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

6

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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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52 # Introduction

53 Weaning is considered one of the most critical periods of pig production because
54 of its highly negative impact on health and productive performance of piglets, mainly in
55 the first post-weaning days. During this period, the animals are exposed to
56 physiological, immunological, microbiological, social, environmental and nutritional
57 factors that lead to post-weaning stress (1–3). In order to overcome this situation a
58 common, though not rational practice, has been the prophylactic use of antibiotics in
59 intensive pig production. Fosfomycin ((cis 1-2 epoxy propyl) phosphonic acid, FOS) is
60 a broad spectrum bactericide antibiotic, widely used in pig farms in Central and South
61 America, South Africa and Southeast Asia. At weaning FOS is indicated for the
62 treatment of several bacterial infections (*Haemophilus parasuis, Streptococcus suis,*
63 *Pasteurella multocida, Bordetella bronchiseptica, Staphylococcus hyicus, Escherichia*
64 *coli*, etc.) associated to stress (4).

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

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

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84 of feed contaminated with low DON concentrations, which is highly likely to occur
85 (19,23,24).

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

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

91

92 Materials and methods

93 Animals

94 The study was carried out according to guidelines of the Animal Welfare
95 Committee of the Faculty of Veterinary Sciences UNCPBA, Argentina, for animal
96 handling and experimentation. One hundred and sixty, healthy, 21 days old weaned
97 piglets (6.26 ± 0.4 kg body weight [b.w.]) of the same genetic line from a commercial
98 farm were used. Piglets were housed in an environmentally controlled barn (22±5°C;
99 light: dark cycle 12:12 h; relative humidity 45-65%), given free access to feed
100 (commercial feed: 3.0 Kcal/Kg of metabolizable energy) and water, and were checked
101 daily.

102 Antibiotic, natural extract and mycotoxin

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

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

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112 *Deoxynivalenol (DON):* The mycotoxin was produced in our laboratory by
113 growing *Fusarium graminearum* NRRL 28063 in corn at 25°C for 25 days. For DON
114 quantification, samples of ground corn were extracted twice with water/acetonitrile and
115 then with hexane by liquid-liquid extraction. Extracts were passed through DONPREP
116 columns (R-Biopharm, Acre Road, Glascow, Scotland) and evaporated to dryness at
117 40°C. The dry extract was reconstituted with MilliQ water and filtered through 0.22 µm
118 nylon membranes before Injection into HPLC UV/VIS for quantification. A Gilson
119 HPLC system equipped with a Gilson 151 UV-Vis detector and Gilson 712 software
120 was used for data analysis (Gilson, Inc., Middleton, USA). The column was a C18; 250
121 mm × 3.00 mm Sinergy Hydro RP 4 µm (Phenomenex, Torrance, United States)
122 maintained in at 35°C. The mobile phase was water: acetonitrile (90:10) at 0.5 ml/min
123 flow rate. DON was detected at 222 nm and its retention time was 8.7 min.

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

126 Experimental groups

127 Weaning piglets were randomly assigned to one of eight groups, which were
128 subjected to different treatments for a 15 days period. The dietary treatments were as
129 follows: A) balanced diet containing DON (1mg/kg of feed), B) balanced diet and FOS
130 administered into the drinking water (30 mg/kg b.w.), C) balanced diet containing CSE
131 (300 g/ton of feed), D) balanced diet containing DON (1mg/kg of feed) and FOS (30
132 mg/kg b.w.) into the drinking water, E) balanced diet containing DON (1mg/kg of feed)
133 plus CSE (300 g/ton of feed), F) balanced diet containing CSE (300 g/ton of feed) and
134 FOS (30 mg/kg b.w.) into the drinking water, G) balanced diet containing DON
135 (1mg/kg of feed) plus CSE (300 g/ton of feed) and FOS (30 mg/kg b.w.) into the
136 drinking water and, H) balanced diet without FOS, CSE or DON.

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

139 pH determination

140 As soon as each sample was obtained, pH was measured with a pH meter (UP-
141 25, Denver Instrument Company, Denver, Colorado, EE. UU.) in the following portions

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

144 Enterobacteriaceae/Lactic acid bacteria ratio (E/L)

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

148 The intestinal contents from ileum (15 cm proximal to the ileocaecal valve),
149 caecum and colon (20 cm distal from caecum) were collected and kept at 4°C until
150 arrival to the laboratory. One g of sample was diluted in 9 ml of peptone water and
151 homogenized by continuous agitation. Counting of viable bacteria was performed by
152 plating serial 10-fold dilutions (in 1% peptone water) onto MRS agar (Britania S.A.) for
153 *Lactic acid bacteria* (LAB) representative of beneficial bacteria in pigs, and onto Mac
154 Conkey agar (Britania S.A.) for *Enterobacteriaceae* representative of commensal Gram
155 negative bacteria (29–32). Colonies were counted, log transformed and expressed as
156 colony forming units per gram of digesta (CFU/g).

157 Volatile fatty acids (VFAs)

158 The caecal content was immediately diluted with phosphoric acid (in a 4:1
159 proportion) for preservation and kept at -70°C until analyzed. Concentrations of VFAs
160 were determined using gas liquid chromatography according to the method described by
161 Jouany (33). A Shimadzu chromatograph (Model GC–17A, Kyoto, Japan) with a
162 19091N-133 Innowax 30M column (Agilent, Santa Clara, CA, USA) was used. A
163 mixture of 10 mM Supelco VFAs (C2 to C10) and 2-ethyl-butyric acid (Fluka) as
164 internal standard were used to build calibration curves.

165 Disaccharidases activity

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

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

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172 solution to eliminate the mucus and remaining intestinal contents. The mucosa was
173 scraped off with a scalpel and 1.000 g of this material was weighed. Then, saline
174 solution (2 ml) was added to the intestinal mucosa and it was ground with a dispersing
175 instrument (Ultra-Turrax®) and a Potter homogenizer. Samples were then cold-
176 centrifuged at 4°C and 6630 rpm for 10 min. The supernatant was used as crude enzyme
177 solution and it was stored at – 20ºC until analysis. The protein concentration of each
178 homogenate was determined by Bradford method using bovine serum albumin as
179 standard (37). The activity of sucrase, lactase and maltase was determined by
180 quantification of released glucose, according to Dahlqvist method (38). Briefly, the
181 homogenate supernatants were diluted, added to an equal volume of 0.1 M sodium
182 maleate buffer (pH 6.0) containing 56 mM lactose, sucrose or maltose, and incubated
183 for 1 h at 37ºC. Then, the mixtures were added to the glucose oxidase–peroxidase
184 reagents (Sigma Chemical Company, USA) containing O-dianisidine as chromogen.
185 The absorbance was measured using a spectrophotometer (Dupont, Sorvall Instruments)
186 at 450 nm. The activity of disaccharidases was expressed as U/mg protein. One U is
187 defined as the amount of enzyme that hydrolyses 1 mmol of lactose, sucrose or maltose
188 in 1 min under the standard assay conditions.

189 Histological study

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

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

199 The intestinal mucosa was examined under light microscope and measured by
200 the Image Analysis Software (ToupTek™ ToupView™). The length of villi and width
201 of villi and crypts were measured in H&E- stained sections. The goblet cells count in
202 villi and crypts (expressed as goblet cells/ 100 villi or crypts) was determined using
203 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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234 for 24 h at 37 °C for colonies count. Results were expressed as percentage of adhered
235 bacteria to the intestinal mucus.

236 Statistical analyses

237 A 2x2x2 factorial arrangement was used to evaluate interactions between FOS (0
238 *vs.* 30 mg/kg b.w.), CSE (0 *vs.* 300 g/ton of feed) and DON (0 *vs.* 1 mg/kg feed) on the
239 intestinal health of weaned piglets. The response variables (pH, intestinal bacteria,
240 VFAs, dissacharidases activity, IAA, goblet cells and percentage of adhered bacteria to
241 intestinal mucus) were subjected to analysis of variance (ANOVA) by GLM procedure
242 of SAS V9.3 (SAS Institute Inc., Cary, NC, USA). Differences between treatments were
243 declared significant when $p < 0.05$. When significant interactions were observed,
244 contrasts were used to compare the different levels of each treatment. Data are presented
245 in tables as means and mean standard error (SEM).

246

247 Results

248 pH

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

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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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258 **Enterobacteriaceae, lactic acid bacteria and E/L**

259 There was no effect of none of the treatments on the studied bacteria at ileum
260 level. LAB counts from caecum and colon did not show any significant differences
261 among treatments and effects of DON on *Enterobacteriaceae* in these intestinal portions
262 were neither detected. In caecum and colon, FOS and CSE treated groups showed lower
263 *Enterobacteriaceae* population and E/L regardless the presence of DON (Table 2). A
264 significant antagonistic interaction was observed between FOS and CSE on
265 *Enterobacteriaceae* count (p= 0.0004) and consequently on the E/L (p= 0.0016) at
266 caecum level. In this case, the effect of both treatments was less pronounced than the
267 effect they produced as individual factors. An indifferent interaction was observed for
268 *Enterobacteriaceae* count (p= 0.0004) and E/L (p= 0.0114) at colon level when FOS
269 and CSE were combined, i.e., the effect produced by the combination of FOS and CSE
270 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) 0		FOS (mg/kg) 30		*p*- value		
		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.

271

272 # Volatile fatty acids

273 Concentrations of VFAs were not modified in FOS treated groups and
274 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) 0	FOS (mg/kg) 30	*p* - value	CSE (g/ton) 0	CSE (g/ton) 300	*p* - value	DON (mg/kg) 0	DON (mg/kg) 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 ($p > 0.05$).

278

279 **Disaccharidases activity**

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

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

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293

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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294 **Intestinal absorptive area and goblet cells**

295 There was an evident increase in the IAA of medium jejunum in the presence of
296 FOS, CSE and the combination of both factors ($p < 0.05$). The co-administration of FOS
297 and CSE showed an indifferent type interaction at this level. IAA of ileum increased in
298 piglets that received CSE and an antagonistic interaction between FOS and CSE was
299 detected ($p < 0.05$). The IAA of medium jejunum and ileum was not affected by the
300 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) 0		FOS (mg/kg) 30		*p*- value		
		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.

301

302 Generally, the number of intestinal goblet cells increased with FOS and CSE
303 treatments, whereas a decrease was evident in goblet cells from villi after DON
304 treatments. Goblet cells count in crypts of ileum increased in FOS treated groups (p=
305 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).

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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]

Item	**FOS** (mg/kg)			**CSE** (g/ton)			**DON** (mg/kg)		
	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 ± 121.0	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 ($p < 0.001$, $p = 0.0133$ 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		**FOS** (mg/kg)				*p*- value		
		0		30				
	CSE (g/ton)	0	300	0	300	FOS	CSE	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.

321

322 # Discussion

323 FOS, CSE and DON are commonly found together in the weaning diet. These
324 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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390 could antagonize bactericidal effect of FOS that requires bacteria to grow at log phase to
391 exert it´s action; i.e. antagonistic or indifference effects may be due to inhibition of
392 bacterial growth by static agents (104).

393 Intestinal morpho-physiology

394 Clinical symptoms characteristic of DON intoxication were not observed in the
395 animals under study. In order to evaluate intestinal health, morphological and
396 physiological integrity of intestinal mucosa was studied. The mycotoxin DON
397 administered at 1mg/Kg of feed in our experiment did not affect IAA, which is in
398 agreement with studies that indicate that higher DON concentrations are needed to
399 deteriorate the tissue at this level (105). However, in the present study, treatments with
400 DON adversely affected the number of goblet cells. Similarly, Obremski *et al.* obtained
401 a lower goblet cells count in jejunum of piglets maintained on diets contaminated with
402 DON for 14 days (106). In addition, Bracarense *et al.* and Gerez *et al.* also reported
403 lower goblet cell counts in jejunum after administration of 1.5 to 3 mg/kg DON
404 respectively in the diet of animals during 4 to 5 weeks (107,108). Apart from a lower
405 goblet cells count, a lower expression of mucins (mainly MUC1, MUC2 and MUC3) by
406 these cells would be expected after the ingestion of low DON concentrations (109–111).
407 In our study this effect was reflected by a lower adherence of *E. coli* to mucus ($p > 0.05$;
408 data not shown). Moreover, disaccharidases activity decreased with DON treatments,
409 particularly maltase and sucrose, in the different portions of the intestine. The
410 undesirable effect of the mycotoxin could be a consequence of its mechanism of action
411 as a potent inhibitor of protein synthesis, including the synthesis of disaccharidases (21).

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

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421 CSE as an additive in the diet significantly increased IAA and goblet cells.
422 These findings are consistent with previous works which reported that using different
423 sources of natural extracts increased villi height and villi: crypts ratio in the small
424 intestine of weaned piglets (95,119,120). In pigs, the action of bile acids on the G
425 protein–coupled bile acid receptor (TGR5) found in enteroendocrine cells stimulates
426 secretion of glucagon like peptides (GLP)-1 and 2, which function respectively as the
427 major incretin hormone involved in glucose homeostasis and key trophic hormone in
428 intestinal adaptation and growth in response to food ingestion. In fact, the induction of
429 GLP-2 secretion, by TGR 5, is involved in the trophic action of bile acids in the
430 intestinal lumen (121,122). The observed increase in IAA and goblet cells in our study
431 could be explained by the direct trophic effect of the increased bile production (73,123)
432 when CSE is added to the diet. As stated before, the increased bacterial adherence to
433 mucus would be a direct consequence of the increased number of goblet cells rendering
434 a better mucus quality. Maltase activity, which plays an important role at weaning, was
435 increased in CSE treated groups at ileum level. This could be related to the trophic
436 effect of bile acids, augmented after CSE administration, in this portion of the intestine
437 through interaction with specific nuclear receptors (10,12,73,124).

438 Beneficial effects observed after co-administration of FOS and CSE on IAA and
439 bacterial adherence to mucus did not exceed the benefits of individual treatments
440 (antagonistic or indifferent interactions). It could be possible that anti-inflammatory
441 mechanisms exerted by FOS and biliary acids, that involve cytokines produced by
442 intestine immune cells, interfere at different levels (117,125–135).

443 Conclusions

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

448 In the present study, we have demonstrated the impact of FOS, CSE and DON
449 on intestinal health parameters. DON showed a deleterious effect at different levels of
450 the intestinal epithelium at sub- toxic concentrations. This could represent a
451 predisposing factor to progressive weight loss, digestive problems and diarrhea as well
452 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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