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Effect of Two Doses of Different Zinc Sources (Inorganic vs. Chelated form) on the Epithelial Proliferative Activity and the Apoptotic Index of Intestinal Mucosa of Early-weaned Pigs Orally Challenged with *E. coli* K88

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ABSTRACT : The effect of two doses of different sources of zinc, inorganic (zinc oxide) or chelated (zinc glutamate chelate), on morphology and turn-over of the small intestine was assessed in early-weaned pigs orally challenged with enterotoxigenic *E. coli* K88 (ETEC). Sixty pigs weaned at 21 days were assigned to one of the following 5 diets: control (C); C+Zinc oxide (ZnO), either a 200 or a 2,500 mg Zn/kg dose; or C+zinc chelate with glutamic acid (Glu-Zn), either a 200 or a 2,500 mg Zn/kg dose. On d 2, the pigs were orally inoculated with 1.5 ml of a 10^{10} CFU/ml *E. coli* K88ac O148 suspension. Zinc supplements did not improve the performance of the pigs, but on d 5 faecal excretion of ETEC was reduced, and this was mainly due to high zinc doses ($p<0.05$). The villous height in the duodenum was improved by the zinc supplements ($p<0.01$) whatever the source and the level, whereas no effect was seen in the other two tracts of small intestine. The diet did not affect apoptosis and mitosis counts, while ETEC-susceptible pigs had more mitotic cells in the villi than non-susceptible pigs, particularly in the jejunum ($p<0.01$). The duodenum had fewer mitotic cells in the villi ($p<0.05$) and in the crypts ($p<0.01$) and more apoptotic cells in the villi. High dietary doses of ZnO or Zn-Glutamate improve villous height of the duodenum, but not of the jejunum and the ileum, and do not affect the epithelial proliferative activity and apoptotic index of intestinal mucosa of early-weaned pigs orally challenged with ETEC. (**Key Words :** Weaning Pig, Zinc Oxide, Small Intestine, Apoptosis, Mitosis)

INTRODUCTION

Current research is increasingly being focused on the supplementation of piglet diets with zinc oxide (ZnO) at levels exceeding the nutritional requirements (3 g/kg). This is a result of the fact that this "pharmacological" addition often improves growth performance and controls diarrhea during the weaning period (Hill et al., 2001). Particularly relevant are studies which try to identify the mechanism of action of supranutritional doses of Zn on piglet gut health. This knowledge could help to find different chemical/physical forms of Zn active in the gut at lower dietary levels, to respect the legislation of some areas (European Union), and reduce concerns about zinc transfer to the soil, and signs of toxicity in the liver (Jensen-Waern et al., 1998).

The effect of ZnO on bacteria, (mainly gram positive bacteria), is well known. Roselli et al. (2005) found that ZnO inhibited the increase of tight junction permeability induced by enterotoxigenic *Escherichia coli* K88 (ETEC) on intestinal CaCo2 cells, at a dose significantly lower than the dose which reduced ETEC growth. Zinc also contrasted the ETEC-induced effects on different cytokines. Broom et al. (2006), after supplementation with 3,100 mg/kg for 20 days post-weaning, found that bacteria counts in intestinal digesta were not affected, but anaerobes and lactic acid bacteria in the mesenteric lymph nodes were reduced compared to the control groups. A trend of reduction was observed on lactic acid bacteria with only 6 days of supplementation, but there was no variation in the lymph nodes. This confirms that the local antibacterial effect is not sufficient to explain the mechanism of action of the zinc

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and that time is required to control microbe translocation. Innate or acquired immunity could be variously stimulated.

Højberg et al. (2005) observed that 2,500 ppm ZnO in newly weaned pigs reduced lactic acid bacteria (but increased coliforms and enterococci). This action, similar to the action of the antibiotic growth promoter, can explain the improved growth performance after ZnO addition, but not the reduction of diarrhea. Again these data support the hypothesis that more than one mechanism is acting during the dietary supply of supra-nutritional doses of Zn.

A local effect of ZnO is also plausible, considering its wound-healing action in topic use and after oral supplementation (in pigs, Bhar et al., 2003), and the concomitant increase of gene expression of IGF-1 (Tarnow et al., 1994). Furthermore Li et al. (2006) recently found that a high level of ZnO in the diet of weanling pigs increased the expression of the IGF-I and IGF-IR genes at the mRNA and protein levels in the small intestine. The growth-stimulating action of this molecule can favor a better recovery of the intestinal epithelium after the stress of weaning or reduce its negative effects, and can explain the frequent observation of a positive effect of high zinc doses on villous height (Li et al., 2001; Owusu-Asiedu et al., 2003; Li et al., 2006). Thus it is hypothesized that the growth promoting effect of "supra-nutritional" doses of zinc is explained by its effect on the maintenance of the integrity and the function of the epithelium. But after weaning, mucosal integrity is frequently negatively affected by the colonization of typical enteropathogenic bacteria, such as *Escherichia coli* K88 (Pluske et al., 1993), and this could affect the repair response after zinc supplementation. The variations of the expression of some proteins which are markers of the turn-over, recently observed after ZnO (Wang et al., 2009) may support this hypothesis. However it has never been verified if growth stimulation by zinc oxide is correlated with a reduced apoptosis or increased mitosis in the villi and crypts. Cell proliferation is often evaluated using Ki67 monoclonal antibody (clone MIB1) which recognizes a nuclear protein expressed by actively cycling cells but not by resting G0 cells (Gerdes et al., 1992). Apoptosis, or programmed cell death, is an active process controlled by inducers and repressors and is morphologically characterized by nuclear condensation and cytoplasmic shrinkage (Liu et al., 2001). For the detection of apoptosis, the TUNEL technique is often used (Wijsman et al., 1993; Lutgens et al., 1999).

Zinc oxide is the most widely studied source of zinc used in studies on weaning pigs, however there are indications that other zinc sources can substitute this additive at the same concentration (tetrabasic zinc chloride, Mavromichalis et al., 2001), while various zinc organic sources fed between 300 and 500 mg Zn/kg increased (Buff et al., 2005) or did not increase (Hollis et al., 2005) growth as did ZnO at 2,000 mg Zn/kg. Glutamate is strongly used by the intestinal mucosa (Reeds et al., 2000) and its supplementation enhanced both total and mucosal growth in several sections of the small intestine (Ewtushik et al., 2000). Furthermore, reason for using Zn-Glu chelates would be their high absorbability, expecting some of them will be absorbed through amino acid transport systems whereas ZnO would be poorly absorbed. So the expectation from ZnO and Zn-Glu chelates is different: ZnO would affect gut microbes and the mucosal environment whereas Zn-Glu chelates would be absorbed and contribute to enterocyte and whole body metabolism. In that sense, ZnO may affect gut microflora more than Zn-Glu. So it can be hypothesized that chelating zinc with this amino acid can be a way of targeting the small intestinal tissue. However, the efficacy of a zinc glutamate chelate on the development and on the health of the intestinal mucosa has never been tested.

Our goal was to study the effects of two doses of zinc from two different source, inorganic (ZnO) or chelated (zinc glutamate chelate) on gut characteristics of weaned pigs challenged with ETEC.

MATERIALS AND METHODS

Animals, experimental procedure and diets

The procedures on the pigs were conducted respecting Italian laws on experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

On day 0, 60 newly weaned pigs (age 21 days, average live weight: 5.95 kg), were allotted to one of five dietary treatments based on body weight and litter origin: control (C); zinc oxide (ZnO) at a dose of 200 mg Zn/kg or 2,500 mg Zn/kg; or zinc-glutamate chelate (Zn-Glu) at a dose of 200 mg Zn/kg or 2,500 mg Zn/kg. A basal diet was formulated to satisfy the requirements of early weaned pigs, according to NRC (1998) and it is presented in Table 1. The diets differed from each other by virtue of the presence of a mixed supplement included in the base diet at 1% (Table 2). Zn-Glu was obtained from the reaction of glutamic acid in solution with zinc oxide and was described by Gramaccioli (1966). In addition to different zinc sources and supplementation, free glutamic acid was added to the diets not supplemented with Zn-Glu, in order to keep its total presence in the feed constant. Sepiolite was used to complete the supplementation.

On d 2, the subjects were orally challenged with 1.5 ml of a 10^{10} CFU/ml *E. coli* K88ac O148 suspension. The pigs were penned separately on a mesh floor.

Samplings

The feces were collected on d 5. On d 7 or 8 the pigs were euthanized: they were anesthetized using sodium

Table 1. Base ingredient composition of the diets (% of diet)

Ingredients or additives	%
Corn meal	26.00
Corn grain (extruded)	23.00
Spray-dried whey	12.50
Herring meal	9.00
Soybean meal (dehulled)	8.00
Spray-dried whey (with 50% lard)	8.00
Potato protein concentrate	4.00
Dextrose	4.00
Beet pulp	2.00
Dicalcium phosphate	1.00
Calcium carbonate	0.50
L-lysine HCl	0.21
L-threonine	0.14
Dl-methionine	0.10
L-tryptophan	0.05
Vitamin mineral premix	0.50
Different supplementation*	1.00

Vitamin and mineral mixture, provided per kg diet: vitamin A, 15,000 IU; vitamin D_3, 1,500 IU; vitamin K_3, 2 mg; vitamin E, 35 mg; vitamin B_1, 6 mg; vitamin B_2, 8 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.04 mg; niacin, 55 mg; biotin, 0.15 mg; d-pantothenic acid, 30 mg; folacin, 1 mg; iron ($FeSO_4$), 150 mg; copper ($ZnSO_4$), 150 mg; manganese ($MnSO_4$), 80 mg, I, 2.5 mg; cobalt ($CoCO_4$), 2 mg; selenium (Na_2SeO_4), 0.2 mg.
* See Table 2 for different supplementations.

Table 2. Supplementation of each diet with different additives (% of diet)

	ZnO	Glutamic acid	Zn-Glu chelated	Sepiolite
C	-	0.640	-	0.360
ZnO 200	0.027	0.640	-	0.333
ZnO 2500	0.330	0.640	-	0.030
Zn-Glu 200	-	0.590	0.080	0.330
Zn-Glu 2500	-	-	1.000	-

thiopenthal (10 mg/kg body weight), and euthanasia was carried out by means of intracardiac injection of Tanax® (0.5 ml/kg body weight; Intervet Italia, Peschiera Borromeo, Italy).

The duodenum (about 10 cm from the pyloric-duodenal junction, mid-jejunum and ileum (about 10 cm from the ileal-cecal junction) ileal-cecal junction were sampled for histological analysis. The mid- jejunum was also sampled for the collection of villi to perform the *in vitro* villous-adhesion assay with the same *E. coli* K88 (Van den Broeck et al., 1999). Pigs can be genetically resistant to ETEC infection and the adhesion of E. coli K88 to the host intestinal wall is variable due to the presence of receptors for the presence of its fimbriae (van den Broeck et al., 1999).

Microbiological analyses

Total *E. coli* and *E. coli* K88 were counted in the feces using the procedure reported by Bosi et al. (2004). The *in vitro* villous-adhesion assay with *E. coli* K88 was carried out as reported by Bosi et al. (2004). After this test, animals were classified as K88 positive-receptor or negative-receptor pigs, *i.e.* susceptible to *E. coli* K88 disease or not.

Histological analysis

Samples were pinned tautly to balsa wood and immersed in 10% buffered formalin (pH 7.4). This procedure allows a good distension of the wall and consequently of the villi. Formalin-fixed, paraffin wax-embedded 4 micron thick sections were deparaffinized in xylene and stained with hematoxylin-eosin. For each subject, the height of 10 villi and the depth of 10 crypts were measured; only villi and crypts perpendicular to the mucosal surface were considered suitable for morphometry. The sections were examined at low magnification with a conventional microscope interfaced to a digital camera and PC computer equipped with Cytometric software (Byk Gulden, Milan). The dimensions of the digitalized images were 513x463 pixels.

Villous height was measured as the distance from the crypt opening to the top of the villous whereas crypt depth was measured from the base of the crypt to the level of the crypt opening.

Ki 67 immunohistochemistry : Ki 67 immunohisto-chemistry was performed with the clone MIB1 (Immunotech, Int, Marseilles, France) using a highly sensitive streptavidin-biotin-peroxidase technique with a commercial kit (BIO SPA, Milan, Italy).The sections were dewaxed in toluene and rehydrated in a graded acetone series. Endogenous peroxidase was blocked by means of 3 per cent hydrogen peroxide for 30 minutes. The sections were then rinsed in Tris buffer, immersed in citrate buffer (2·1 g citric acid monohydrate/l distilled water), pH 6.0 and incubated for four periods of 5 minutes each in a microwave oven at 750 W. After microwave irradiation, the sections were allowed to cool to room temperature (RT) (approximately 20 minutes). In the control sections, the primary antibody was substituted by an isotype-matched non-specific antibody.

Detection of apoptosis in tissues : The sections were stained with the Apoptag Kit (Oncor, Resnova, Rome, Italy). The kit utilizes reagents for non-isotopic DNA end-extension *in situ* (digoxigenin-11-dUTP), and other reagents for immunohistochemical staining (antidigoxigenin-peroxidate antibody) of the extended DNA. Residues of digoxigenin-nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase (TdT) which generates tails of digoxigenin d-UTP to the 3'-OH ends of double or single stranded DNA. The latter was revealed

immunohistochemically by means of the antidigoxigenin antibody. In control slides, TdT-enzyme solution was substituted by distilled water.

Scoring method : Quantification of cell proliferation and apoptosis was performed with the image cytometer Cytometrica (Byk Gulden, Milano, Italy).

MIB1 index : For each case, 10 villi and 10 crypts were selected and the positivity scored along all the length using a 25x objective. In each field an initial image of the total nuclear area was mapped with a green (575/10 nm) bandpass filter; a second area consisting of all the MIB1 positive nuclei, was mapped with a blue (490/10 nm) bandpass filter. In each field, inflammatory or stromal cells were masked. The MIB1 index was calculated as the percentage of labelled nuclei compared with total nuclear area (MIB1 index = total nuclear area of the positively stained nuclei in ten fields/total nuclear area in ten fields per 100).

Apoptotic index : The apoptotic fraction was quantitated choosing, in each case, 10 villi and 10 crypts where apoptosis was recognizable. All images were obtained using a 40x objective. In each field, the number of apoptotic nuclei, the total nuclear area and the mean area of a nucleus were evaluated. Apoptosis was expressed as the number of positive nuclei per 100 cells, named apoptotic index and calculated as follows:

Total nuclear area of the fields/mean area of a nucleus = N

N = Number of cells present in the total nuclear area of the chosen fields

Apoptotic index = Total number of apoptosis/N×100

Statistical analysis

The data were analyzed by analysis of variance using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) with a 3-factor design, including diet, susceptibility of the intestinal villous to ETEC adhesion, diet x susceptibility, and litter. ETEC adhesion to intestinal villi was included in the model as a classification factor due to its relevance for the health of the piglet gut.

For morphometric analysis and the mitosis and apoptosis counts in the villi and crypts of the different intestinal tracts, we used the MIXED procedure of SAS with the REML estimation method. The point of measurement along the small intestine (as a repeated measurement), and the effect of the individual subjects were included. The other fixed factors were: diet, litter, ETEC adhesion to the intestinal villi, and diet with the point of measurement, ETEC adhesion with the point of measurement, and diet with ETEC adhesion to the intestinal villi. The following orthogonal and preplanned comparisons were tested: “C vs. Zinc additions”, “ZnO vs. Zn-Glu”, “Between Zn doses”, “C vs. High Zn doses”.

RESULTS

***In vivo* performance and *E. coli* fecal excretion**

Whatever the source and the level of addition, zinc did not affect growth performance and feed intake, which on average were 32.2 g/d and 105.4 g/d respectively (Table 3). K88 positive-receptor pigs were 6; 6; 7; 5; 5 in C; ZnO 200; ZnO 2,500; Zn-Glu 200; Zn-Glu 2,500. No interaction was seen between the diet and the susceptibility to K88, for growth performance.

K88 positive-receptor pigs had lower live daily gain, compared to negative (12.9 g vs. 51.6 g) ($p<0.05$).

On d 5 (Table 4), total *E. coli* faecal excretion was reduced by the high Zn supplementations, as compared with

Table 3. Effect of diet and K88 susceptibility on growth performance

	Diet					SEM	K88 susceptibility		SEM
	C	ZnO 200	ZnO 2500	Zn-Glu 200	Zn-Glu 2500		No	Yes	
Initial LW (kg)	6.02	5.90	5.88	6.06	5.87	0.25	5.98	5.91	0.16
Final LW (kg)	6.38	6.19	6.30	6.16	6.08	0.27	6.42	6.03	0.17**
ADG (g)	43.51	32.65	49.41	11.08	24.52	20.2	51.6	12.9	13.0**
Feed intake (g/d)	97.57	119.65	109.82	110.83	89.27	14.97	130.71	80.15	9.65**

Table 4. Effect of diet and K88 susceptibility on faecal *E. coli* excretion on d 5

	Diet					SEM	K88 susceptibility		SEM
	C	ZnO 200	ZnO 2500	Zn-Glu 200	Zn-Glu 2500		No	Yes	
Faecal excretion (ln CFU/g)									
Total *E. coli* [1]	17.8	16.9	14.6	18.0	15.3	1.1	16.2	16.8	0.7*
E. coli K88[2]	14.5	9.7	9.4	12.7	9.4	1.7	8.6	13.6	1.1**

[1] C vs. High Zn dose, $p<0.05$; Between Zn doses, $p<0.05$. [2] C vs. Zn additions, $p<0.05$; C vs. Zn High doses, $p<0.05$.
* $p = 0.10$; ** $p<0.01$.

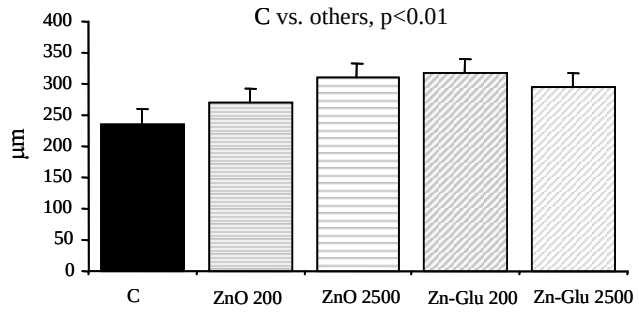

Figure 1. Effect of diet on the duodenal villous (µm) of piglets challenged with *E. coli* K88 (Means±SEM).

control and with low Zn doses ($p<0.05$). Zinc supplementations reduced the *E. coli* K88 faecal excretion, and this was mainly due to high zinc doses ($p<0.05$). K88 positive-receptor pigs tended to excrete more total *E. coli* ($p = 0.10$) and excreted more *E. coli* K88 ($p<0.01$).

Villous height and crypt depth

Villous height was higher in the duodenum than in the jejunum and the ileum (287 vs. 254 and 232 µm, respectively, SEM = 7.9, $p<0.01$). However the point of measure interacted with the diet ($p<0.05$), while the diet and the sensitivity of the intestinal villous to ETEC adhesion did not interact with each other.

In the duodenal tract, the villous height was improved by zinc supplementation (Figure 1, $p<0.01$) whatever the source and level, whereas no effect was seen in the other two tracts. The pigs susceptible to ETEC had increased villous height wherever the point was (Figure 2).

The crypts were also deeper in the duodenum than in the jejunum and the ileum (256 vs. 194 and 188 µm, respectively, SEM = 5.7, $p<0.01$). The crypt depth was not affected by zinc nor by susceptibility to ETEC.

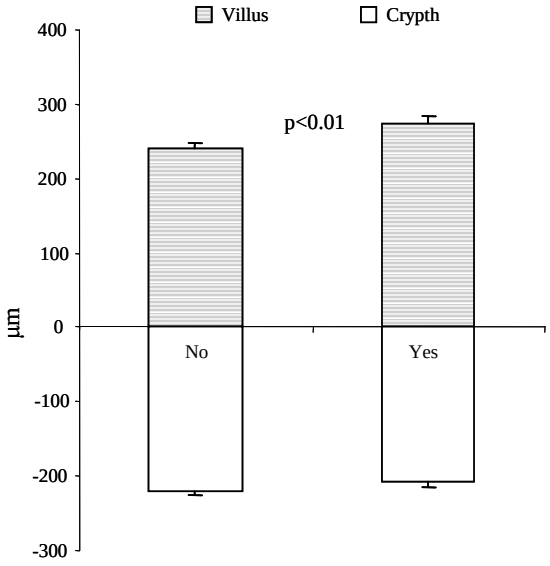

Figure 2. Effect of *E. coli* K88 susceptibility on the villous and crypt morphology (µm) of the small intestine of piglets challenged with *E. coli* K88 (Means±SEM).

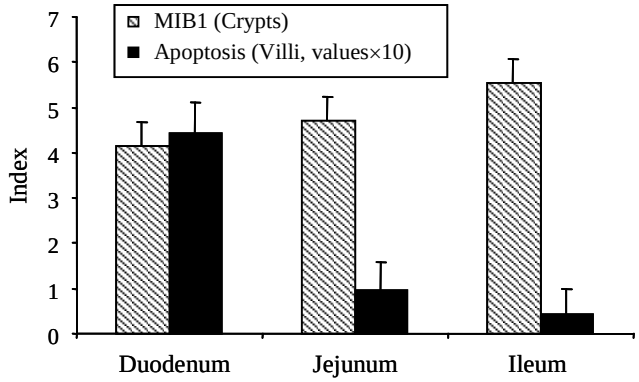

Figure 3. Effect of small intestine segment on the MIB1 index of the crypts, and on the apoptotic index in the villi of the small intestine of piglets challenged with *E. coli* K88 (Means±SEM). For MIB1, duodenum vs. (jejunum and ileum), $p<0.05$. For apoptosis: duodenum vs. (jejunum and ileum), $p<0.01$.

Intestinal mitosis and apoptosis

In general the samples obtained from the duodenum had a different pattern compared with the other two segments. The duodenum had less mitotic cells in the villi ($p<0.05$) and in the crypts ($p<0.01$) and more apoptotic cells in the villi (Figure 3, 4, 5 and 6). However, a statistically significant interaction of the intestinal point with the sensitivity of the intestinal villous to ETEC adhesion was seen for mitosis in the villi ($p<0.01$) (Figure 4 and 7). ETEC-susceptible pigs had more mitotic cells in the villi than non- susceptible pigs, particularly in the jejunum ($p<0.01$), and with less relevance in the ileum ($p<0.05$) and in the duodenum ($p = 0.10$). The sensitivity of the intestinal villous to ETEC adhesion did not affect mitosis counts in the crypts and apoptosis counts in the villi.

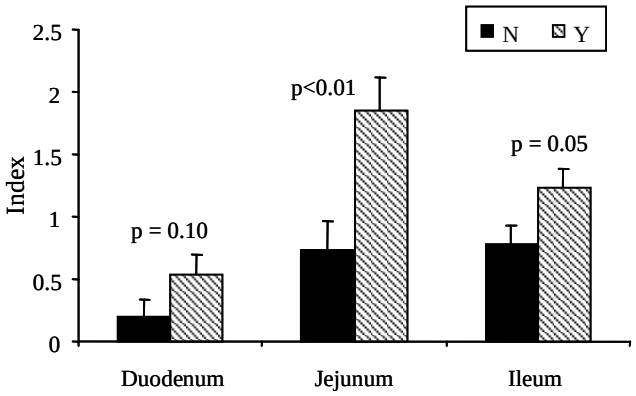

Figure 4. Effect of the susceptibility to *E. coli* K88 on the MIB1 index in the villi of different small intestinal tracts of piglets challenged with *E. coli* K88 (Means±SEM).

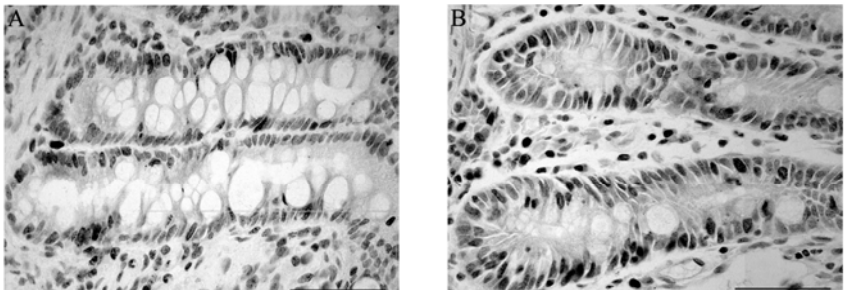

Figure 5. Piglet. MIB1 immunohistochemistry in the duodenum (A) and ileum (B). Proliferating cells (MIB1 positive), showing brown stained nuclei, scattered along the crypts. Note the different positivity between duodenum (lower) and ileum (higher). In the *lamina propria*. some fibroblasts and lymphocytes are also positive. Scale bar = 50 μm.

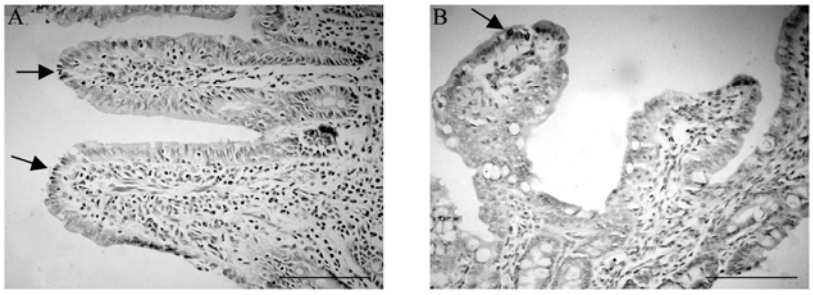

Figure 6. Piglet. Staining for apoptosis in the villi of duodenum (A) and ileum (B). Note the highest number of the positive nuclei (brown in colour, arrows) at the top of the villi in the duodenum than in the ileum. Scale bar = 100 μm.

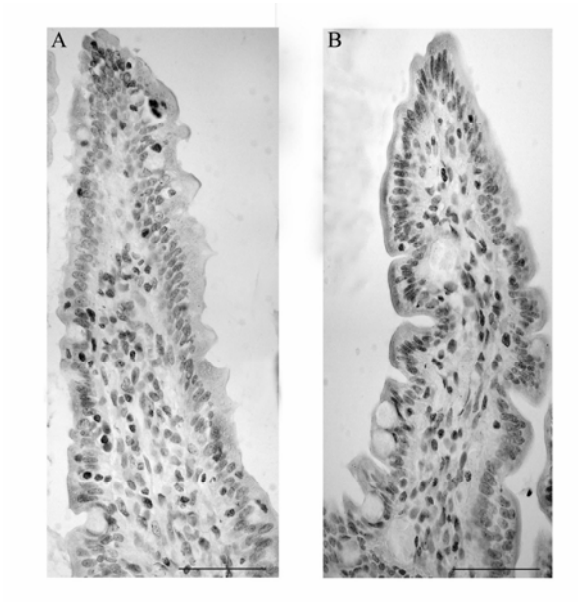

Figure 7. Piglet. High magnification of a jejunal villous (A and B), with proliferating cells (MIB1 positive). Positive cells are localized at the top and, along the villous, and in the *lamina propria* of the mucosa. Note the different positivity between the ETEC- susceptible (many positive cells, A) and non- susceptible pigs (few positive cells, B). Occasionally, some fibroblasts and lymphocytes are also positive. Scale bar = 50 μm.

The diet never interacted with the other factors, nor did it affect apoptosis and the mitosis counts (Figure 8).

DISCUSSION

Our results confirm that the short-term addition of high levels of zinc (from zinc oxide) to the weaning pig diet may have an important role in the resistance to infection by reducing the coliform multiplication and *E. coli* K88 gut colonization.

The absence of differences between the Zn sources for *E. coli* excretions indicates that zinc-glutamate chelate is also effective in reducing ETEC colonization. This indirectly confirms the observations that the growth promoting action of supra-nutritional doses of zinc is not related only to ZnO (Mavromichalis et al., 2001; Case et al., 2002; Zhang and Guo, 2007). However, our results are not conclusive regarding the possibility of reducing the effective doses by the use of an organic form of zinc, and this is consistent with the observations of Hollis et al. (2005).

The contemporary reduction of fecal excretion suggests that zinc has a local action, but after one week of Zn supplementation, the favorable effect on *E. coli* colonization was not reflected in the intestinal morphology, with the exception of the increased villous height in the duodenum. With a reduction of ETEC colonization, a more

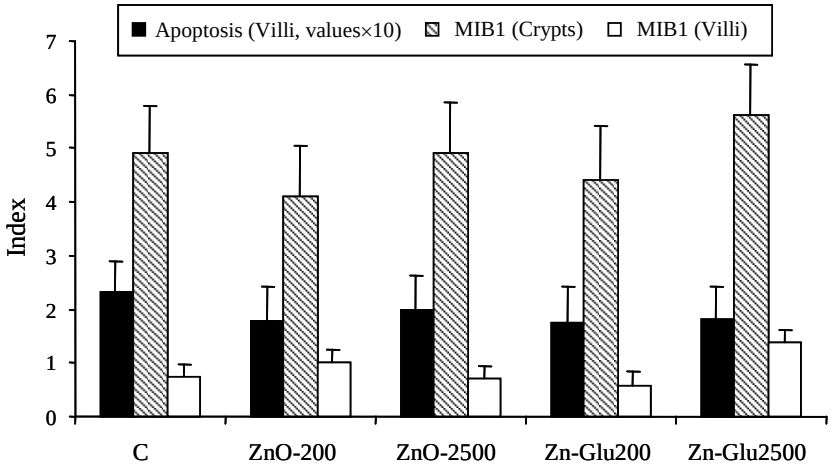

Figure 8. Effect of diet on the MIB1 index in the crypts and villi, and on the apoptotic index in the villi of the small intestine of piglets challenged with *E. coli* K88 (Means±SEM).

consistent reduction of villous atrophy was expected. However also Thymann et al. (2007), supplementing weaning pigs with antimicrobials (Amoxicillin+ZnO), did not find any effect on the small intestine villi, notwithstanding a dramatic reduction of intestinal microbial diversity and of *E. coli* colonization. Conversely Hedemann et al. (2006), observed a reduction of villi in the first 10% of the small intestine in ZnO-supplemented piglets, as compared to the controls.

Furthermore, there was no reduction of apoptosis nor an increase in the mitotic rate seen. This indicates that at this time point there was no effect of Zn addition. To our knowledge, apoptotic and mitotic cells were never counted in pigs supplemented with high ZnO. The absence of an effect of high zinc doses contrasts with the observations of a decreased expression of active caspase-3, a marker of apoptosis, in pigs fed 3,000 mg/kg Zn as ZnO (Wang et al., 2009).

Pigs receptive to ETEC intestinal adhesion responded to the challenge with greater ETEC faecal excretion. The ETEC adhesion to the intestinal epithelium may have stimulated an inflammatory response. This, in turn, can explain the higher villous height of ETEC-susceptible pigs and the fact that their greater mitosis is particularly concentrated in the mid-jejunum, that being the site where typically the ETEC receptors are more concentrated. Conversely the increased multiplication of ETEC in susceptible pigs did not affect the crypts, where no significant effect on cell recruitment into mitosis was seen. This can be because of the time which passed from the early infection with ETEC.

It is also interesting to observe that in the duodenum the mitosis rate in the crypts and in the villi were lower than in the other small intestinal tracts, and that also apoptosis was higher. Notwithstanding this, in the duodenum, the villi were higher and the crypts were deeper. This can be explained by an increased number of crypts, in the crypts to villi ratio in the duodenum, as compared with the other tracts as was observed in the mouse and rat (Alferez and Goodlad, 2007). In fact, if many crypts contribute to the development of the same villous, this can explain its great height.

In our trial, zinc addition had no effect on growth performance. Longer periods of observations were used in general when growth was positively related to high doses of zinc oxide in the diet of weaning pigs (Hill et al., 2001). Seven days may not have been sufficient to evaluate the influence of zinc supplementation.

Villous development and adaptation to nutritional stress is a very dynamic and complex phenomenon. Our data indicate that one week after ETEC challenge, ETEC-susceptible pigs show an increase of the height of the intestinal villi. However, this observation is presumably the result of an adaptation which was almost complete, as indicated by the absence of an effect on the rate of in the crypts. Moreover, a residual sign of this effect can be identified in the higher mitotic index of cells, presumably still migrating, observed in the jejunum villi, were the ETEC adhesion is higher in susceptible pigs.

In this context, the absence of indications of an effect of zinc, except for the higher villi in the duodenum, should be considered carefully. In particular, the absence of an effect on the mitotic and apoptotic indexes should be regarded as a clinical picture five days post-challenge but this does not exclude the possibility that some effects could occurred earlier. More dynamic research tools should be implemented to improve our knowledge of how the gut progressively adapts to post-weaning changes and to the stress of the intestinal microbiota.

The results do not support a specific effect of zinc in the chelated for on small intestine morphology, and particularly on enterocyte turnover, as compared to an inorganic source.

The data still confirm that supranutritional doses of zinc can effectively reduce the intestinal colonization of enterobacteria and particularly of ETEC, but also that more than one week may be necessary to fully exploit the auxinic effect of a 3,000 mg/kg zinc dietary addition.

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