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

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Abstract

Intestinal health of weaning piglets was studied after oral treatments with fosfomycin (FOS), *Cynara scolymus* extract (CSE), deoxynivalenol (DON) and their combinations. Piglets were divided in groups and received different treatments during 15 days, namely DON (1mg/kg of feed), FOS administered into the drinking water (30 mg/kg b.w.), CSE (300 g/ton of feed) and all possible combinations including a control group that received clean balanced diet. At day 15, three piglets from each group were euthanized and gastrointestinal tract samples were immediately taken to evaluate pH, bacteriology (enterobacteria and lactic acid bacteria), volatile fatty acids concentration (VFAs), disaccharidases activity (lactase, sucrase and maltase), histology (intestinal absorptive area [IAA] and goblet cells count) and adherence of bacteria to intestinal mucus. Animals receiving FOS and CSE treatments exhibited evident beneficial intestinal effects compared to animals receiving diets free from these compounds. This was revealed by a lower enterobacteria population together with a lower E/L, an enhanced production of butyric acid, an increased enzymatic activity (particularly maltase), and a greater IAA and goblet cells count along with an increase in pathogenic bacteria adherence to intestinal mucus. Interactions between both treatments resulted in similar beneficial effects as their individual administration. On the contrary, DON produced detrimental effects on intestinal health as a decrease was observed on volatile fatty acids production, enzymatic activity and goblet cells count in animals receiving diets containing sub- toxic concentrations of this mycotoxin. The knowledge of the intestinal effects of these compounds contributes to understand the physiological and pathological gut changes and their potential productive consequences.

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Introduction

Weaning is considered one of the most critical periods of pig production because of its highly 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 (1–3). In order to overcome this situation a common, though not rational practice, has been the prophylactic use of antibiotics in intensive pig production. 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 (4).

In addition, vegetable extracts, particularly *Cynara scolymus* extract (CSE), have long been used in different species for their hepatoprotective and digestive roles, exerting a choleretic– cholagogue effect, increasing bile concentrations at small intestine level and thus enhancing fat and lipophilic vitamins absorption. In animal production, these compounds are used as feed additives to improve zootechnical parameters (5–8) and they have shown further beneficial consequences on intestine and liver functions. Nowadays enteroprotective, trophic, antitoxic and antimicrobial effects are ascribable to bile action (9–12). CSE is used in intensive pig and avian productions. It is obtained from the leaves of the plant and contains caffeolquinic acid derivatives which are known for their choleretic– cholagogue effect in different species (7,13,14), including pigs (15).

Among weaning stress factors, the presence of anti-nutritional compounds in feed, such as mycotoxins, negatively influences the productive performance of animals. Deoxynivalenol (DON) is a mycotoxin produced by *Fusarium* species, being pigs the most susceptible species to its toxic effects (16,17). Formerly DON was also called vomitoxin, referring to its emetic effect (18,19). Other clinical signs that have been described include reduction in feed intake and complete feed refusal, immunosuppression, haemorrhage and eventually, circulatory shock (20–22). However there is little information on the possible subclinical effects associated to the ingestion

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of feed contaminated with low DON concentrations, which is highly likely to occur (19,23,24).

In the productive reality, in innumerable situations, but mostly during weaning, antibiotics, natural extracts and mycotoxins coexist in the animals´ diet, and consequently in the gut, regardless the potential interactions among them.

The aim of this study was to evaluate the effect of FOS, CSE, DON and their interactions on the intestinal health of weaning piglets.

Materials and methods

Animals

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. One hundred and sixty, healthy, 21 days old weaned piglets (6.26 ± 0.4 kg body weight [b.w.]) of the same genetic line from a commercial farm were used. Piglets were housed in an environmentally controlled barn (22±5°C; light: dark cycle 12:12 h; relative humidity 45-65%), given free access to feed (commercial feed: 3.0 Kcal/Kg of metabolizable energy) and water, and were checked daily.

Antibiotic, natural extract and mycotoxin

Fosfomycin (FOS): Calcium fosfomycin was provided by Bedson S.A. laboratory (Fosbac®, Pilar, Buenos Aires, Argentina). The antibiotic dose was 30 mg/kg b.w. administered via drinking water. Water consumption was measured by a water flow meter installed at the entrance pipeline of the weaning room two days before the beginning of the trial. Medicated water was prepared daily at 8.00 am, considering water consumption and mean piglets weight.

Cynara scolymus extract (CSE): This natural extract was provided by Bedson S.A. laboratory (Bedgen40®, Pilar, Buenos Aires, Argentina). Three hundred grams of CSE were uniformly mixed with one ton of feed (15 mg/kg b.w.).

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Deoxynivalenol (DON): The mycotoxin was produced in our laboratory by growing *Fusarium graminearum* NRRL 28063 in corn at 25°C for 25 days. For DON quantification, samples of ground corn were extracted twice with water/acetonitrile and then with hexane by liquid-liquid extraction. Extracts were passed through DONPREP columns (R-Biopharm, Acre Road, 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 was a C18; 250 mm × 3.00 mm Sinergy Hydro RP 4 µm (Phenomenex, Torrance, United States) maintained in at 35°C. The mobile phase was water: acetonitrile (90:10) at 0.5 ml/min flow rate. DON was detected at 222 nm and its retention time was 8.7 min.

Convenient aliquots of ground contaminated corn were uniformly mixed with feed in order to obtain 1 mg DON/ Kg (50 µg/kg b.w.).

Experimental groups

Weaning piglets were randomly assigned to one of eight groups, which were subjected to different treatments for a 15 days period. The dietary treatments were as follows: A) balanced diet containing DON (1mg/kg of feed), B) balanced diet and FOS administered into the drinking water (30 mg/kg b.w.), C) balanced diet containing CSE (300 g/ton of feed), D) balanced diet containing DON (1mg/kg of feed) and FOS (30 mg/kg b.w.) into the drinking water, E) balanced diet containing DON (1mg/kg of feed) plus CSE (300 g/ton of feed), F) balanced diet containing CSE (300 g/ton of feed) and FOS (30 mg/kg b.w.) into the drinking water, G) balanced diet containing DON (1mg/kg of feed) plus CSE (300 g/ton of feed) and FOS (30 mg/kg b.w.) into the drinking water and, H) balanced diet without FOS, CSE or DON.

After 15 days of treatment, three piglets of each group were randomly selected and euthanized for sampling of the gastrointestinal tract.

pH determination

As soon as each sample was obtained, pH was measured with a pH meter (UP-25, Denver Instrument Company, Denver, Colorado, EE. UU.) in the following portions

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of the gastrointestinal tract: caudal portion of the stomach, ileum (15 cm proximal to ileocaecal valve), caecum and colon (20 cm distal from caecum).

Enterobacteriaceae/Lactic acid bacteria ratio (E/L)

The E/L has traditionally been used to determine balance of intestinal microbiota in pigs (25). It has been demonstrated that a greater resistance to gastrointestinal diseases is acquired when animals show a lower E/L (26–28).

The intestinal contents from ileum (15 cm proximal to the ileocaecal valve), caecum and colon (20 cm distal from caecum) were collected and kept at 4°C until arrival to the laboratory. One g of sample was diluted in 9 ml of peptone water and homogenized by continuous agitation. Counting of viable bacteria was 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 (29–32). Colonies were counted, log transformed and expressed as colony forming units per gram of digesta (CFU/g).

Volatile fatty acids (VFAs)

The caecal content was immediately diluted with phosphoric acid (in a 4:1 proportion) for preservation and kept at -70°C until analyzed. Concentrations of VFAs were determined using gas liquid chromatography according to the method described by Jouany (33). 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 mM Supelco VFAs (C2 to C10) and 2-ethyl-butyric acid (Fluka) as internal standard were used to build calibration curves.

Disaccharidases activity

The digestive function of the intestine can be evaluated by the activity of disaccharidases present in the microvilli or brush border of the enterocytes (34,35). The evaluation of these enzymes gives information on the physio pathological status of the intestinal mucosa (36).

The four portions of the small intestine (duodenum, proximal jejunum, medium jejunum and ileum) were opened along the mesenteric border and washed with saline

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solution to eliminate the mucus and remaining intestinal contents. The mucosa was scraped off with a scalpel and 1.000 g of this material was weighed. Then, saline solution (2 ml) was added to the intestinal mucosa and it was ground with a dispersing instrument (Ultra-Turrax®) and a Potter homogenizer. Samples were then cold-centrifuged at 4°C and 6630 rpm for 10 min. The supernatant was used as crude enzyme solution and it was stored at – 20ºC until analysis. The protein concentration of each homogenate was determined by Bradford method using bovine serum albumin as standard (37). The activity of sucrase, lactase and maltase was determined by quantification of released glucose, according to Dahlqvist method (38). Briefly, the homogenate supernatants were diluted, added to an equal volume of 0.1 M sodium maleate buffer (pH 6.0) containing 56 mM lactose, sucrose or maltose, and incubated for 1 h at 37ºC. 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. The activity of disaccharidases 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.

Histological study

Different measures on villi and crypts can be correlated to nutrient absorption capacity, possible structural alterations of intestinal mucosa and consequent productive yield (34,39–47).

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

The intestinal mucosa was examined under light microscope and measured by the Image Analysis Software (ToupTek™ ToupView™). The length of villi and width of villi and crypts were measured in H&E- stained sections. The goblet cells count in villi and crypts (expressed as goblet cells/ 100 villi or crypts) was determined using PAS staining (48,49). Means were calculated for each group. The mathematical model

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proposed by Kisielinski *et al* was used to estimate the intestinal absorptive area (IAA) using the following equation (50):

$$IAA = (villusW \times villusL) + \left(\frac{villusW}{2} + \frac{cryptW}{2}\right)^2 - \left(\frac{villusW}{2}\right)^2 / \left(\frac{villusW}{2} + \frac{cryptW}{2}\right)^2$$

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

Goblet cells count was used as index of the secretory capacity and the production of protective intestinal mucus (51).

Adherence of bacteria to intestinal mucus

Mucus quality has been evaluated by its ability to adhere *E. coli*, since bacterial adhesion is associated with the protective and antimicrobial functions of mucus favoring bacterial elimination by the rapid removal of mucus by peristaltic movements (52,53). The interaction between the glycoproteins of the outer layer of the intestinal mucus and *E. coli* would prevent the attachment of bacteria to epithelial cells and subsequent damage (51,53–58).

Ileum samples (15 cm proximal to ileocaecal valve) were opened along the mesenteric border. The mucus was carefully scraped off with a scalpel (leaving intestinal mucosa intact), collected into sterile tubes and kept at -70°C until analyzed.

The adherence of bacteria to the intestinal mucus was analyzed according to Bai *et a.* (59). One hundred milligrams of mucus were diluted with 1.5 ml of saline solution and centrifuged (12.000 rpm, 10 min, 4ºC) to remove cell debris and bacteria. The supernatant was sterilized by filtration (13 mm x 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 *Escherichia coli* O157:H7 was incubated with supernatant containing crude mucus for 30 min, at 37°C under continuous agitation. Then the tubes were centrifuged (12.000 rpm, 10 min, 4ºC) and pellets (with adhered and not adhered bacteria) were resuspended in 400 µl saline solution and further centrifuged (2000 rpm, 4ºC, 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

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for 24 h at 37 °C for colonies count. Results were expressed as percentage of adhered bacteria to the intestinal mucus.

Statistical analyses

A 2x2x2 factorial arrangement was used to evaluate interactions between FOS (0 *vs.* 30 mg/kg b.w.), CSE (0 *vs.* 300 g/ton of feed) and DON (0 *vs.* 1 mg/kg feed) on the intestinal health of weaned piglets. The response variables (pH, intestinal bacteria, VFAs, dissacharidases activity, IAA, goblet cells and percentage of adhered bacteria to intestinal mucus) were subjected to analysis of variance (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 significant interactions were observed, contrasts were used to compare the different levels of each treatment. Data are presented in tables as means and mean standard error (SEM).

Results

pH

pH values are shown in Table 1. No statically significant differences on pH were found neither in gastrointestinal (GI) portions studied for groups treated with CSE and DON nor in interactions between the different factors. In FOS treated groups, no statically significant effects were found in caudal portion of stomach and ileum, but piglets that received FOS showed a lower pH ($p< 0.01$) in caecum and colon. The mean caecal pH was 5.51±0.33 in FOS treated groups and 6.90±0.29 in FOS free groups. The mean pH in the colon was 6.21±0.30 in FOS treated groups and 7.36±0.26 in FOS free groups.

Table 1. Effect of fosfomycin (FOS), *C. scolymus* extract (CSE), deoxynivalenol (DON) and their combinations on the gastrointestinal pH of weaned piglets[1]

pH	FOS (mg/kg)								*p* - value
	0				30				
CSE (g/ton)	0		300		0		300		FOS
DON (mg/kg)	0	1	0	1	0	1	0	1	
stomach	4.08 ± 0.12	3.69 ± 0.34	3.34 ± 0.54	2.98 ± 0.34	3.27 ± 0.28	3.18 ± 0.37	4.15 ± 0.82	3.34 ± 0.06	NS
ileum	7.48 ± 0.14	7.29 ± 0.15	6.68 ± 0.99	7.51 ± 0.17	6.75 ± 0.90	6.27 ± 0.95	6.83 ± 0.72	7.92 ± 0.32	NS
caecum	6.77 ± 0.51	7.69 ± 0.29	6.40 ± 0.69	6.73 ± 0.52	5.70 ± 0.06	5.18 ± 0.11	4.97 ± 0.25	6.19 ± 1.00	0.0041
mean pH in FOS free or treated groups	6.90 ± 0.29				5.51 ± 0.33				
colon	7.78 ± 0.28	7.63 ± 0.25	6.42 ± 0.74	7.62 ± 0.07	6.40 ± 0.75	6.24 ± 0.93	5.16 ± 0.35	7.05 ± 0.96	0.0081
mean pH in FOS free or treated groups	7.36 ± 0.26				6.21 ± 0.30				

NS= not significant ($p > 0.05$).

[1] No significant effect of CSE and DON on the gastrointestinal pH were detected. Interaction between the different factors was not significant ($p > 0.05$).

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Enterobacteriaceae, lactic acid bacteria and E/L

There was no effect of none of the treatments on the studied bacteria at ileum level. LAB counts from caecum and colon did not show any significant differences among treatments and effects of DON on *Enterobacteriaceae* in these intestinal portions were neither detected. In caecum and colon, FOS and CSE treated groups showed lower *Enterobacteriaceae* population and E/L regardless the presence of DON (Table 2). A significant antagonistic interaction was observed between FOS and CSE on *Enterobacteriaceae* count (p= 0.0004) and consequently on the E/L (p= 0.0016) at caecum level. In this case, the effect of both treatments was less pronounced than the effect they produced as individual factors. An indifferent interaction was observed for *Enterobacteriaceae* count (p= 0.0004) and E/L (p= 0.0114) at colon level when FOS and CSE were combined, i.e., the effect produced by the combination of FOS and CSE was similar to the one observed when they were administered individually.

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Table 2. Effect of the interaction between fosfomycin (FOS) and *C. scolymus* extract (CSE) on the intestinal bacteria of weaned piglets[1]

Item	CSE (g/ton)	FOS (mg/kg)				*p*- value		
		0		30				
		0	300	0	300	FOS	CSE	FOS x CSE
***Enterobacteriaceae* (log_{10} CFU/g)**								
caecum		6.21 ± 0.55	2.93 ± 0.32	2.42 ± 0.84	4.35± 0.62	0.0071	0.0331	0.0004
colon		6.67 ± 0.40	2.74 ± 0.37	2.53 ± 0.86	3.39 ± 0.48	0.0055	0.0136	0.0004
***Lactic acid bacteria* (log_{10} CFU/g)**								
caecum		7.54 ± 0.30	6.83 ± 0.14	6.52 ± 0.27	7.45 ± 0.50	NS	NS	NS
colon		7.23 ± 0.45	7.11 ± 0.27	6.72 ± 0.19	8.03 ± 0.33	NS	NS	NS
E/L								
caecum		0.82 ± 0.06	0.43 ± 0.04	0.37 ± 0.13	0.60 ± 0.09	0.0106	0.0313	0.0016
colon		0.96 ± 0.11	0.39 ± 0.06	0.38 ± 0.13	0.44 ± 0.07	0.0253	0.0337	0.0114

NS= not significant ($p > 0.05$).

[1] Effects of mycotoxin treatments on the studied intestinal bacteria were not significant.

Volatile fatty acids

Concentrations of VFAs were not modified in FOS treated groups and interactions between different factors were not significant ($p > 0.05$). CSE treated

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275 groups increased the concentrations of butyric acid (p= 0.033). For DON treated groups
276 lower acetic (p= 0.0104) and butyric (p= 0.0001) acids and lower total VFAs
277 concentrations (p= 0.0021) were detected (Table 3).

Table 3. Effect of fosfomycin (FOS), *C. scolymus* extract (CSE) and deoxynivalenol (DON) on VFAs (mmol/L) in the caecum of weaned piglets[1]

VFAs	FOS (mg/kg)			CSE (g/ton)			DON (mg/kg)		
	0	30	*p* - value	0	300	*p* - value	0	1	*p* - value
Acetic acid	49.71 ± 4.5	60.35 ± 7.31	NS	50.29 ± 4.75	58.77 ± 6.88	NS	62.35 ± 5.55	43.01 ± 4.33	0.0104
Propionic acid	13.62 ± 1.27	16.44 ± 1.60	NS	13.95 ± 1.30	15.79 ± 1.60	NS	17.74 ± 1.10	10.84 ± 1.16	NS
Butyric acid	5.87 ± 0.60	6.78 ± 1.03	NS	5.83 ± 0.65	6.76 ± 0.93	0.033	8.00 ± 0.65	3.92 ± 0.36	0.0001
Total VFAs	71.49 ± 6.42	85.53 ± 9.69	NS	72.55 ± 6.72	83.07 ± 9.25	NS	90.76 ± 7.14	59.26 ± 5.81	0.0021

NS= not significant ($p > 0.05$).

[1] Interaction between the different factors was not significant ($p > 0.05$).

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279 **Disaccharidases activity**

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There were not significant interactions between FOS, CSE and DON on disaccharidases activity ($p > 0.05$).

It was found that the activity of maltase in the different intestinal regions in piglets from FOS treated groups was significantly higher ($p < 0.05$) than that observed in FOS free groups. FOS treatments also increased sucrose and lactase activity in proximal and medium jejunum and ileum though this effect was not statistically significant. Treatments with CSE produced higher maltase activity in ileum ($p = 0.0020$). However, an effect on the activity of sucrase and lactase was not observed. DON showed negative effects for all enzymes in all intestinal portions, being enzymatic activity lower for pigs fed diets supplemented with DON when compared to those without DON supplementation. P value< 0.05 was observed for maltase and lactase activity in duodenum and proximal jejunum, sucrase and lactase in medium jejunum and maltase in the ileum (Table 4).

Table 4. Effect of fosfomycin (FOS), the *C. scolymus* extract (CSE) and deoxynivalenol (DON) on disaccharidases activity

Item	FOS (mg/kg)			CSE (g/ton)			DON (mg/kg)		
	0	30	*p* - value	0	300	*p* - value	0	30	*p* - value
Duodenum									
Maltase	1427.85 ± 169.23	2422.91 ± 338.39	0.0008	1951.87 ± 350.73	1862.65 ± 246.43	NS	2462.37 ± 339.66	1391.43 ± 147.09	0.0004
Sucrase	37.02 ± 15.26	36.86 ± 8.85	NS	41.32 ± 15.17	32.90 ± 10.07	NS	41.69 ± 10.17	32.56 ± 14.43	NS
Lactase	86.84 ± 28.70	88.19 ± 27.68	NS	72.60 ± 18.06	101.23 ± 34.11	NS	141.09 ± 33.98	38.01 ± 8.36	0.0092
Proximal jejunum									
Maltase	1964.33 ± 232.52	3419.02 ± 456.61	0.0028	2727.34 ± 465.01	2602.81 ± 362.69	NS	3420.65 ± 461.72	1962.83 ± 223.01	0.0028
Sucrase	89.26 ± 29.52	133.11 ± 51.55	NS	133.87 ± 28.64	88.55 ± 49.20	NS	145.62 ± 53.67	77.71 ± 23.98	NS
Lactase	162.66 ± 49.17	257.84 ± 91.37	NS	214.62 ± 55.72	202.56 ± 84.92	NS	324.52 ± 93.05	101.12 ± 22.84	0.041
Medium jejunum									
Maltase	2712.30 ± 293.58	4333.48 ± 493.03	0.0064	3854.78 ± 549.21	3154.17 ± 349.49	NS	4038.04 ± 432.94	2985.01 ± 440.51	NS
Sucrase	164.87 ± 48.53	284.39 ± 66.33	NS	259.65 ± 63.40	187.70 ± 55.14	NS	319.49 ± 69.41	132.47 ± 34.25	0.0147
Lactase	201.64 ± 62.08	342.47 ± 79.00	NS	316.65 ± 72.98	225.47 ± 71.34	NS	407.83 ± 80.99	141.30 ± 39.20	0.0105
Ileum									
Maltase	1047.08 ± 129.29	1464.89 ± 265.51	0.0337	947.73 ± 122.61	1524.46 ± 239.42	0.002	1623.23 ± 259.01	900.92 ± 75.58	0.0007
Sucrase	14.20 ± 9.13	59.01 ± 45.49	NS	15.72 ± 9.81	54.16 ± 42.12	NS	57.68 ± 45.56	15.42 ± 9.34	NS
Lactase	10.52 ± 3.42	18.26 ± 5.51	NS	14.16 ± 4.36	14.30 ± 4.87	NS	15.69 ± 4.86	12.89 ± 4.42	NS

NS= not significant ($p > 0.05$).

[1] Interaction between the different factors was not significant ($p > 0.05$).

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Intestinal absorptive area and goblet cells

There was an evident increase in the IAA of medium jejunum in the presence of FOS, CSE and the combination of both factors ($p< 0.05$). The co-administration of FOS and CSE showed an indifferent type interaction at this level. IAA of ileum increased in piglets that received CSE and an antagonistic interaction between FOS and CSE was detected ($p< 0.05$). The IAA of medium jejunum and ileum was not affected by the treatments with DON ($p> 0.05$), (Table 5).

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Table 5. Effect of the interaction between fosfomycin (FOS) and *C. scolymus* extract (CSE) on the intestinal absorptive area (IAA, µm^2) in weaned piglets[1]

IAA	CSE (g/ton)	FOS (mg/kg)				*p*- value		
		0		30				
		0	300	0	300	FOS	CSE	FOS x CSE
Medium jejunum		5.67 ± 0.24	7.35 ± 0.46	8.44 ± 0.88	6.98 ± 0.44	0.0034	0.0192	0.0006
Ileum		5.49 ± 0.26	6.29 ± 0.23	5.13 ± 0.31	4.95 ± 0.33	NS	0.0296	0.0205

NS= not significant ($p > 0.05$).

[1] The effects of the mycotoxin treatments on the IAA were not significant.

Generally, the number of intestinal goblet cells increased with FOS and CSE treatments, whereas a decrease was evident in goblet cells from villi after DON treatments. Goblet cells count in crypts of ileum increased in FOS treated groups (p= 0.0120). The treatments with CSE increased the count of these cells in villi (p= 0.0159)

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306 and crypts (p= 0.0143) of medium jejunum. A negative effect of DON was observed in
307 goblet cells count in villi of medium jejunum (p= 0.0125) and ileum (p= 0.0336) (Table
308 6). No significant interactions were detected between FOS, CSE and DON on goblet
309 cells count (p> 0.05).

Table 6. Effect of fosfomycin (FOS), *C. scolymus* extract (CSE) and deoxynivalenol (DON) on goblet cells/100 villi and goblet cells/100 crypts in the small intestine of weaned piglets[1]

	FOS (mg/kg)			CSE (g/ton)			DON (mg/kg)		
Item	**0**	**30**	***p* - value**	**0**	**300**	***p* - value**	**0**	**30**	***p* - value**
Medium jejunum									
villi	871.69 ± 84.47	1153.33 ± 120.80	NS	886.47 ± 107.87	1114.62 ± 93.93	0.0159	1087.80 ± 103.30	882.31 ± 102.77	0.0125
crypts	1155.69 ± 78.55	1291.67 ± 86.14	NS	1095.73 ± 70.53	1350.38 ± 83.63	0.0143	1221.07 ± 82.77	1205.77 ± 85.74	NS
Ileum									
villi	1045.94 ± 72.8	954.58 ± 75.52	NS	947.33 ± 81.14	1075.38 ± 61.25	NS	1102.67 ± 75.37	896.15 ± 61.61	0.0336
crypts	1421.31 ± 94.7	1677.08 ± 81.43	0.012	1437.73 ± 103.07	1638.46 ± 78.63	NS	1526.73 ± 75.23	1535.77 ± 121.08	NS

NS= not significant ($p > 0.05$).

[1] Interaction between different factors was not significant ($p > 0.05$).

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311 # Adherence of bacteria to the intestinal mucus

312 Treatments with FOS, CSE and the combination of both resulted in a statistically
313 significant increase in the percentages of adhesion of bacteria to intestinal mucus ($p<$
314 0.001, $p=$ 0.0133 and $p=$ 0.0049, respectively) compared to FOS and CSE free groups.
315 In the latter, the adhesion percentage of *E. coli* was 45.71%, whereas FOS or CSE
316 treated groups increased the percentage of adhesion to 83.67% and 72.75%,
317 respectively. The combination of treatments evidenced an indifferent type interaction. In
318 this case, the adhesion percentage of bacteria was 81.61%. The percentage of bacteria
319 adhered to intestinal mucus was not affected by treatments with DON ($p>$ 0.05), (Table
320 7).

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Table 7. Effect of the interaction between fosfomycin (FOS) and *C. scolymus* extract (CSE) on the percentages (%) of adherence of bacteria to intestinal mucus of weaned piglets[1]

Bacterial adhesion to the ileum mucus	CSE (g/ton)	FOS (mg/kg) 0	FOS (mg/kg) 300	FOS (mg/kg) 0	FOS (mg/kg) 300	*p*- value FOS	*p*- value CSE	*p*- value FOS x CSE
Adhered		45.71 ± 4.45	72.25 ± 6.42	83.67 ± 4.02	81.61 ± 6.08	<0.0001	0.0133	0.0049

[1] Mycotoxin treatments produced no significant effects on the adherence of bacteria to intestinal mucus.

Discussion

FOS, CSE and DON are commonly found together in the weaning diet. These compounds, individually or combined, may impact on the important morphological,

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325 histological and microbiota modifications produced during weaning, affecting the
326 animals´ productive outcome.

327 Bacteria, VFAs and pH

328 LAB populations were not affected by none of the treatments in any of the
329 intestine portions studied. Natural resistance of LAB strains to antibiotics and bile salts,
330 increased by CSE consumption, has been largely demonstrated (17,60–62), The
331 influence of mycotoxins on intestinal microbiota of pigs have been poorly investigated.
332 Available data on the interaction of mycotoxins with bacteria are mainly related to the
333 ability of the intestinal microbiota to detoxify mycotoxins (63–67,24). Results obtained
334 in a study conducted by Waché *et al.* showed that cultivable bacteria diversity in fecal
335 samples was conserved in animals that consumed feed naturally contaminated with
336 DON (2.8 mg/kg) (24). Accordingly, in our study, when piglets received diets
337 containing DON at 1mg/kg, alone or in combination with the other factors, significant
338 changes in CFU counts were neither observed for LAB nor for *Enterobacteriaciae*. In
339 addition, pH values were conserved in all gastrointestinal tract portions after DON
340 treatments. It is likely that gut bacteria possess resistance mechanisms against this
341 mycotoxin, in fact *in vitro* studies identified intestinal bacterial strains that promote
342 metabolism, binding or detoxification of DON (64,67). By contrast, VFAs
343 concentrations were lowered. The normal concentration of VFAs in the caecum varies
344 according to the content and composition of the raw material in the diet, being around
345 80 mmol/L for this stage of pig rearing (68–70). In the present study, the decrease in
346 VFAs at caecum level, where the mycotoxin is metabolized, could be explained by a
347 detrimental effect of DON on the metabolism of culture independent bacterial
348 populations as it has been previously demonstrated (17,24).

349 A lower count of *Enterobacteriaciae* population and E/L in caecum and colon
350 was observed in pigs treated with CSE. It has been recently demonstrated by our
351 research group that using CSE as feed additive substantially increases bile production in
352 pigs (15). Important bile effects on the intestinal microbiota have been described
353 involving two main mechanisms: direct detergent action on bacterial cell membranes
354 (mainly in proximal intestine) and an indirect action by interacting with specific nuclear
355 receptors (FXR, TGR 5, mainly in large intestine) and thus inducing antimicrobial
356 peptides synthesis (10,71–73). Furthermore, Cremers *et al.* indicated that bile acid salts

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357 have profound effects on many key proteins in bacteria (74). Results from different
358 studies suggest that bile salts could potentially induce DNA damage through oxidative
359 stress in *E. coli* (75–79). Therefore bile acids are thought to have destructive effects on
360 gut microbes except for some bile acid tolerant bacteria. LAB can tolerate biliary acids
361 by expressing bile salts hydrolases (80). This might have contributed to lower
362 *Enterobacteriaceae* count in CSE treated groups in our study without altering LAB.

363 The administration of CSE in the piglets´ diet increased the concentration of
364 butyric acid, an important energetic VFA in large intestine (81–84). This finding is in
365 agreement with other scientific studies that detected an increase in the proportion of
366 butyrate and an equal or lower concentration of acetate in diets containing other natural
367 extracts (85–90). The no significant change in the levels of acetate could be attributed to
368 the fact that butyrate producing bacteria are able to use acetate as a substrate. In this
369 way, acetic acid constitutes a type of substrate for cross-feeding interactions that occur
370 among colonic bacteria (91–95).

371 FOS reduced *Enterobacteriaceae* populations in caecum and colon exerting a
372 bactericide effect, related to its low oral bioavailability (96). LAB populations, capable
373 of resisting relatively high bactericide antibiotic concentrations through different
374 adaptive mechanisms (97,98), were not affected by FOS. Further, a reduction in pH was
375 observed in caecum and colon as consequence of the diminished E/L in FOS treated
376 groups. These findings, together with an increase of butyrate production ($p > 0.05$)
377 represent an important favorable aspect of intestinal health in weaning piglets.

378 Interactions observed between FOS and CSE treatments could be explained by a
379 possible interference of their mechanisms of action: The modes by which antimicrobial
380 intestinal peptides kill bacteria are varied. The cytoplasmic membrane is a frequent
381 target, but peptides may also interfere with DNA and protein synthesis, protein folding,
382 and cell wall synthesis. Thereby, some peptides form a complex with different cell wall
383 precursors inhibiting cell wall biosynthesis. On the other hand, FOS is transported into
384 bacteria via both glycerol-3-phosphate and hexose phosphate membrane transporter
385 systems. Besides, it interferes with the cytoplasmic step of bacterial cell wall
386 biosynthesis, the formation of the peptidoglycan precursor UDP *N*-acetylmuramic acid
387 (99–103). Interference between the action of FOS and intestinal peptides (induced by
388 biliary acids through nuclear receptors), at cytoplasmatic or membrane transporter level
389 could occur. Moreover, some cytoplasmic peptides show bacteriostatic effects that

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could antagonize bactericidal effect of FOS that requires bacteria to grow at log phase to exert it´s action; i.e. antagonistic or indifference effects may be due to inhibition of bacterial growth by static agents (104).

Intestinal morpho-physiology

Clinical symptoms characteristic of DON intoxication were not observed in the animals under study. In order to evaluate intestinal health, morphological and physiological integrity of intestinal mucosa was studied. The mycotoxin DON administered at 1mg/Kg of feed 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 (105). However, in the present study, treatments with DON adversely affected the number of goblet cells. Similarly, Obremski *et al.* obtained a lower goblet cells count in jejunum of piglets maintained on diets contaminated with DON for 14 days (106). In addition, Bracarense *et al.* and Gerez *et al.* 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 weeks (107,108). Apart from a lower goblet cells count, a lower expression of mucins (mainly MUC1, MUC2 and MUC3) by these cells would be expected after the ingestion of low DON concentrations (109–111). In our study this effect was reflected by a lower adherence of *E. coli* to mucus ($p > 0.05$; data not shown). Moreover, disaccharidases activity decreased with DON treatments, particularly maltase and sucrose, 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 (21).

After FOS treatments, IAA and goblet cells were considerably increased. In a previous study, Pérez Gaudio *et al.* demonstrated a protective effect of the antibiotic FOS on in vitro cell cultures that would favor a trophic effect on intestinal mucosa (112). On the other hand, certain antibiotics modulate physiological inflammation decreasing the catabolic cost of maintaining immune response, thereby favoring mucosal anabolic processes (113–117). A greater goblet cells count improved mucus production which was revealed by a greater pathogenic bacterial adhesion. Enzymatic activity was also increased in FOS treated groups, being maltase the most active disaccharidase, as expected for the age and diet of the animals (118).

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CSE 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 (95,119,120). 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 TGR 5, is involved in the trophic action of bile acids in the intestinal lumen (121,122). The observed increase in IAA and goblet cells in our study could be explained by the direct trophic effect of the increased bile production (73,123) when CSE is added to the diet. 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 (10,12,73,124).

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 (antagonistic or indifferent interactions). 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 (117,125–135).

Conclusions

The gastrointestinal mucosa is the first biological barrier that makes contact to different compounds present in feed, and consequently, it could be exposed to dietary toxins. Thereby the intestinal epithelial cells are target for antibiotic, natural extracts used as additives and mycotoxins.

In the present study, we have demonstrated the impact of FOS, CSE and DON on intestinal health parameters. DON showed a deleterious effect at different levels of the intestinal epithelium at sub- toxic concentrations. This could represent a predisposing factor to progressive weight loss, digestive problems and diarrhea as well as a reduction in the intestinal barrier function.

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453 The antibiotic FOS and CSE improved all studied parameters in relation with the
454 intestinal health. Interactions between both treatments resulted in similar beneficial
455 effects as the individual administration, there remains work to be done investigating the
456 specific mechanisms which contribute to this type of interactions.

457 Finally, the knowledge of the intestinal effects of these compounds contributes
458 to understand the physiological/physio-pathological gut changes and their potential
459 productive consequences. Particularly, CSE could be considered as a nutritional strategy
460 to prevent enteric disorders and improve intestinal health in post-weaned piglets,
461 emerging as a possible alternative to preventive use of antibiotics. In addition, the
462 presence of mycotoxins in feed even at sub-toxic concentrations may cause detrimental
463 gastrointestinal effects and should not be underestimated.
464

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470

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