Piglets' Intestinal Microflora Fed with a Plants Mix

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Abstract

The objective of this study was to evaluate the effect of a plant mix (bilberry, black currant, quince, peppermint and fennel essential oil) inclusion into the diets of piglets (18-45 kg) on intestinal microflora equilibrium. An experiment was performed on 8 castrated hybrid TOPIGS (18.69±1.25 kg) divided in 2 groups (C and E). The piglets were kept in an experimental house in individual metabolic cages. Compared to the conventional diet (18% crude protein and 3214 kcal/kg metabolic energy) of group C, the diet of E group had included 789 mg mixture of plants/kg feed. At the final of the experiment the piglets were slaughtered and digesta samples were collected from jejunum and ileum for microbiological analysis. For jejunum, a significant ($P<0.05$) decrease of *Staphylococci* spp. (log_{10} CFU/g) concentration in E group was noticed correlated strongly negative with a statistically significant ($P<0.05$) increasing concentration of *Lactobacillus* spp. (log_{10} CFU/g) concentration. The results were similar for ileum. This dietary mixture of plants had some effects on microbial population of piglets' jejunum and ileum to help positively the intestinal changes of microbiota.

Keywords: diet, microbiota, jejum, ileum , swine

INTRODUCTION

Overall health of young piglets has serious implications on performances, efficiency and economic losses. Gut microflora has significant effects on animal nutrition, health, and growth performance by interacting with nutrient utilization and the development of gut system of the animal (Hashemi and Davoodi, 2011).

The pigs' gastrointestinal microbiota has been studied to increase production efficiency, to improve products quality and help attempt to reduce disease. Gastrointestinal parameters and intestinal microflora are important markers of health status and nutrients metabolism in the animal organism (Lokesh *et al*., 2012).

Understanding the variations of microflora composition is crucial for a reduction antibiotics use in animal production, which are the main points of interest for improved animal health care and welfare and for consumer health protection (Vincenzo Motta *et al*., 2017).

To eliminate the suspicions concerning the antibiotics usage as growth promotor, to decrease residual meat contamination, to lower the environmental pollution, lots of studies focused on a wide variety of herbs, spices, and essential oils (Windisch *et al*., 2008).

Therefore as a result of these studies, antioxidant/antimicrobial/growth-promotor effects in pigs were demonstrated corroborated with an enhanced imunity and a digestibility increasing (Basmacioglu *et al*., 2004, Wei and Shibamoto, 2007). It is considered that natural feed components and herbs can sustain animal health, diminishing this way the number of disease outbreaks on a farm and helping to restrict the use of antibiotics.

Bilberry (*Vaccinium myrtillus L.) h*ave been reported to show direct antimicrobial effects against human pathogens, including *Salmonella* and *Staphylococcus aureus* (Puupponen-Pimiä *et al*., 2005 a,b). In a recent study of wild berries, bilberry juice inhibited adhesion of *Streptococcus pneumoniae* to human bronchial (Calu-3) cells, and bilberry juice also inhibited growth of *S. pneumoniae* (Huttunen *et al*., 2010).

Esposito *et al*. (2015) consider black currant (*Ribes nigrum L*.) a rich source of anthocyanins; however, the relationship between their apparently limited bioavailability and significant protection against metabolic pathologies is poorly understood.

Quince (*Cydonia oblonga*) was to little investigate as fruit waste source for animal feeding, although numerous nutritional human studies were done concerning the identification and quantification of the phenolic compounds. Fattouch *et al*. (2007) examined the antimicrobial activity of fruit aqueous acetone extracts against different microorganism strains and stated that quince peel extract was the most active for inhibiting bacteria growth with minimum inhibitory and bactericide concentrations in the range of 10^2–5×10^3 μg polyphenol/mL.

Essential oils and aromatic plants are well known to exert antibacterial, antifungal and antiviral activity in *in vitro* and *in vivo* experiments, inhibiting effects against pathogens such as *C. perfringens*, *E. coli* or *Eimeria* species and contributing to an improved intestinal integrity and protection against enteric disease (Windish *et al*., 2008).

Fennel (*Foeniculum vulgare mil*.) essential oil has very strong antioxidant, antimicrobial, and hepatoprotective activity (Ruberto *et al*., 2000; Ozbek *et al*., 2003). According to Schöne *et al*. (2006) in some nutritional studies fennel oil significantly decreased the feed intake of piglets.

Peppermint (*Mentha piperita*) essential oil was often used in pigs' experiment in oil blends (peppermint, anise and clove) as Maenner *et al*. (2011) used in weaner pigs diets with no effect on body weight gain, feed intake and gastrointestinal microbiota. Improvements of feed conversion ratio and amino acids digestibility were observed.

The objective of this study was to examine the effect of a plant mix inclusion (bilberry, black currant, quince, peppermint and fennel essential oil) with antibacterial properties into the diets of piglets (18-45 kg) on their intestinal microflora equilibrium, improving/maintaining the production parameters.

MATERIALS AND METHODS

The study protocol was approved by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotesti, IBNA Romania. The experiment has been carried out within IBNA experimental center facilities for 28 days, on 8 hybrid Topigs castrated males [(Large White×Pietrain)-female × (Talent)-male]. Pigs were divided into 2 homogenous groups (C, E). The animals were housed in individual metabolic cages (Agrico, Rybarska, Czech Republic) with an area of 0.87 m^2 with an average initial weight 18.75±1.71 kg for C group and 18.63±0.85 kg for E group. The compound feeds formulations (Tab. 1) were isoenergetic and isoprotein and consisted of corn, wheat, rice, soybean meal, rapeseed meal, gluten, powder milk, sunflower oil, choline premix, Zoofort (a vitamin-mineral premix IBNA's brand) in agreement with TOPIGS requirements.

The E group diet aimed to improve intestinal gut dysfunctions of young piglets using a mixture of plants and oils added into the basic feeding formula of C group (789 mg/kg feed).

The plants mixture (a mix of bilberry dried fruits, black currant dried fruits, quince dried fruits, peppermint and fennel essential oil) are originated from Romania so it could be easy to provide them for current animal feeding practices by the small farmers. Their chemical composition was analysed within laboratory of chemistry and nutrition physiology of INCDBNA (tab. 2).

Pigs were given *ad libitum* access to water and feed. They were individually weighed at the beginning, weekly and at the end of trial. The average daily gain (ADG), average daily feed intake (ADFI), and gain-feed ratio (G:F) were calculated. The microclimate parameters (temperature and humidity) were monitored. Feed consumption was recorded daily per pig.

The chemical analyses of the feed ingredients were done in the laboratory of chemistry and nutrition physiology of INCDBNA. The following physical-chemical methods have been used to characterise the feed ingredients and the compound feeds samples: dry matter (DM) - gravimetric method SR ISO 6498:2001; crude

Table. 1. Formulation of the experimental compound feed

Specification	Feeding formula
Corn, %	45.96
Wheat, %	10
Rice bran, %	10
Soybean meal, %	8
Rapeseed meal, %	13
Powder milk	5
Gluten, %	2
Oil, %	1
Lysine, %	0.5
Methionine, %	0.1
Calcium carbonate, %	1.75
Monocalcic phosphate, %	1.4
Salt, %	0.2
Choline, %	0.09
Zoofort*, %	1.00
Total	**100.00**
Calculated nutrients (g/kg feed)	
Dry matter, %	90.94
Metabolisable energy, kcal/kg	3214.00
Crude protein, %	18.00
Crude fat, %	5.00
Crude fiber, %	5.42
Calcium, %	1.02
Phosphorus,%	0.75

Note: * Vitamin mineral-premix; Ingredients per kilogram of diet: 10000 IU vitamin A, 2000 IU vitamin D_3, 30 IU vitamin E, 3 mg vitamin K_3, 2 mg vitamin B_1, 6 mg vitamin B_2, 13.5 mg d-pantothenic acid, 20 mg nicotinic, 3 mg vitamin B_6, 0.06 mg vitamin B_7, 0.8 mg vitamin B_9, 0.05 mg vitamin B_{12}, 10 mg vitamin C, 30 mg vitamin Mn, 110 mg Fe, 25 mg Cu, 100 mg Zn, 0.38 mg I, 0.36 mg Se, 0.3 mg Co, 60 mg antioxidant.

Table. 2. Chemical composition of analysed plants mixture

Specification (%)	Plant mixture
Dry matter	92.89
Crude protein	7.03
Ether extract	3.74
Crude fibre	14.02
Crude fat	11.96
Calcium	0.24
Phosphorus	0.25

protein (PB) - Kjeldhal method, Regulation (CE) nr. 152 /2009 and SR EN ISO 5983-2:2009, using a semiautomatic KJELTEC auto 2300 – Tecator (Sweden); ether extractives (EE) – extraction in organic solvents – Regulation (CE) nr. 152 /2009 and SR ISO 6492:2001, using an automatic system SOXTEC 2055 – Tecator (Sweden); crude fibre (CF) – method with intermediary filtration – Regulation (CE) nr. 152 /2009 and SR EN ISO 6865:2002, using FIBERTEC 2010 system–Tecator (Sweden); crude ash (Ash) – gravimetric method – Regulation (CE) nr. 152 /2009 and SR EN ISO 2171:2010, using calcination oven Caloris CL 1206; metabolisable energy (ME) – was calculated from the chemical composition. Microbial populations from jejunum and ileum content samples were determined by counting the colonies on selected media for each microorganism. The analysis of *Escherichia coli* (*E. coli*) consists of the successive sowing of the sample in Durham fermentation tube tubes and the band for identifying the indole in the lauryl sulphate medium followed by incubation at 44°C for 24-48 h. It is usually run 3 dilutions/sample and seed three test tubes/dilution. The positive tubes were scored and the number of *E. coli* colonies according to the McCrady table. The result is expressed as the number of *E. coli* colonies/1g produced.

The analysis of *Salmonella* bacteria consists in sowing the sample to be analysed on preimmigration media (selenite-cystine medium) followed by incubation for 24 h at 37°C, then performing grooving with selective media (Mac Conckey medium and Rambach medium), at least 2 Petri dishes each medium, and incubated for 24-48 h at 37°C. After incubation, the boxes are analysed on Mac Conckey *Salmonella* environment, transparent, colourless (slightly colourless) transparent are formed on Rambach's medium form red, violet, round, humid, slightly bulging colonies.

The analysis of *Staphylococcus* spp. consists in submerging 10 g of the product in liquid hyperchlorinated medium and in sowing from successive dilutions (10^{-3}-10^{-8}) either on solid medium or on selective medium for *Staphylococcus* spp. (Baird Parker medium with egg yolk and sodium tellurite); incubation at 37°C for 24-48 hours, followed by counting of colonies developed (only boxes containing 30-300 colonies are considered), the arithmetic mean of the number of colonies in the boxes of the same dilution is calculated, multiplied by the dilution said. The result is expressed as number of germs/1 g of product.

The *Lactobacillus* spp. analysis consists in immersing 10 g of the product in liquid MRS medium and then sowing from successive dilutions (10^{-8}-10^{-9}) on MRS agar and incubating at 37°C for 24-48 hours, followed by counting of the developed colonies, calculate the arithmetic mean of the number of colonies in the boxes of the same dilution, multiply with that dilution. The result is expressed as the number of lactic bacteria/1 g of product. All microbiological concentrations were subjected to base-10 logarithm transformation before analysis.

The data obtained were analysed using StatView software. One-way analysis of variance (ANOVA) was used to evaluate statistical significance of differences between dietary treatments. The results are given as means and standard error of the mean (SEM). Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSIONS

Figure 1 shows no significant differences ($P>0.05$) between the two groups concerning the final weight of the piglets, C group had 45±2.68 kg and E group had 45.25 ±0.96 kg.

The feed conversion had a slightly lower value for E group (2.25±0.33), but without significant differences ($P>0.05$) compared to C group (2.30±0.36). In general, the plants mix inclusion appear to have no effect on final body weight nor feed conversion. In a literature review, Panagiotis (2017) observed a feed conversion improvement when essential oils were used in monogastric experiments. Improve nutrient utilization and animal performance were observed by Viveros *et al.*, (2011); Dueñas *et al.*, (2015); Brenes *et al.*, (2016) when using phenolic compounds sources in piglets feed.

Weight gain, feed conversion of pigs fed essential oils was essentially equal to that of pigs fed antibiotics. The improved performance appeared to be mediated by improvements in dry matter and protein digestibility arising from improvements in intestinal morphology (Li *et al.*, 2012).

When plant extract are used a swine gut health is correlated with growth performances. Regarding this matter, Vigors *et al*. (2016) reported a correlation between high feed efficiency and increased abundance of *Lactobacillus* spp. in the caecum of pigs.

Within table 3 it was noticed a significant (P=0.026) decrease in jejunum of *Staphylococcus* spp. concentration and a statistically very significant (P<0.0001) increasing concentration of *Lactobacillus* spp. on E group compared to C group. On the entire experimental period, regarding both groups a strongly negative correlation (r=-0.864) it was noticed between the decreasing concentration of *Staphylococcus* spp. and the increasing concentration of *Lactobacillus* spp. concentration. Also, it was registered a lower value of E. coli concentration on E group but without statistical significance (P>0.05).

It is proven that phenolic compounds (cyanidin, pelargonidin, delphinidin, etc) from sources as black currant, elderberry fruit, blackberries have bactericidal (Gordon and Wareham, 2010) and bacteriostatic properties (Etxeberria *et al*., 2013), they minimize the adhesion of pathogenic bacteria (*E. coli, Clostridium*), inhibit the progression of infections in the digestive tract.

By enhancing the proliferation of beneficial bacteria (*Bacillus* spp., *Lactobacillus* spp.) and stabilizing gut microflora, polyphenols indirectly

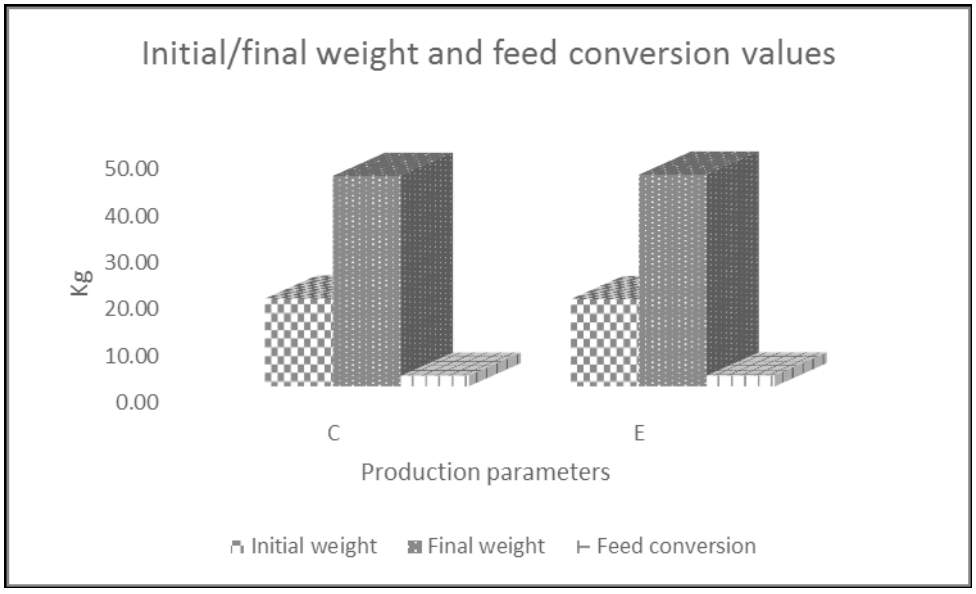

Figure 1. Growth performances values of the experiment

Table. 3. The effect of plants mixture from pigs' diet on jejunum microflora

Specification	Control	Experimental	SEM	*P-value*
E. coli (log_{10}CFU/g)	4.096	4.081	0.008	*0.364*
Salmonella spp. (log_{10}CFU/g)	absent	absent	-	-
Staphylococcus spp. (log_{10}CFU/g)	3.637 [a]	3.519 [b]	0.028	*0.026*
Lactobacillus spp.(log_{10}CFU/g)	5.018[b]	5.105[a]	0.017	*<0.0001*

Note: *Values with the different superscript in the same raw are significantly different (*P* <0.05).

enhance the host's immune system and overall health (Hashemi and Davoodi, 2011; Paszkiewicz *et al.*, 2012). They can exert a positive influence on gut morphology and improve nutrient absorption in monogastric animals (Kamboh *et al*., 2015). Also, *Lactobacillus* spp., are the most commonly used microorganisms as probiotics because of the perception that they are desirable members of the intestinal microflora and because these bacteria have "Generally Recognized As Safe" (GRAS) status (Shokryazdan, 2014).

Within table 4 the microbiological analyses from ileum showed a very significant statistically (P<0.0001) lower concentration of *Staphylococcus* spp. and a significantly higher concentration of *Lactobacillus* spp. (P=0.01) in E compared with C group. On the entire trial, regarding both groups, a strongly negative correlation (r=-0.8447) it was noticed between the decreasing concentration of *Staphylococcus* spp. and the increasing concentration of *Lactobacillus* spp. concentration. Also, a reduction tendency of *E. coli* concentration in the E group was noticed (P=0.062).

A very strong relation between production of antimicrobial compounds by lactobacilli and, therefore, prevention of pathogenic micro-organisms such as *Salmonella*, *E. coli* and *Clostridia* was also observed in previous studies (Naaber *et al*., 2004; Coconnier-Polter *et al*., 2005; Bernbom *et al*., 2006). Succession of *Lactobacillus* spp. in the ileum largely depends on their growth rate and ability to adhere the intestinal mucus layer, thus avoiding the "wash-out" effect of the digesta flow (Tannock, 1995).

Jamroz and Kamel (2002) suggested that supplementation with plant extracts increases the digestibility of nutrients and promotes a balanced microflora. This reduces the potential for adhesion of pathogens to the intestinal epithelium, representing one of mechanisms by which the symbiotic acts.

According to Fuller *et al.* (1989), beneficial bacteria would be favoured in the gastrointestinal tract, preventing colonization of pathogenic bacteria in the mucosa.

CONCLUSION

The experiment demonstrated that the pigs ate the feed with the plant mixture without any sign of taste dislike or reduction of feed intake reflected in similar performances with control group. Also, this experiment demonstrated that this mixture of plants included in piglets' diet had some effects on microbial population of jejunum and ileum, therefore it can be used to help positively the changes of the intestinal microbiota.

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Table. 4. The effect of plants mixture from pigs' diet on ileum microflora

Specification	Control	Experimental	SEM	*P-value*
E. coli (log_{10}CFU/g)	6.258	5.586	0.186	*0.062*^{T}
Salmonella (log_{10}CFU/g)	absent	absent	-	-
Staphylococcus spp. (log_{10}CFU/g)	6.183 ^{a}	5.708 ^{b}	0.090	*<0.0001*
Lactobacillus spp. (log_{10}CFU/g)	6.212 ^{b}	6.240 ^{a}	0.006	*0.01*

Note: *Values with the different superscript in the same raw are statistically different (P <0.05);
^{T} denote a tendency that the results were influence by treatment (P <0.10).

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