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Original Research Article

Effect of fosfomycin, *Cynara scolymus* extract, deoxynivalenol and their combinations on intestinal health of weaned piglets

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ABSTRACT

Weaning is a challenging stage of pig farming. Animals undergo environmental, social and dietary changes leading to weaning stress syndrome. In order to compensate for the detrimental effects of weaning stress, antibiotics and natural extracts are used as feed additives, sometimes without fully understanding the interactions between them or even with low concentrations of mycotoxins that are frequently present in feed. The aim of this study was to evaluate the effect of fosfomycin (FOS), *Cynara scolymus* extract (CSE), deoxynivalenol (DON) and their combined administration on intestinal health of weaned piglets. The experiment was designed as a $2 \times 2 \times 2$ factorial arrangement with 3 factors (FOS, CSE and DON treatments), 2 levels each (presence and absence) and 3 repeats. Weaned piglets ($n = 24$) were randomly divided in groups to receive the different treatments, namely DON administered in diet (50 μg/kg BW), FOS administered into the drinking water (30 mg/kg BW), CSE administered in diet (15 mg/kg BW) and all their combinations. After 15 d, the animals were euthanized and gastrointestinal tract samples were immediately taken to evaluate gastrointestinal pH, *Enterobacteriaceae* to lactic acid bacteria (E:L) ratio, volatile fatty acid (VFA) concentrations, disaccharidase (lactase, sucrase and maltase) activity, histology (intestinal absorptive area [IAA] and goblet cells count) and mucus ability to adhere pathogenic *Escherichia coli*. From our results, FOS and CSE treatments, individually or combined, produced a lower E:L ratio, an enhanced production of butyrate, increased disaccharidase activity (particularly maltase), and a greater IAA and goblet cells count along with an increase in pathogenic bacteria adherence to intestinal mucus. Deoxynivalenol did not show interactions with the other factors and its administration produced decreases on VFA, disaccharidase activity and goblet cells count. In conclusion, weaning piglets receiving diets containing FOS, CSE or both exhibited evident beneficial intestinal effects compared to animals receiving diets free from these compounds. On the contrary, the presence of DON at sub-toxic concentrations produced detrimental effects on intestinal health. The knowledge of the

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physiological and pathological gut changes produced by these compounds contributes to understand their potential productive consequences.

1. Introduction

Weaning is considered one of the most critical periods of pig production because of its high negative impact on health and productive performance of piglets, mainly in the first post-weaning days. During this period, the animals are exposed to physiological, immunological, microbiological, social, environmental and nutritional factors that lead to post-weaning stress (Hampson and Kidder, 1986; Lallès et al., 2007; Heo et al., 2013). In order to overcome this situation, the prophylactic use of antibiotics has become a common practice. Fosfomycin (cis 1-2 epoxy propyl phosphonic acid, FOS) is a broad spectrum bactericide antibiotic, widely used in pig farms in Central and South America, South Africa and Southeast Asia. At weaning, FOS is indicated for the treatment of several bacterial infections (*Haemophilus parasuis, Streptococcus suis, Pasteurella multocida, Bordetella bronchiseptica, Staphylococcus hyicus, Escherichia coli*, etc.) associated to stress (Martineau and Morvan, 2010).

On the other hand, vegetable extracts, particularly *Cynara scolymus* extract (CSE), have long been used in different species for their hepatoprotective and digestive roles. In animal production, these compounds are used as feed additives to improve zootechnical parameters as body weight gain, feed intake and feed conversion ratio (Abbasi and Samadi, 2014; Martínez and Uculmana, 2016; Saeed et al., 2017). In addition, they have shown further beneficial consequences on intestine and liver functions. *C. scolymus* extract is obtained from the leaves of the plant and contains caffeolquinic acid derivatives which are known for their choleretic – cholagogue effect in different species (Speroni et al., 2003; Martínez and Uculmana, 2016), including pigs (Martínez et al., 2018). As a consequence, increased bile concentrations at small intestine level enhance fat and lipophilic vitamins absorption. Furthermore, entero-protective, trophic, antitoxic and antimicrobial effects are ascribable to bile action (Bertók, 2004; Inagaki et al., 2006; Mikov et al., 2006; Chiang, 2009). These functions provide important benefits during stressful periods like weaning.

Among weaning stress factors, the presence of anti-nutritional compounds in feed, such as mycotoxins, negatively influence productive performance of animals. Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium* species, being pigs the most susceptible species to its toxic effects (Rotter, 1996; Piotrowska et al., 2014). Formerly DON was also called vomitoxin, referring to its emetic effect (Vesonder et al., 1973; Eriksen and Pettersson, 2004). Other clinical signs of intoxication with DON include reduction in feed intake and complete feed refusal, immunosuppression, haemorrhage and eventually, circulatory shock (Lawlor and Lynch, 2001; Pestka, 2007; Alizadeh et al., 2015). However, there is little information on the possible subclinical effects associated to the ingestion of feed contaminated with low DON concentrations, which is highly likely to occur (Eriksen and Pettersson, 2004; Roigé et al., 2009; Waché et al., 2009).

In intensive pig production, in innumerable situations, but mostly during weaning, antibiotics, natural extracts and low concentrations of mycotoxins coexist in the animals' diet, and consequently in the gut, regardless the potential interactions among them. We hypothesize that the presence of these compounds, individually or combined, influence intestinal health. The aim of this study was to evaluate the effect of FOS, CSE, DON and their combined administration on the intestinal health of weaned piglets.

2. Materials and methods

The study was carried out according to guidelines of the Animal Welfare Committee of the Faculty of Veterinary Sciences UNCPBA, Argentina, for animal handling and experimentation.

2.1. Experimental design

The experiment was designed as a $2 \times 2 \times 2$ factorial treatment arrangement with 3 factors (FOS, CSE and DON treatments), 2 levels each: presence and absence and 3 repeats.

Twenty-four weaned piglets were randomly distributed in order to receive, daily, one of the following combinations of factors: DON (50 μg/kg BW); FOS (30 mg/kg BW); CSE (15 mg/kg BW); DON (50 μg/kg BW) + FOS (30 mg/kg BW); DON (50 μg/kg BW) + CSE (15 mg/kg BW); CSE (15 mg/kg BW) + FOS (30 mg/kg BW); DON (50 μg/kg BW) + CSE (15 mg/kg BW) + FOS (30 mg/kg BW) and a balanced diet without FOS, CSE or DON. Thus, each level of each factor was assigned to 12 piglets.

Hereunder, animals receiving any of the treatments will be referred to as FOS, CSE or DON treated animals and as FOS, CSE or DON free animals in the absence of the corresponding factors.

2.2. Animals

Twenty-four, healthy, 21-d-old, just weaned piglets (6.26 ± 0.4 kg BW) of the same genetic line from a commercial farm were used. Piglets receiving each combination of factors were housed in pens inside an environmentally controlled barn (22 ± 5 °C; 12:12 light–dark cycles; relative humidity 45% to 65%), given free access to feed (commercial feed, Perfecto Transición, Biofarma S.A.: 3,325 kcal/kg of metabolizable energy; Table 1) and water, and checked daily for clinical symptoms of DON intoxication.

Feed and water consumption were monitored in order to assure that total doses of each treatment were taken. After 15 d of treatment, piglets of each group were euthanized for sampling of the gastrointestinal tract.

2.3. Antibiotic, natural extract and mycotoxin

Fosfomycin (FOS): calcium fosfomycin (Fosbac) was provided by Bedson S.A. laboratory (Pilar, Buenos Aires, Argentina). The antibiotic was dissolved in drinking water. Water consumption was measured by a water flow meter installed at the entrance pipeline of the weaning room 2 d before the beginning of the trial. Medicated water was prepared daily at 08:00, considering water consumption and mean piglets weight in order to obtain the adequate dosage. A FOS dose of 30 mg/kg per d was used as this is the recommended prescription for the treatment of systemic infections at weaning (Pérez et al., 2012; Soraci et al., 2014).

C. scolymus extract (CSE): this natural extract (Bedgen40) was provided by Bedson S.A. laboratory (Pilar, Buenos Aires, Argentina). Three hundred grams of CSE were uniformly mixed with 1 t of feed.

Table 1
Ingredients and analysed composition of the basal diet (as-fed basis, %).

Item	Content
Ingredients[1]	
Corn	66.67
Soybean pellet	23.33
Soybean expeller	6.67
Premix[2]	3.33
Analysed composition	
Metabolizable energy, kcal/kg	3,325
Minimum protein content	20.00
Maximum humidity	12.00
Maximum mineral content	3.8
Ether extract	5.00
Maximum crude fibre	2.00
Minimum calcium content	8.00
Minimum total phosphorous content	4.00
Total lysine	1.62
Total methionine	0.437
Total tryptophan	0.275
Total threonine	1.085
Lactose	12.00
Sodium	0.28
Chloride	0.25
Average available phosphorous	3.80

[1] Ingredients contents meet nutritional requirements of weaning pigs (Rostagno et al., 2017).
[2] Premix includes vitamins, minerals and other protein and carbohidrates sources.

In this way, each piglet received 15 mg/kg per d. This dose was based on studies in which choleretic and cholagogue effects of CSE in pigs have been demonstrated (Martínez et al., 2018).

Deoxynivalenol (DON): the mycotoxin was produced in our laboratory by growing *Fusarium graminearum* NRRL 28063 in corn at 25 °C for 25 d. For DON quantification, samples of ground corn were extracted twice with water or acetonitrile and then with hexane by liquid–liquid extraction. Extracts were passed through columns (DONPREP, Glascow, Scotland) and evaporated to dryness at 40 °C. The dry extract was reconstituted with MilliQ water and filtered through 0.22 μm nylon membranes before injection into HPLC UV/VIS for quantification. A Gilson HPLC system equipped with a Gilson 151 UV–Vis detector and Gilson 712 software was used for data analysis (Gilson, Inc., Middleton, USA). The column (250 mm × 3.00 mm, 4 μm; Sinergy Hydro-RP C18, Phenomenex, Torrance, USA) was maintained at 35 °C. The mobile phase was a water-acetonitrile mixture (90%, wt/wt) at 0.5 mL/min flow rate. Deoxynivalenol was detected at 222 nm and its retention time was 8.7 min.

Feed was previously analyzed in order to confirm the absence of mycotoxins using the method described by Roigé et al. (2009). Aliquots of ground contaminated corn were uniformly mixed with feed in order to obtain 1 mg DON/kg. This is considered a low level of this mycotoxin, which is likely to occur in feed, but for which no clinical signs have been observed other than reduced feed intake (Eriksen and Pettersson, 2004; Yeong-hsiang et al., 2006; Chaytor et al., 2011).

2.4. Gastrointestinal tract study

2.4.1. pH determination

As soon as each sample was obtained, pH was measured with a pH meter (UP-25, Denver Instrument Company, Denver, Colorado) in the caudal portion of the stomach, ileum (15 cm proximal to ileocecal valve), caecum and colon (20 cm distal from caecum). The pH meter was calibrated in the range of pH 4 to 7 strictly following manufacturer's instructions.

2.4.2. Enterobacteriaceae to lactic acid bacteria (E:L) ratio

The intestinal contents from caecum and colon (20 cm distal from caecum) were collected and kept at 4 °C until arrival to the laboratory, within a maximum period of 4 h. One gram of sample was immediately diluted in 9 mL of peptone water and homogenized by continuous agitation. Bacteria cultures were performed by plating serial 10-fold dilutions (in 1% peptone water) onto MRS agar (Britania S.A.) for lactic acid bacteria (LAB), representative of beneficial bacteria in pigs, and onto Mac Conkey agar (Britania S.A.) for *Enterobacteriaceae*, representative of commensal Gram negative bacteria (Macconkey, 1900; De Man et al., 1960; White et al., 2002; Mikkelsen and Jensen, 2004). Colonies were counted, log transformed and expressed as $\log_{10}$ of colony forming units per gram of digesta ($\log_{10}$ CFU/g).

2.4.3. Volatile fatty acids (VFA)

One gram of caecal contents was immediately diluted with phosphoric acid (4:1, wt./wt.) for preservation and kept at −70 °C until analyzed. Concentrations of VFA were determined using gas liquid chromatography according to the method described by Jouany (1982). A Shimadzu chromatograph (Model GC–17A, Kyoto, Japan) with a 19091N-133 Innowax 30M column (Agilent, Santa Clara, CA, USA) was used. A mixture of 10 mmol/L Supelco VFA (C2 to C10) and 2-ethyl-butyric acid (Fluka) as internal standard were used to build calibration curves.

2.4.4. Disaccharidases activity

Four portions of the small intestine (duodenum, proximal and medium jejunum and ileum) were opened along the mesenteric border and washed with saline solution to eliminate the mucus and remaining intestinal contents. The mucosa was carefully scraped off with a scalpel and 1 g of this material was weighed. Then, saline solution (2 mL) was added and the intestinal mucosa was ground with a dispersing instrument (Ultra-Turrax) and a Potter homogenizer. Samples were then cold-centrifuged at 4 °C and 4,825 × *g* for 10 min. The supernatant represented the crude enzyme solution and it was stored at −70 °C until analysis. Protein concentration of each homogenate was determined by Bradford method using bovine serum albumin as standard (Bradford, 1976). The activity of sucrase, lactase and maltase was determined by quantification of released glucose, according to Dahlqvist method (Dahlqvist, 1964). Briefly, the homogenate supernatants were diluted, added to an equal volume of 0.1 mol/L sodium maleate buffer (pH 6.0) containing 56 mmol/L lactose, sucrose or maltose, and incubated for 1 h at 37 °C. Then, the mixtures were added to the glucose oxidase–peroxidase reagents (Sigma Chemical Company, USA) containing O-dianisidine as chromogen. The absorbance was measured using a spectrophotometer (Dupont, Sorvall Instruments) at 450 nm. Disaccharidases activity was expressed as U/mg protein. One U is defined as the amount of enzyme that hydrolyses 1 mmol of lactose, sucrose or maltose in 1 min under the standard assay conditions.

2.4.5. Histological study

Samples of medium jejunum (1.5 m from stomach) and ileum (20 cm proximal to ileocecal valve) were washed with saline solution to remove intestinal contents, transversally cut and fixed in Bouin solution (75% saturated picric acid, 20% formaldehyde and 5% acetic acid). After 24 h, the samples were embedded in paraffin and stained with haematoxylin-eosin (H&E) and periodic acid-Schiff (PAS).

The intestinal mucosa was examined under light microscope and analyzed using the Image Analysis Software (ToupTek ToupView). The length and width of villi and width of crypts were measured in H&E-stained sections. Fifty villi and 50 crypts were

measured from each section, and mean villi widths and heights and crypt widths were calculated. The mathematical model proposed by Kisielinski et al. (2002) was used to estimate the intestinal absorptive area (IAA) using the following equation:

$$IAA = (villusW \times villusL) + (villusW/2 + cryptW/2)^2 - (villusW/2)^2 \Big/ (villusW/2 + cryptW/2)^2,$$

where, *IAA* = intestinal absorptive surface area, *villusW* = villi mean width, *villusL* = villi mean length, and *cryptW* = crypts mean width.

Goblet cells in villi and crypts (expressed as goblet cells/100 villi or crypts) were determined using PAS staining and mean values were calculated (Buddle and Bolton, 1992; Burrin et al., 2001).

2.4.6. Adherence of bacteria to intestinal mucus

Ileum samples (15 cm proximal to ileocecal valve) were opened along the mesenteric border. Mucus was carefully scraped off with a scalpel (leaving intestinal mucosa intact), collected into sterile tubes and kept at −70 °C until analyzed. Adherence of bacteria to mucus was assessed according to Bai et al. (2000). Briefly, 100 mg of mucus were diluted with 1.5 mL of saline solution and centrifuged (14,400 × *g*, 10 min, 4 °C) to remove cell debris and bacteria. The supernatant was sterilized by filtration (13 mm × 0.22 μm nylon filter membranes) and the filtered solution was defined as the original crude mucus that contained glycoproteins responsible for bacteria adherence. A concentration of 10^3 CFU/mL of *E. coli* O157:H7 was incubated with supernatant containing crude mucus for 30 min, at 37 °C under continuous agitation. Then the tubes were centrifuged (14,400 × *g*, 10 min, 4 °C) and pellets (with adhered and not adhered bacteria) were resuspended in 400 μL saline solution and further centrifuged (400 × *g*, 4 °C, 2 min). Two fractions were obtained, the pellet which contained adhered bacteria and the supernatant which contained not adhered bacteria. Aliquots from pellet and supernatant were spread on Mac Conkey Agar with Sorbitol (Britania S.A.) and incubated under aerobic condition for 24 h at 37 °C for colonies count. Results were expressed as percentage of adhered bacteria to the intestinal mucus.

2.5. Statistical analyses

The response variables (pH, intestinal bacteria, VFA, disaccharidases activity, IAA, goblet cells and percentage of adhered bacteria to intestinal mucus) were analyzed by ANOVA by GLM procedure of SAS V9.3 (SAS Institute Inc., Cary, NC, USA). Differences between treatments were declared significant when $P < 0.05$. When statistically significant interactions were observed, contrasts (using Tukey's test) were carried out to compare the different levels of each treatment. Data are presented in tables as means and mean standard error (SEM).

3. Results

3.1. pH

Effects of interactions among different factors on pH values were not observed ($P > 0.05$).

Mean pH values obtained for each treatment are shown in Table 2. No statistically significant differences were obtained between CSE or DON treated and CSE or DON free animals in the gastrointestinal portions analyzed. Additionally, FOS treated animals showed no statistically significant effects in caudal portion of stomach and ileum, but they showed a lower pH ($P < 0.01$) in caecum and colon, (5.51 ± 0.26 and 6.21 ± 0.39, respectively), compared to FOS free animals (6.66 ± 0.32 and 7.25 ± 0.27, respectively).

3.2. Enterobacteriaceae, lactic acid bacteria and Enterobacteriaceae to lactic acid bacteria (E:L) ratio

3.2.1. Lactic acid bacteria

Effects of interactions among different factors on LAB $\log_{10}$ CFU/g values were not observed ($P > 0.05$).

Results did not show statistically significant differences between FOS, CSE or DON treated and FOS, CSE or DON free animals ($P > 0.05$).

3.2.2. Enterobacteriaceae

The co-administration of CSE and FOS at colon level resulted in a reduction of *Enterobacteriaceae* $\log_{10}$ CFU/g and E:L ratio ($P < 0.05$) being the outcome similar to that observed when the factors were administered individually. At caecum level, an interaction was found between CSE and FOS ($P < 0.05$) but the reduction in *Enterobacteriaceae* $\log_{10}$ CFU/g and E:L ratio was less evident than in colon. No further interactions were observed among factors.

No statistically significant differences were found for *Enterobacteriaceae* $\log_{10}$ CFU/g and E:L ratio between DON treated and DON free animals.

Fosfomycin and CSE treated animals exhibited lower *Enterobacteriaceae* $\log_{10}$ CFU/g and lower E:L ratio, in caecum and colon, than FOS and CSE free animals ($P < 0.05$) (regardless the presence of DON). Results are shown in Table 3.

3.3. Volatile fatty acids

Effects of interactions among different factors on VFA concentrations were not observed ($P > 0.05$).

C. scolymus extract and FOS treated animals showed numerically higher concentrations of acetate, propionate, butyrate and total VFA than CSE and FOS free animals ($P > 0.05$ except for butyrate concentrations in CSE treatment where $P < 0.05$). Deoxynivalenol treated animals showed significantly lower acetate, propionate and butyrate concentrations, and consequently lower total VFA concentrations than DON free animals ($P < 0.05$ for all acids). Results are shown in Table 4.

3.4. Disaccharidases activity

Effects of interactions among different factors on disaccharidase activity were not observed ($P > 0.05$).

Disaccharidases activity mean values for each treatment are shown in Table 5. It was found that maltase activity in all intestinal portions from FOS treated animals was significantly higher than that observed for FOS free animals ($P < 0.05$). Sucrase and lactase activity also increased in jejunum and ileum of FOS treated animals, though this effect was not statistically significant.

C. scolymus extract treated animals showed a higher maltase activity in ileum compared to CSE free animals ($P < 0.05$). However, an effect on sucrase and lactase activity was not observed ($P > 0.05$).

Deoxynivalenol treated animals showed lower disaccharidase activity than DON free animals in all intestinal portions. $P < 0.05$ was observed for maltase activity in duodenum, proximal jejunum and ileum, sucrase activity in medium jejunum and lactase activity in duodenum and jejunum.

Table 2
Effect of fosfomycin (FOS), Cynara scolymus extract (CSE) and deoxynivalenol (DON) on the gastrointestinal pH ($n = 12$).

Item	FOS, mg/kg BW		*P*-value	CSE, mg/kg BW		*P*-value	DON, μg/kg BW		*P*-value
	0	30		0	15		0	50	
Stomach	3.50 ± 0.18	3.49 ± 0.27	0.9613	3.48 ± 0.15	3.51 ± 0.28	0.9458	3.64 ± 0.25	3.35 ± 0.19	0.4063
Ileum	7.24 ± 0.24	6.95 ± 0.37	0.5329	6.94 ± 0.32	7.24 ± 0.31	0.5283	6.93 ± 0.34	7.25 ± 0.29	0.4947
Caecum	6.66 ± 0.32	5.51 ± 0.26	0.0123	6.18 ± 0.34	5.99 ± 0.34	0.6372	5.81 ± 0.29	6.36 ± 0.37	0.1962
Colon	7.25 ± 0.27	6.21 ± 0.39	0.0376	6.90 ± 0.35	6.56 ± 0.38	0.4816	6.33 ± 0.37	7.13 ± 0.34	0.1124

Table 3
Effect of fosfomycin (FOS), Cynara scolymus extract (CSE) and their combination on the Enterobacteriaceae and Enterobacteriaceae to lactic acid bacteria (E:L) ratio ($n = 6$).

Item	0 FOS		30 mg/kg BW FOS		*P*-value
	0 CSE	15 mg/kg BW CSE	0 CSE	15 mg/kg BW CSE	FOS × CSE
Enterobacteriaceae, log10 CFU/g					
Caecum	5.85 ± 0.60[a]	3.01 ± 0.37[b]	2.42 ± 0.84[b]	4.35 ± 0.62[ab]	0.0012
Colon	6.49 ± 0.44[a]	2.87 ± 0.41[b]	2.53 ± 0.86[b]	3.39 ± 0.48[b]	0.001
E:L ratio					
Caecum	0.79 ± 0.07[a]	0.43 ± 0.05[b]	0.37 ± 0.13[b]	0.60 ± 0.09[ab]	0.0056
Colon	0.85 ± 0.04[a]	0.41 ± 0.07[b]	0.38 ± 0.13[b]	0.44 ± 0.07[b]	0.0114

FOS × CSE = combination of FOS and CSE.
[a, b] Within a row, mean values with different superscript letter differ significantly ($P < 0.05$).

Table 4
Effect of fosfomycin (FOS), Cynara scolymus extract (CSE) and deoxynivalenol (DON) on volatile fatty acid (VFA) concentrations (mmol/L) in the caecum of weaned piglets ($n = 12$).

Item	FOS, mg/kg BW		*P*-value	CSE, mg/kg BW		*P*-value	DON, μg/kg BW		*P*-value
	0	30		0	15		0	50	
Acetate	47.36 ± 6.11	60.35 ± 7.31	0.1363	46.51 ± 6.29	61.20 ± 7.00	0.0945	63.58 ± 7.78	44.12 ± 4.55	0.0307
Propionate	13.27 ± 1.71	16.44 ± 1.60	0.0698	13.19 ± 1.74	16.51 ± 1.56	0.0588	18.56 ± 1.41	11.15 ± 1.21	0.0002
Butyrate	5.44 ± 0.80	6.78 ± 1.03	0.1493	5.17 ± 0.85	7.05 ± 0.96	0.0481	8.24 ± 0.91	3.98 ± 0.39	0.0001
Total VFA	68.47 ± 8.72	85.53 ± 9.69	0.1291	67.39 ± 8.96	86.61 ± 9.29	0.0897	93.20 ± 9.93	60.81 ± 6.08	0.0070

Table 5
Effect of fosfomycin (FOS), Cynara scolymus extract (CSE) and deoxynivalenol (DON) on disaccharidases activity (U/mg protein; $n = 12$).

Item	FOS, mg/kg BW		*P*-value	CSE, mg/kgBW		*P*-value	DON, μg/kg BW		*P*-value
	0	30		0	15		0	50	
Duodenum									
Maltase	1,440.60 ± 183.45	2,422.91 ± 338.39	0.0012	1,951.87 ± 350.73	1,911.64 ± 262.55	0.8743	2,462.37 ± 339.66	1,401.15 ± 159.56	0.0006
Sucrase	39.82 ± 16.30	36.86 ± 8.85	0.8841	41.32 ± 15.17	35.36 ± 10.62	0.7688	41.69 ± 10.17	34.99 ± 15.46	0.7414
Lactase	88.42 ± 31.15	88.19 ± 27.68	0.9951	72.60 ± 18.06	104.00 ± 36.96	0.4024	141.09 ± 33.98	35.51 ± 8.68	0.0106
Proximal jejunum									
Maltase	2,001.63 ± 249.50	3,419.02 ± 456.61	0.0043	2,727.34 ± 465.01	2,693.32 ± 381.81	0.9374	3,420.65 ± 461.72	2,000.01 ± 239.05	0.0042
Sucrase	96.48 ± 31.11	133.11 ± 51.55	0.5497	133.87 ± 28.64	95.72 ± 52.92	0.5334	145.62 ± 53.67	83.97 ± 25.16	0.3190
Lactase	171.45 ± 52.60	257.84 ± 91.37	0.3935	214.62 ± 55.72	214.67 ± 91.38	0.9996	324.52 ± 93.05	104.78 ± 24.51	0.0383
Medium jejunum									
Maltase	2,795.56 ± 306.05	4,333.48 ± 493.03	0.0097	3,854.78 ± 549.21	3,274.27 ± 356.80	0.2844	4,038.04 ± 432.94	3,091.00 ± 464.81	0.0896
Sucrase	174.12 ± 51.79	284.39 ± 66.33	0.1369	259.65 ± 63.40	198.85 ± 58.71	0.4006	319.49 ± 69.41	139.02 ± 36.55	0.0208
Lactase	212.66 ± 66.42	342.47 ± 79.00	0.1627	316.65 ± 72.98	238.48 ± 76.25	0.3931	407.83 ± 80.99	147.30 ± 42.11	0.0087
Ileum									
Maltase	1,071.56 ± 138.01	1,464.9 ± 265.51	0.0456	947.73 ± 122.61	1,588.72 ± 250.73	0.0028	1,623.23 ± 259.01	913.22 ± 81.07	0.0012
Sucrase	15.27 ± 9.86	59.01 ± 45.49	0.3441	15.72 ± 9.81	15.33 ± 5.18	0.3539	57.68 ± 45.56	16.59 ± 10.07	0.3733
Lactase	11.24 ± 3.64	18.26 ± 5.51	0.1875	14.16 ± 4.36	58.55 ± 45.54	0.8208	15.69 ± 4.86	13.80 ± 4.70	0.7159

3.5. Intestinal absorptive area and goblet cells

3.5.1. Intestinal absorptive area

At medium jejunum and ileum, interactions between DON and the other factors were not observed ($P > 0.05$). Deoxynivalenol treated animals showed no statistically significant differences compared to DON free animals.

At medium jejunum, the co-administration of CSE and FOS resulted in a lower effect than both individual factors ($P < 0.05$). In this way, FOS and CSE treated animals exhibited higher IAA compared to FOS and CSE free animals ($P < 0.05$).

At ileum level, effects of interactions between FOS and CSE on IAA were not observed ($P > 0.05$). Values were higher in CSE treated animals than in CSE free animals ($P < 0.05$). No differences were observed between FOS treated, FOS free animals ($P > 0.05$). Results are shown in Table 6.

3.5.2. Goblet cells

Interactions among different factors on goblet cells counts were not significant ($P > 0.05$).

Fosfomycin and CSE treated animals exhibited higher goblet cells counts in crypts than FOS and CSE free animals ($P < 0.05$) in

both intestinal portions, while no significant differences were found in villi ($P > 0.05$). For DON treated animals, lower goblet cells counts were obtained in villi of both intestinal portions ($P < 0.05$) but no effect was observed at crypts level. Results are shown in Table 7.

3.6. Adherence of bacteria to the intestinal mucus

The co-administration of CSE and FOS resulted in an increase of adherence of *E. coli* to intestinal mucus ($P < 0.05$) being the outcome similar to that observed when the factors were administered individually (81.61%). Interactions between DON and the other factors were not observed ($P > 0.05$).

Adhesion increased from 44.57% in FOS and CSE free animals to 83.67% and 73.14% for FOS and CSE treated animals, respectively ($P < 0.05$). Results are shown in Table 8. No significant differences in *E. coli* adhesion to mucus were observed between DON treated (67.53%) and DON free animals (73.96%) ($P > 0.05$).

4. Discussion

Weaning is the stage of pig farming where the greatest use of natural extracts and synthetic compounds occurs. When incorporated as feed additives, focus is made on their individual benefits without considering, sometimes without knowing, their mechanisms of action and possible interactions among different additives or even with other anti-nutritional factors that may be present in feed. Fosfomycin, CSE and DON are commonly found in the weaning diet. These compounds, individually or combined, may impact on important morphological, histological and microbiota modifications produced during weaning, affecting the animals' productive outcome.

4.1. Bacteria, volatile fatty acids and pH

Lactic acid bacteria in the gastrointestinal tract were not influenced by any of the treatments in the present study. Accordingly, natural resistance of LAB strains to antibiotics and bile salts, increased by feed consumption, has been largely demonstrated (Piotrowska et al., 2014; Dowarah et al., 2017; Liao and Nyachoti, 2017).

Deoxynivalenol showed no effect on *Enterobacteriaceae* populations and pH values were conserved in all gastrointestinal tract portions. The influence of mycotoxins on intestinal microbiota of pigs has been poorly investigated. Available data relies mainly on the ability of bacteria to detoxify mycotoxins (Eriksen et al., 2002; Niderkorn et al., 2006; Young et al., 2007; Waché et al., 2009). In agreement with our results, a study conducted by Waché et al. (2009) showed that cultivable bacteria diversity in fecal samples was conserved in animals that consumed feed that was naturally contaminated with DON (2.8 mg/kg). It is likely that gut bacteria possess resistance mechanisms against this mycotoxin. In fact, *in vitro* studies identified intestinal bacterial strains that promote metabolism, binding or detoxification of DON (Kollarczik et al., 1994; Young et al., 2007). By contrast, our results showed that VFA concentrations were lowered in DON treated animals. This outcome at caecum level, where the mycotoxin would be metabolized by microorganisms, could be explained by a detrimental effect of DON on the metabolism of culture independent bacterial populations (Waché et al., 2009; Piotrowska et al., 2014).

C. scolymus extract treated animals showed a decrease in *Enterobacteriaceae* and E:L ratio in caecum and colon. It has been recently demonstrated by our research group that using CSE as feed additive substantially increased bile production in pigs (Martínez et al., 2018). Important bile effects on the intestinal microbiota have been described involving 2 main mechanisms: a direct detergent action on bacterial cell membranes (mainly in proximal intestine) and an indirect action by interacting with specific nuclear receptors (FXR, TGR 5, mainly in large intestine) and thus inducing antimicrobial peptides synthesis (Inagaki et al., 2006; D'Aldebert et al., 2009; Burrin et al., 2013; Nie et al., 2015). Furthermore, Cremers et al. (2014) indicated that bile acid salts have profound effects on many key proteins in bacteria. Results from different studies suggest that bile salts could potentially induce DNA damage through oxidative stress in *E. coli* (Bernstein et al., 1999a, 199b; Oh et al., 2000). Therefore, bile acids are thought to have destructive effects on gut microbes except for some bile acid tolerant bacteria. Lactic acid bacteria can tolerate biliary acids by expressing bile salts hydrolases (Begley et al., 2005), active efflux of bile acids/salts and changes in composition and architecture of cell membrane and cell wall (Ruiz et al., 2013; Nie et al., 2015). Thus, higher bile acids concentration in gastrointestinal tract of CSE treated animals in our study might have contributed to lower *Enterobacteriaceae* without altering LAB. Moreover, the use of CSE as an additive of the piglets' diet increased the concentration of butyrate, an important energetic VFA in large intestine (Steer et al., 2000; Blottière et al., 2003; Bederska-Lojewska and Pieszka, 2011; Samanta et al., 2013). This finding is consistent with other scientific studies that detected an increase in the proportion of butyrate and an equal or lower concentration of acetate when diets containing other natural extracts are consumed (Kleessen et al., 2001; Poulsen et al., 2002; Gibson et al., 2004; Loh et al., 2006). Even if all VFA concentrations were higher in CSE treated animals than in CSE free animals, statistically significant differences were only observed for butyrate. This could be attributed to the fact that butyrate producing bacteria are able to use other VFA as a substrate. As an example, acetate constitutes a type of substrate for cross-feeding interactions that occur among colonic bacteria (Duncan et al., 2004; Mølbak et al., 2007; Patterson et al., 2010; Liu et al., 2012a).

Fosfomycin treatments reduced *Enterobacteriaceae* in caecum and colon exerting a bactericide effect, related to its low oral bioavailability (Pérez et al., 2012). Lactic acid bacteria populations, capable of resisting relatively high bactericide antibiotic concentrations through different adaptive mechanisms (Bernier and Surette, 2013; Munita et al., 2016), were not affected by FOS. Further, a reduction in pH was observed in caecum and colon that can be explained as a consequence of the diminished E:L ratio in FOS treated animals. On the other hand, the observed significant increase in caecal VFA concentrations could be indicating that the development of FOS resistant bacteria (other than LAB) has not been affected by the antibiotic (Frimondt-Møller, 2010; Clausen

Table 6
Effect of fosfomycin (FOS), Cynara scolymus extract (CSE) and their combination the intestinal absorptive area (IAA, μm^2; $n = 6$).

Item	0 FOS		30 mg/kg BW FOS		*P*-value
	0 CSE	15 mg/kg BW CSE	0 CSE	15 mg/kg BW CSE	FOS × CSE
Medium jejunum	5.47 ± 0.26^{a}	7.36 ± 0.55^{b}	8.44 ± 0.88^{b}	6.98 ± 0.44^{ab}	0.0065
Ileum	5.19 ± 0.34^{a}	6.26 ± 0.27^{b}	5.13 ± 0.31^{a}	4.95 ± 0.33^{ab}	0.0205

FOS × CSE = combination of FOS and CSE.
[a, b] Within a row, mean values with different superscripts differ significantly ($P < 0.05$).

Table 7
Effect of fosfomycin (FOS), Cynara scolymus extract (CSE) and deoxynivalenol (DON) on goblet cells/100 villi and goblet cells/100 crypts in the small intestine of weaned piglets ($n = 12$).

Item	FOS, mg/kg BW		*P*-value	CSE, mg/kg BW		*P*-value	DON, μg/kg BW		*P*-value
	0	30		0	15		0	50	
Medium jejunum									
Villi	882.42 ± 105.74	1,153.33 ± 120.80	0.0802	924.08 ± 130.74	1,111.67 ± 102.07	0.2165	1,175.75 ± 112.63	860.00 ± 109.05	0.0441
Crypts	1,053.75 ± 71.57	1,291.67 ± 86.14	0.0249	1,035.83 ± 73.56	1,309.58 ± 79.36	0.0116	1,192.50 ± 98.27	1,152.92 ± 73.40	0.6859
Ileum									
Villi	1,080.83 ± 76.02	954.58 ± 75.52	0.1717	968.75 ± 86.17	1,066.67 ± 65.91	0.2847	1,162.92 ± 67.44	872.50 ± 61.84	0.0039
Crypts	1,287.58 ± 88.99	1,677.08 ± 81.43	0.0013	1,375.92 ± 122.47	1,588.75 ± 66.24	0.0499	1,487.17 ± 90.16	1,477.50 ± 115.38	0.9244

Table 8
Effect of fosfomycin (FOS) Cynara scolymus extract (CSE) and their combination on the percentages (%) of adherence of bacteria to intestinal mucus of weaned piglets ($n = 6$).

Item	0 FOS		30 mg/kg BW FOS		*P*-value
	0 CSE	15 mg/kg BW CSE	0 CSE	15 mg/kg BW CSE	FOS × CSE
Bacterial adhesion to the ileum mucus adhered	44.57 ± 5.09[a]	73.14 ± 7.53[b]	83.67 ± 4.02[b]	81.61 ± 6.08[b]	0.0049

FOS × CSE = combination of FOS and CSE.
[a, b] Within a row, mean values without a common superscript letter differ significantly ($P < 0.05$).

et al., 1991). These findings represent an important favorable aspect of intestinal health in weaned piglets.

When FOS and CSE were combined in the piglets' diet, the reduction of *Enterobacteriaceae* in colon was similar to the effect obtained when the treatments were administered individually, though it was less pronounced at caecum level. This could be explained by a possible interference of their mechanisms of action: as a consequence of CSE consumption, bile acids concentrations increase at intestine level (Martínez et al., 2018) and this would trigger antimicrobial peptides synthesis (induced by biliary acids through nuclear receptors). The modes by which antimicrobial intestinal peptides kill bacteria are varied: the cytoplasmic membrane is a frequent target, but they may also interfere with DNA and protein synthesis, protein folding and cell wall synthesis (Jenssen et al., 2006; Bahar and Ren, 2013; Mahlapuu et al., 2016). On the other hand, FOS is transported into bacteria via both glycerol-3-phosphate and hexose phosphate membrane transporter systems and it interferes with the cytoplasmic step of bacterial cell wall biosynthesis, the formation of the peptidoglycan precursor UDP-N-acetylmuramic acid (Kahan et al., 1974; Gobernado, 2003; Castañeda-García et al., 2009; Popovic et al., 2010; Pérez et al., 2014). Interference between FOS and intestinal peptides' mechanisms of action, at cytoplasmic or membrane transporter level, could occur. Moreover, some cytoplasmic peptides show bacteriostatic effects that could antagonize bactericidal effect of FOS that requires bacteria to grow at log phase to exert its action; i.e. antagonistic effects may be due to inhibition of bacterial growth by static agents (Nguyen et al., 2011).

4.2. Intestinal morpho-physiology

Clinical symptoms characteristic of DON intoxication were not observed in the animals under study. The mycotoxin DON administered at 1 mg/kg of feed (50 μg/kg BW) in our experiment did not affect IAA, which is in agreement with studies that indicate that higher DON concentrations are needed to deteriorate the tissue at this level (Grenier and Applegate, 2013). However, in the present study, treatments with DON adversely affected the number of goblet cells. Similarly, Obremski et al. (2008) obtained a lower goblet cells count in jejunum of piglets maintained on diets contaminated with DON for 14 d. In addition, Bracarense et al. (2012) and Gerez et al. (2015) also reported lower goblet cell counts in jejunum after administration of 1.5 to 3 mg/kg DON respectively in the diet of animals during 4 to 5 wk. Apart from a lower goblet cells count, a lower expression of mucins by these cells would be expected after the ingestion of low DON concentrations (Laparra and Sanz, 2009; Pinton et al., 2015; Robert et al., 2017). In our study, this effect was reflected by a lower adherence of *E. coli* to mucus. In addition, disaccharidase activity decreased with DON treatments in the different portions of the intestine. The undesirable effect of the mycotoxin could be a consequence of its mechanism of action as a potent inhibitor of protein synthesis, including the synthesis of disaccharidases (Pestka, 2007; Xiao et al., 2015).

After FOS treatments, IAA and goblet cells were considerably increased. In a previous study, Pérez Gaudio et al. (2016) demonstrated a protective effect of the antibiotic FOS on *in vitro* cell cultures that would favor a trophic effect on intestinal mucosa. On the other hand, certain antibiotics modulate physiological inflammation decreasing the catabolic cost of maintaining immune response, thereby favoring mucosal anabolic processes (Niewold 2007, 2013; Costa et al., 2011; Brown et al. 2016, 2017). A greater pathogenic bacteria adhesion, obtained in the present work, can be explained by an improved mucus production given by a greater goblet cells count. Maltase activity was also increased in FOS treated groups, being the most active disaccharidase, as expected for the age and diet of the animals (Collington et al., 1990).

C. scolymus extract used as an additive in the diet significantly increased IAA and goblet cells. These findings are consistent with previous works which reported that using different sources of natural extracts increased villi height and villi:crypts ratio in the small intestine of weaned piglets (Touchette et al., 2002; Liu et al., 2008; Liu et al., 2012; Yuan et al., 2017). In pigs, the action of bile acids on the G protein–coupled bile acid receptor (TGR5) found in enteroendocrine cells stimulates secretion of glucagon like peptides (GLP)-1 and 2, which function respectively as the major incretin hormone involved in glucose homeostasis and key trophic hormone in intestinal adaptation and growth in response to food ingestion. In fact, the induction of GLP-2 secretion, by TGR5, is involved in the trophic action of bile acids in the intestinal lumen (le Roux et al., 2010; Jain et al., 2012). The observed increase in IAA and goblet cells in our study could be explained by the direct trophic effect given by an increased bile production (Burrin et al., 2013; de Diego-Cabero et al., 2015). As stated before, the increased bacterial adherence to mucus would be a direct consequence of the increased number of goblet cells rendering a better mucus quality. Maltase activity, which plays an important role at

weaning, was increased in CSE treated groups at ileum level. This could be related to the trophic effect of bile acids, augmented after CSE administration, in this portion of the intestine through interaction with specific nuclear receptors (Inagaki et al., 2006; Chiang, 2009; Stojancevic et al., 2012; Burrin et al., 2013).

Beneficial effects observed after co-administration of FOS and CSE on IAA and bacterial adherence to mucus did not exceed the benefits of individual treatments. It could be possible that anti-inflammatory mechanisms exerted by FOS and biliary acids, that involve cytokines produced by intestine immune cells, interfere at different levels (Tullio et al., 2008; Shen et al., 2009; Buret, 2010; Yokota et al., 2010; Michalopoulos et al., 2011; Zhang et al., 2014; Brown et al., 2017).

5. Conclusions

In the present study, we have demonstrated the impact of FOS, CSE and DON on intestinal health parameters.

Deoxynivalenol showed a deleterious effect at different levels of the intestinal epithelium at sub-toxic concentrations. Thereby, the presence of low levels of this mycotoxin in feed should not be underestimated.

Fosfomycin and CSE improved all studied parameters in relation to intestinal health. Particularly, CSE could be considered as a nutritional strategy to prevent enteric disorders and improve intestinal health in post-weaned piglets, emerging as a possible alternative to preventive use of antibiotics.

The knowledge of the intestinal effects of the studied compounds contributes to understand the physiological/physiopathological gut changes and their potential productive consequences.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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