2002), who determined the number of *Enterobacteriaceae* bacteria in the faeces of piglets following feed supplementation with *Bacillus cereus* var. *toyoi*. A beneficial effect has also been obtained in fattening pigs using a feed supplement containing a strain of *Pediococcus acidilactici*, which resulted in a decrease in the number of *Enterobacteriaceae* bacteria and an increase in lactic acid bacteria (Rekiel and Gajewska, 2006). The reduction in the number of harmful bacteria from the *Enterobacteriaceae* family may be due to competition between probiotic strains and pathogenic microorganisms for nutrients and receptor sites, known as competitive exclusion (Liao and Nyachoti, 2017; Nowak et al., 2010). Substances produced by strains of bacteria of the genus *Bacillus*, including bacteriocins (e.g. cerein and subtilin) with confirmed bactericidal and bacteriostatic properties, as well as antibiotics such as surfactin, bacilysin and subtilisin, are also antagonistic to pathogenic microorganisms (Elshagabee et al., 2017). On the other hand, enzymes, vitamins and peptides produced outside the cell stimulate the growth of commensal and beneficial gut flora and influence its composition, helping to maintain homeostasis in the gut. This is confirmed by our own research.

The feed additive used in our study significantly increased the number of *Lactobacillus* bacteria in the faeces. A similar effect has been reported by Napiórkowska et al. (2014), who used a preparation containing an *Enterococcus faecium* strain in the diet of piglets. The use of a preparation containing bacterial strains of the genus *Lactobacillus* in piglets has also been found to increase their resistance to infections caused by pathogenic *E. coli* bacteria two weeks after weaning (Huang et al., 2004). The administration of probiotic and prebiotic preparations has been shown to have a favourable effect on the intestinal microbial population in piglets by increasing the number of beneficial microbes and reducing the number of pathogenic ones (Mojka, 2014; Shim et al., 2005). Probiotic substances, by exhibiting the ability to adhere to the intestinal epithelium, protect the intestinal wall against harmful agents forming a biofilm and stimulate immunoglobulin production and phagocytosis (Mojka, 2014).

The qualitative bacteriological testing of the faeces showed a slight decrease in the percentage of *E. coli* bacteria after 28-day administration of the probiotic and vitamin C, but seven days after the supplement was discontinued, a percentage increase in these bacteria was observed. Thirty-day administration of a probiotic containing *Enterococcus faecium* to pigs has been found to significantly reduce the number of *E. coli* and *Clostridium perfringens* in their faeces (Pospíšková et al., 2013). In addi-

tion, supplementation with probiotics or xylooligosaccharides has been found to reduce the number of *E. coli* bacteria in the faeces of weaned piglets (Liu et al., 2018).

Prior to the start of administration of the probiotic and vitamin C, pathogenic *Salmonella choleraesuis* were found in the faeces of animals from both groups, although the piglets showed no disease symptoms. However, these bacteria were not detected in subsequent samples. Similar results have been obtained by Casey et al. (2007), who showed that the use of a mixture of probiotics from the genus *Lactobacillus* caused a reduction in *Salmonella typhimurium* in pigs.

Many probiotic bacteria, including *Bacillus* sp., ferment carbohydrates to produce organic acids thereby dropping the luminal pH to a level that harmful bacteria cannot tolerate (Liao and Nyachoti, 2017). A decrease in gut pH by vitamin C may additionally offset the low secretion of hydrochloric acid in the stomach of weanling piglets.

The results confirm the beneficial effect of supplementation with vitamin C together with a probiotic on the erythrocyte parameters of the blood of piglets. Similar results have been reported by Friedrich (1988), who found that the inclusion of vitamin C alone in the diet of piglets increased the erythrocyte count and haemoglobin and haematocrit levels in the blood. The increase in the number of erythrocytes is probably linked to the involvement of vitamin C in erythropoiesis. In addition, it is present in significant amounts in red blood cells. Other researchers (de Rodas et al., 1998), however, have found that the addition of vitamin C had no effect on haemoglobin and haematocrit levels in weaned piglets. Kunavue and Lien (2012), after administering a probiotic to pigs together with fulvic acid, found no effect of this additive on red blood cell parameters, but noted a beneficial effect on immunity. Our research found no significant differences in leukocyte parameters. In contrast, Szakacs et al. (2016) reported that a probiotic increased the leukocyte count in pig blood, thereby stimulating the immune system.

The use of the supplement in the diet of weaned piglets also caused a significant reduction in lipid profile parameters. This was probably due to the addition of vitamin C, which as an antioxidant may prevent lipid peroxidation (Maćkowiak and Trolński, 2007). Ascorbic acid, by participating in the hydroxylation of cholesterol to bile acids, reduces its level in the blood and tissues, thereby preventing atherosclerosis (Das et al., 2006). According to some authors (Pereira and Gibson, 2002; Pătrăscanu et al., 2011), the presence of a probiotic in a feed additive has also reduced total and LDL cholesterol. A decrease in cholesterol content in the blood vessels of animals and humans is made possible by the assimilation and degradation processes taking place. Most of the cholesterol metabolized in the body is excreted in faeces in the form of bile acids. Probiotic substances probably increase the excretion of cholesterol in the faeces by inhibiting the formation of easily digested fat micelles (Mojka, 2014).

Conclusions

The probiotic in combination with vitamin C added to the diet of the piglets had a beneficial effect on the gut microbiota, causing a decrease in the number of bacteria of the family *Enterobacteriaceae* while increasing the number of lactobacilli. This

was probably due to the effect of vitamin C as a feed additive acidifying the gut microflora and inhibiting the growth of pathogenic bacteria, as well as to the effect of the probiotic protecting the intestinal wall against the penetration of pathogenic microbes. The use of this supplement in the diet also had a beneficial effect on red blood cell and lipid parameters of the blood.

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