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RUNNING TITLE: Dietary copper sulphate for piglets gut**.**

Copper sulphate forms in piglets diet: microbiota, intestinal morphology and enteric nervous system glial cells.

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ABSTRACT

The objective of this study was to evaluate dietary supplementation with different copper sulphate ($CuSO_4$) forms on small intestine microanatomy and large intestine microbiota. Ninety weaned piglets were divided into three experimental groups: control diet (CTR), with no added $CuSO_4$ and diets supplemented with 150 ppm of $CuSO_4$ in protected (150P) and unprotected form (150UP). After 18 days of dietary treatment, six piglets per treatment were randomly selected and sacrificed. Duodenum villi length and crypts depth were higher (P <0.001) in the animals fed 150UP than other groups. The glial fibrillar acidic protein (GFAP), a marker for enteric glial cells, were unaffected by dietary treatments. The total bacteria and *Enterobacteriaceae* bacteria counts were lower (P <0.05) in caecum of animals fed 150P in comparison with the other two groups. In the colon the *Streptococci spp* were lower (P <0.001) in both $CuSO_4$ supplemented groups than controls. The obtained results revealed a modulation of intestinal structure and microbiota exerted by the studied $CuSO_4$ dietary supplementation. The present data show that dietary supplementation with 150UP in the first period post weaning may act restoring the gut morphology, improving duodenal structure.

Keywords: copper sulphate, intestinal microanatomy, microbiota, nutrition, piglets.

INTRODUCTION

Reduction in antimicrobial use and prevention of pathologies is a key point to keep good productivity and animal health (Seal *et al.* 2013). Copper is an essential trace element that plays an important role in many physiological processes. In weaned piglets, copper dietary level of 5 to 6 mg/kg feed is adequate to meet the nutritional requirements (NRC 2012). However, it is usually used in piglet's diet at higher concentrations (100-175 ppm) due its growth promoting effects (Shelton *et al.* 2011). In fact, several studies showed that dietary copper supplementation in weaned piglets can modulate gut health, reducing potential pathogens (Jensen 1998; Højberg *et al.* 2005). Otherwise, Fry *et al.* (2012) reported that dietary copper sulphate ($CuSO_4$) supplementation in weaned piglets increased oxidative stress status in small intestine negatively affecting villi height in duodenum and jejunum. Moreover, Huang *et al.* (2015) observed that the same $CuSO_4$ feed content increased duodenal mucosal malondialdehyde content. Copper is essential for neuron physiological function; in fact, brain astrocytes are deeply involved in copper homeostasis and an imbalance of this latter, may lead to neurodegeneration (Scheiber *et al.* 2014). On this basis, it is also possible that $CuSO_4$ affects intestinal functions due to interactions with enteric nervous system (ENS), which regulates the motor and secretory activities of the gut. The enteric glial cells are analogous to the astrocytes of the central nervous system, they express glial fibrillary acidic protein (GFAP), form complex intramural networks, and are reputed to display multitasking roles in the intestinal barrier that acts between the external environment and the gut (Yu & Li 2014). Enteric glial cells protect against pathogen invasion, too, thus completing the mucosal barrier (Savidge *et al.* 2007). Moreover, dietary $CuSO_4$ supplementation in post weaning piglets positively modulated intestinal microflora, reducing the number of coliforms in both cecum and colon (Højberg *et al.* 2005). Different sources of dietary copper have been shown to display a different effect on intestinal structure and microflora (Armstrong *et al.* 2004; Creech *et al.* 2004). On these bases, the aim of the present study was to determine the

effects of dietary supplementation in post weaning piglets with different forms of copper sulphate on small intestinal micro anatomy and large intestine microbiota.

MATERIALS AND METHODS

Procedures involving animals were carried out in accordance with the European Communities Council Directive (2010/63/EU) and approved by the Italian Ministry of Health (DL 26, 2014 march 4th).

Animals, diets and management

Ninety newly weaned female piglets (Landrace × Large White) of 26 days of age weighing 8.4 ± 0.2 kg, were stratified by weight and randomly assigned to one of the three dietary treatments. The animals were divided in 3 pens per treatment (10 piglets/pen) and reared in an environmentally-controlled room (average temperature 26° C and relative humidity 60%). The dietary treatments consisted in a basal diet (CTR) with no added $CuSO_4$, and two other diets that contained 150 ppm of $CuSO_4$ in protected (150P) and unprotected form (150UP), respectively. The protected form of $CuSO_4$ was microencapsulated in a protective matrix of hydrogenated vegetable lipids manufactured with a spray cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy). None of the diets was medicated. The experimental diets were formulated to meet the nutrient requirements for piglets (NRC 2012) (Table 1). During the experimental period lasting for 18 days, pigs were allowed *ad libitum* access to feed and water. Piglets' growth performance were reported in our previous study (Pastorelli *et al.* 2014). After 18 days, at an average body weight (BW) of 12.3 ± 0.15 kg, six piglets per treatment were randomly selected and slaughtered following the standard procedure. Samples of duodenum (2 cm after the pylorus), jejunum (5 cm after the beginning of the jejunum), and ileum (1 cm before the caecum) were immediately excised from each pig. Fresh samples of caecum and colon contents were collected from each animal for microbial counting as described by Shen *et al.* (2009).

Histology and Histometry

The duodenum, jejunum and ileum samples were transversally sectioned and used for standard paraffin hematoxylin/eosin-stained sections histology. For intestinal histometry, on HE-stained sections the height of intestinal villi (V) (10 villi measured per section), the depth of intestinal crypts (C) (10 crypts measured per section), and the ratio of villi and crypts values (V:C ratio) were measured and calculated by an image analysis software (Image pro Plus 6.3 Media Cybernetics Inc., Silver Springs, USA).

Histochemistry

Sections from duodenum, jejunum, and ileum were used to determine the mucin profile, which was obtained analysing the histochemical reactivity of the intestinal mucous cells. The following histochemical stains were employed: a) Alcian blue 8GX pH 2.5-periodic acid Schiff (AB-PAS) sequence, which reveals neutral (PAS-reactive, purple stained) and acid (AB-reactive, azure stained) glycoconjugates, and b) high iron diamine-Alcian blue 8GX pH 2.5 (HID-AB) sequence, which demonstrates sulphated (diamine-positive, brown-black stained) and sialylated (AB-reactive, azure stained) glycoconjugates respectively.

Immunohistochemistry and cell counts

Sections from duodenum, jejunum and ileum were utilized to evaluate enteric glial cells. Immunohistochemistry was performed with the biotin/avidin system using the anti glial fibrillar acidic protein (GFAP, Dakocytomation, code N1506; 1:1000) (Di Giancamillo et al. 2010). Immunoreactive enteric glial cells were counted in the enteric nervous system of duodenum, jejunum and ileum that is in this species organized as follows: i) inner submucosal plexus (Schabadasch's plexus), ii) outer submucosal plexus (Meissner's plexus), iii) myenteric plexus (Auerbach's plexus).

Quantifications of glial cells were referred to each intestinal section area and extrapolated to mm-^{2} to allow comparison of the data, thus reflecting glial cell density (Di Giancamillo *et al.* 2010). The specificity of the immunostaining was verified by incubating other sections with: (i) PBS instead of the specific anti-GFAP antibody; (ii) pre-immune serum instead of the primary antiserum; (iii) PBS instead of the secondary antibodies. The results of these controls were negative (i.e. staining was abolished). All the microscopic observations were made using an Olympus BX51 light microscope (Olympus, Milan, Italy), equipped with a digital camera. The observer was not aware of the origin of the sections.

Microbiological analyses

The microbiological counts in the large intestinal digesta were determined by the plate-count technique (Shen *et al.* 2009). One gram of digesta was homogenized in salt solution (0.9% NaCl) and serially diluted in sterlile saline ranging from 10^{-1} to 10^{-10}. Then each sample was inoculated on the following agar plates, using standard protocols: for total bacterial count, Bile Esculine Agar (BEA, Oxoid, Milan, Italy) for *Streptococci spp* count, MacConkey medium (Oxoid, Milan, Italy) for *Enterobacteriaceae* count, and Man-Rogosa-Sharpe Agar (MRS Oxoid, Milan, Italy) for *Lactobacillus spp* count. The plates were incubated in an aerobic atmosphere at 20°C for 24 h (for TSA and MacConkey medium), and at 20°C for 48 - 72 h under 5% CO_2 atmosphere (for BEA and MRS medium). The microbial populations were log transformed before statistical analysis and data are expressed as $\log_{10}$ colony-forming units (CFU) / g of digesta.

Statistical analysis

Statistical analyses of the quantitative data were performed using the general linear model of the SAS (version 8.1, Cary Inc., NC, USA). One-way analysis of variance (ANOVA) was used to determine the effect of dietary $CuSO_4$ form on microbiological analyses. Histometrical analyses and cell counts

were analysed by ANOVA using the PROC MIXED of the SAS package. The mixed model included the fixed effects of treatment and the random effect of the piglet. Individual piglets was considered as the experimental unit. The data were presented as least squared means ± SEM. Differences between means were considered significant at $P < 0.05$.

RESULTS

Intestinal Histology and Histometry

Histological evaluation of duodenum, jejunum and ileum revealed that dietary supplementation with 150 ppm of $CuSO_4$ in both forms did not show detrimental effects upon the small intestine structure (Fig. 1). Histometrical analyses of small intestine are reported in Table 2. Histometrical evaluation of jejunum and ileum indicated no effect ($P > 0.05$) of dietary supplementation with $CuSO_4$ in both forms on villus height, crypt depth, and villus height to crypt depth ratio. In the duodenum, villi height and crypt depth resulted higher ($P < 0.001$) in 150UP than in 150P and control groups.

Histochemistry

The histochemistry data showed that the duodenum and jejunum goblet cells contained mixtures of neutral and acidic glycoconjugates in both villi and crypts (Fig. 2 a, d, g - b, e, h) in either control or treated piglets. Limited to the ileum, villi goblet cells prevalently contained neutral glycoconjugates (Fig. 2, c, f, i, arrows) and intestinal glands mucous cells prevalently contained acid glycoconjugates (Fig. 2, c, f, i, asterisks). In addition, duodenal Brünner glands were always PAS-reactive only, irrespective of the treatments (Fig. 2, a, d, g, arrowheads). The sulphated glycoconjugates revealed to be the predominant type in the goblet cells at the top of the villi, otherwise, sialo-glycoconjugates occurred mainly at the bases of the villi and along crypts without differences among experimental groups. (Fig. 2 j-r).

171 **Immunohistochemistry and cell counts**

172 An evident GFAP-immunopositivity was present in small cell bodies and in slender cytoplasmic
173 processes in ganglia belonging to the inner and outer submucosal plexuses, as well as in the myenteric
174 plexus (Fig. 3). In Table 3 data on GFAP-positive cells in small intestine was reported. No differences
175 (P >0.05) on GFAP-positive cells were observed in duodenum, jejunum and ileum among control
176 versus treated groups.

177

178 **Microbial counts in large intestine**

179 Microbiological analyses of caecum and colon contents are reported in Table 4. Total bacteria count
180 in caecum revealed to be lower (P <0.001) in piglets fed 150P than control and 150UP ones. A
181 decrease (P <0.001) in *Enterobacteriaceae* was also evident in piglets fed 150P. In the colon total
182 bacterial count was higher (P <0.001) in group fed 150UP than 150P and control groups. The dietary
183 treatments did not affect the *Enterobacteriaceae* count in the colon. In the caecum the
184 *Enterobacteriaceae* were lower (P <0.05) in animals fed 150P in comparison with the other two
185 groups. The number of *Streptococci* of the treated animals was significantly lower in the colon when
186 compared to the control group (P <0.01), whereas no differences were observed in the caecum. No
187 differences (P >0.05) were observed on the number of *Lactobacilli* in both caecum and colon of the
188 three groups of animals.

189

190 **DISCUSSION**

191 The weaning phase is the most stressful event in pig's life, in fact, the gastrointestinal tract is not yet
192 adequately developed and this could lead to pathogenic bacteria proliferation. Moreover, intestinal
193 morphological changes during weaning are reputed to cause a reduced absorptive capacity (Boudry
194 *et al.* 2004). Usually, copper sulphate dietary supplementation is a common mean to improve growth
195 performances in post weaning piglets due to its antibacterial activity (Cromwell 2002). On the other

196 hand, a study reported that ionic copper might work as a pro-oxidant in the organism, catalysing
197 hydroxyl radicals formation (Bremner 1998). In fact, Fry *et al.* (2012) recently found that in post
198 weaned piglets, dietary copper sulphate increased oxidative stress markers in the duodenum, which
199 negatively affect villi morphologies. In the present study, histological data of duodenum, jejunum
200 and ileum showed a normal structure. Only in duodenum dietary treatment with the copper sulphate
201 in unprotected form increased villi length and crypt depth. Both these structures, and in particular
202 villi height, are important factors to evaluate the digestive capacity in piglets and are positively
203 correlated with weight gain and feed conversion ratio (Pluske *et al.* 1997). In fact, piglets feed
204 conversion was improved in the same experimental group (1.63 kg/kg 150UP *vs* 2.2 kg/kg 150P,
205 P=0.07) (Pastorelli *et al.* 2014). In literature, the data on the relationship between dietary copper
206 sulphate and intestinal histometry are quite controversial. Shurson *et al.* (1990) observed an increase
207 in villus height and crypt depth in weanling piglets fed high level of copper sulphate. No changes in
208 intestinal morphology of weaned piglets with dietary supplementation of 250 ppm of copper sulphate
209 were observed (Radecki *et al.* 1992). In disagreement, Zhao *et al.* (2007) observed decreased crypts
210 depth in the small intestine of weaning piglets treated with dietary supplement of 200 ppm of $CuSO_4$
211 for 35 days. Also Fry *et al.* (2012) reported a reduced duodenal villus height in relation to $CuSO_4$
212 supplementation. This difference should be related to the high dosage of $CuSO_4$ supplementation used
213 by the authors (225 mg/kg feed) and the different length of dietary supplementation (35 days). We
214 speculate that $CuSO_4$ supplementation in the first period post weaning may act by restoring the gut
215 morphology while with a longer supplementation period it may act as pro-oxidant, negatively
216 affecting intestinal mucosa. The histochemical evaluation of mucous cells glycoconjugate content did
217 not show qualitative differences of the cell reactivity among the experimental groups, thus evidencing
218 that the studied dietary interventions did not modify the small intestinal mucin profile. Similar results
219 were obtained by Hedemann *et al.* (2003) who observed that the staining area of neutral, acidic or
220 sulphated mucins of the intestinal mucous cells was not affected by treatments. Moreover, considering

221 that an excess of Cu is associated with oxidative stress (Cerpa *et al.* 2005) we decided to study enteric
222 glial cells (EGC), which are crucial for gut neuronal maintenance (Rühl 2005). In the present study,
223 copper supplementation did not affect the enteric glial cells number in the small intestine, suggesting
224 the absence of toxic effect upon the gut. The EGC form a fundamental component of the enteric
225 nervous system, and their connections with intramural neurons are able to regulate the intestinal
226 barrier function. Actually, it has been observed that EGC are necessary in the maintenance of
227 intestinal homeostasis (Yu *et al.* 2014), considering their essential, even if indirect, role in
228 maintaining the mucosal barrier integrity, which inhibits pathogens and toxins translocation. To our
229 knowledge, it is the first time that the interaction between dietary copper sulphate and EGC has been
230 studied. The data reveal that at the present dosage the dietary supplementation did not affect GFAP-
231 positive cells, whose expression in mature EGC is modulated by cell differentiation, inflammation,
232 and injury (Rühl 2005). The *Enterobacteriaceae* and *Streoptococci* counts in the distal part of
233 gastrointestinal tract are in line with the data reported by Pieper *et al.* (2009) in healthy piglets. The
234 present data showed that dietary supplementation with 150P affects caecum total bacterial counts, as
235 well as *Enterobacteriaceae* counts. In the colon, copper sulphate in both forms decrease
236 *Enterobacteriaceae* and *Streptococci* counts. The *Enterobacteriaceae* including *E. coli* and
237 *Salmonella*, are the main contributors to post weaning diarrhoea, and their reduction is consistent
238 with the health piglets status (Stensland *et al.* 2015). Our data are in agreement with Varel *et al.*
239 (1987) that observed a decreased number of intestinal populations of *Streoptococci* in the colon of
240 piglets treated with 125 ppm of copper sulphate. In addition, Jensen (1998), and Højberg *et al.* (2005)
241 observed that colifoms counts decreased in the colon of animals treated with 175 ppm of copper
242 sulphate. In conclusion, the present study shows that dietary supplementation with 150UP in the first
243 post weaning period may act by restoring the gut morphology, improving duodenal structure and
244 positively modulating large intestinal microbiota. As a last consideration, the dietary treatment with
245 copper in both forms did not stimulate EGC activation. Further studies are required to evaluate the

influence of dosage and length of copper sulphate dietary supplementation on gut oxidative stress markers.

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Table 1. Composition of experimental diet as-fed basis.

Ingredient	g/kg
Steam-rolled corn	280
Corn, yellow	150
Barley	150
Wheat middlings	80
Dried whey	50
Soy protein concentrate	40
Soybean meal 48	60
Fish meal, 70	28
Rice protein meal, 65	24
Dextrose	25
Wheat bran	30
Soy oil	30
Vitamin-mineral premix[1]	35
Dicalcium phosphate	10
L-Lysine·HCl	5
Preservative[2]	3

[1] Provided per kilogram of complete diet: Ca, 2.8 g; P, 0.14 g; Na, 1.33 g; vitamin A, 16,000 IU; vitamin D_3, 2,000 IU; vitamin E, 175 IU; vitamin K (menadione sodium bisulphite), 3.8 mg; vitamin B_1, 4.9 mg; vitamin B_2, 9.8 mg; calcium D-pantothenate, 40 mg; niacin, 57.8 mg; vitamin B_{12}, 0.09 mg; vitamin B_6, 7.7 mg; folic acid, 3.4 mg; biotin, 0.33 mg; choline chloride, 1000.0 mg; Zn (ZnO), 100.0 mg; Cu (CuSO4), 0, 150 mg for control and treated groups; Mn (MnO), 108.0 mg; Fe ($FeCO_3$), 270.0 mg; I (KI), 3.85 mg; Co ($CoSO_4$), 1.40 mg; Se (Na_2SeO_3), 0.49 mg. Premix containing Calcium formiate, sodium chloride, barley, butyric acid, dl-tryptophan, dl-methionine, l-threonine.

[2]Composition per kg of complete feed: formic acid, 0.3 g; lactic acid, 1.1 g; colloidal silica carrier 1.6 g.

Table 2. Effect of dietary copper sulphate, in protected and unprotected form, on histometrical analyses of piglet small intestine.

	Dietary treatment[2]		
Item[1]	CTR	150P	150UP
Duodenum			
Villi height (µm)	$357.35^{A} \pm 6.84$	$351.55^{A} \pm 11.06$	$392.04^{B} \pm 7.98$
Crypts depth (µm)	$436.54^{A} \pm 9.19$	$439.80^{A} \pm 7.92$	$473.37^{B} \pm 9.25$
V:C ratio	0.82 ± 0.011	0.79 ± 0.02	0.83 ± 0.02
Jejunum			
Villi height (µm)	317.04 ± 14.87	345.83 ± 9.19	346.02 ± 10.05
Crypts depth (µm)	394.67 ± 8.06	380.19 ± 8.41	374.34 ± 6.66
V:C ratio	0.81 ± 0.04	0.91 ± 0.03	0.92 ± 0.02
Ileum			
Villi height (µm)	364.93 ± 12.02	350.48 ± 7.39	343.65 ± 6.72
Crypts depth (µm)	367.90 ± 9.79	332.54 ± 8.39	327.27 ± 6.14
V:C ratio	1.00 ± 0.04	1.06 ± 0.03	1.05 ± 0.02

[1]Values are means ± SEM, n =6.

[2]CTR, no added copper sulphate; 150P, 150 ppm of copper sulphate in protected form; 150UP, 150 ppm of copper sulphate in unprotected form.

[A,B,C] Means with different superscripts differ significantly ($P < 0.001$).

388 **Table 3.** Effect of dietary copper sulphate, in protected and unprotected form, on glial fibrillar
389 acidic protein (GFAP) in piglets small intestine.

	Dietary treatment[2]		
Item[1]	CTR	150P	150UP
Duodenum			
Auerbach's plexus	51.89 ± 12.48	75.42 ± 10.81	66.44 ± 12.48
Meissner's plexus sup	58.00 ± 12.01	60.75 ± 10.40	61.00 ± 12.01
Meissner's plexus prof	54.78 ± 6.70	62.25 ± 5.80	67.44 ± 6.70
Jejunum			
Auerbach's plexus	45.44 ± 11.03	54.83 ± 9.55	46.44 ± 11.03
Meissner's plexus sup	56.78 ± 9.97	53.16 ± 8.63	50.44 ± 9.97
Meissner's plexus prof	46.55 ± 5.61	58.50 ± 4.85	48.00 ± 5.61
Ileum			
Auerbach's plexus	43.67 ± 7.39	74.17 ± 6.40	59.89 ± 7.39
Meissner's plexus sup	75.78 ± 15.52	74.00 ± 13.44	72.55 ± 15.52
Meissner's plexus prof	65.89 ±11.64	74.91 ± 10.08	57.00 ± 11.64

390 [1]Values are means ± SEM, *n* =6.

391 [2]CTR, no added copper sulphate; 150P, 150 ppm copper sulphate in protected form; 150UP, 150 ppm
392 copper sulphate in unprotected form.

393

Table 4. Effect of dietary copper sulphate, in protected and unprotected form, on microbiological analyses in piglets caecum and colon.[1]

	Dietary treatment[2]		
Item[1]	CTR	150P	150UP
Caecum			
Total bacterial count	$6.71^{A} \pm 0.40$	$4.70^{B} \pm 0.22$	$7.00^{A} \pm 0.01$
Enterobacteriaceae spp	$6.56^{a} \pm 0.39$	$4.43^{b} \pm 0.23$	$6.30^{a} \pm 0.60$
Streptococcus spp	6.37± 0.25	5.72 ± 0.24	5.77 ± 0.73
Lactobacillus spp	6.54 ± 0.65	6.22 ± 0.60	6.34 ± 1.11
Colon			
Total bacterial count	$6.84^{A} \pm 0.21$	$5.60^{B} \pm 0.26$	$7.00^{A} \pm 0.01$
Enterobacteriaceae spp	6.58 ± 0.30	6.22 ± 0.47	6.90 ± 0.72
Streptococcus spp	$6.92^{A} \pm 0.50$	$5.79^{B} \pm 0.23$	$5.77^{B} \pm 0.73$
Lactobacillus spp	6.54 ± 0.66	5.70 ± 0.71	6.34 ± 0.87

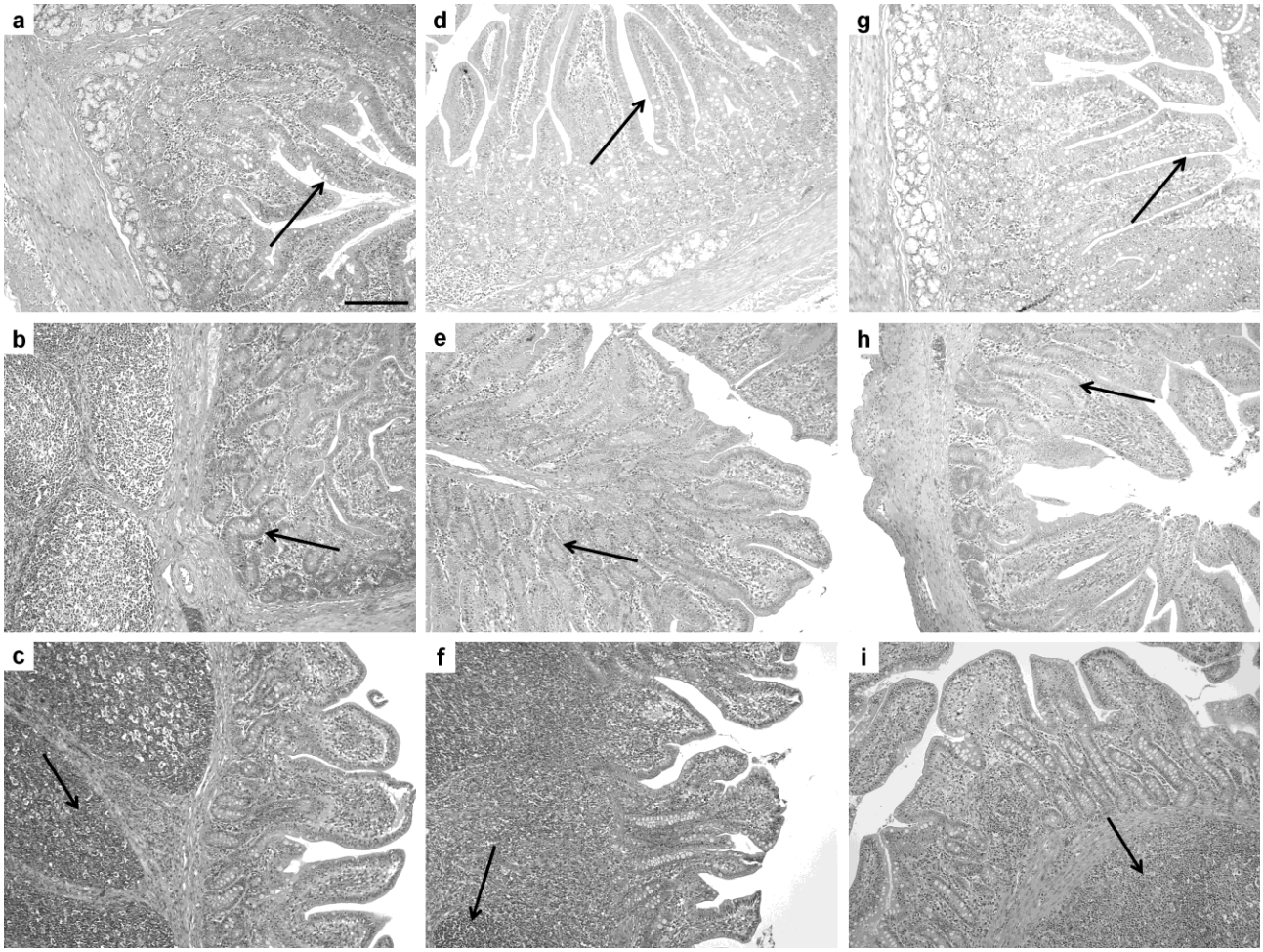
[1]Values are means ± SEM, n =6; data are expressed as $\log_{10}$ colony-forming units / g of digesta)

[2]CTR, no added copper sulphate; 150P, 150 ppm copper sulphate in protected form; 150UP, 150 ppm copper sulphate in unprotected form.

[A,B,C] Means with different superscripts differ significantly ($P < 0.001$).

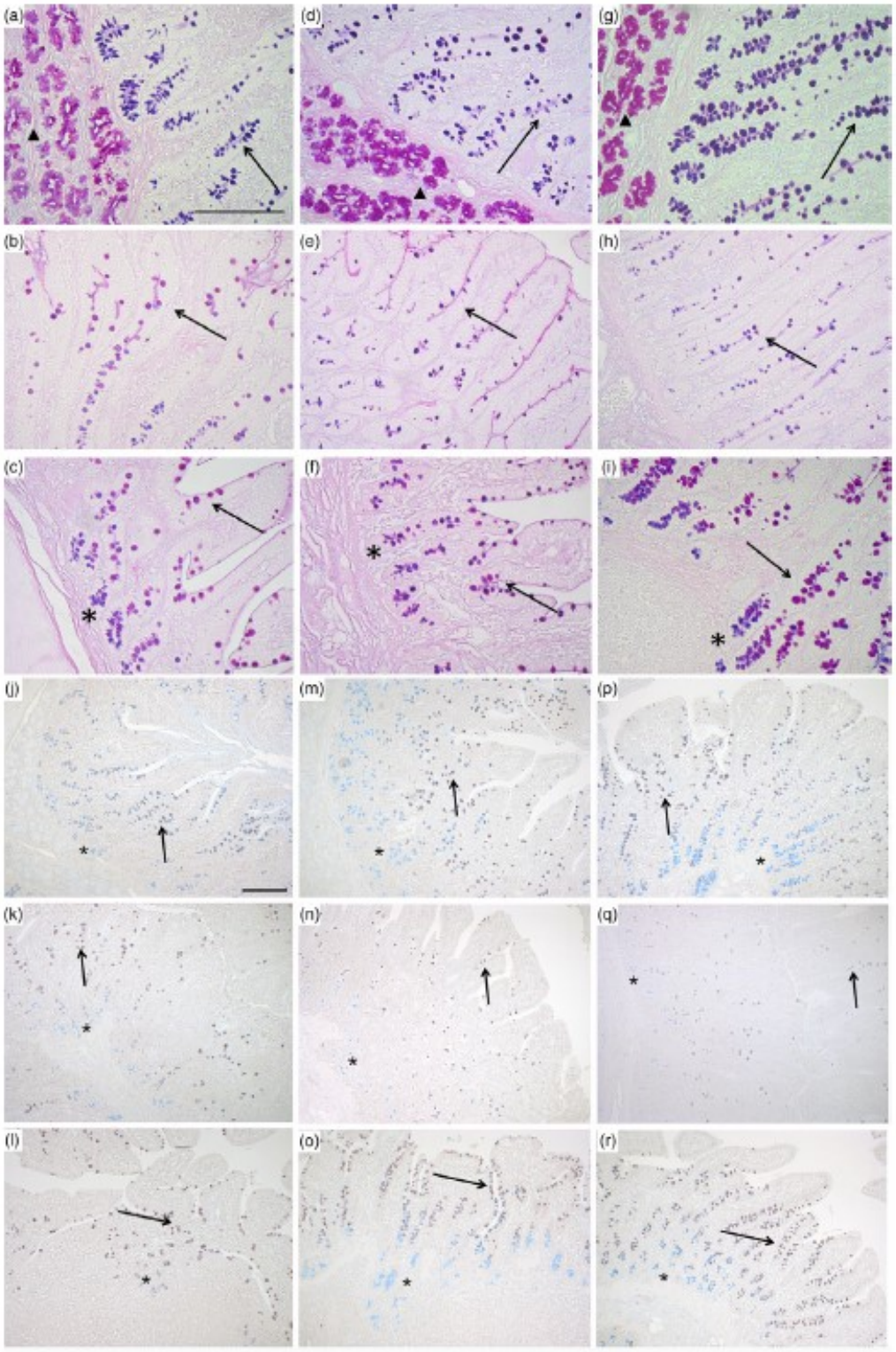
[a,b,c] Means with different superscripts differ significantly ($P < 0.05$).

Figure 1. Histological analyses of duodenum, jejunum and ileum, HE.[1]



[1]Histology of duodenum (a, d, g), jejunum (b, e, h) and ileum (c, f, i). All figures have the same scale bar located in Fig 1A: scale bar: 200 μm. a,d,g) Normal aspect of the small intestine mucosa: note the numerous villi (arrows) which appear to be regularly arranged and shaped in all animal groups (a=CTR; d=150P, g=150UP); b,e,h) Normal aspect of the small intestine mucosa: intestinal crypts (arrows) are evident in all the piglets groups (b=CTR; e=150P, h=150UP); c, f, i) Gut-associated lymphoid tissue is evident in all the piglet groups (c=CTR; f=150P, i=150UP).

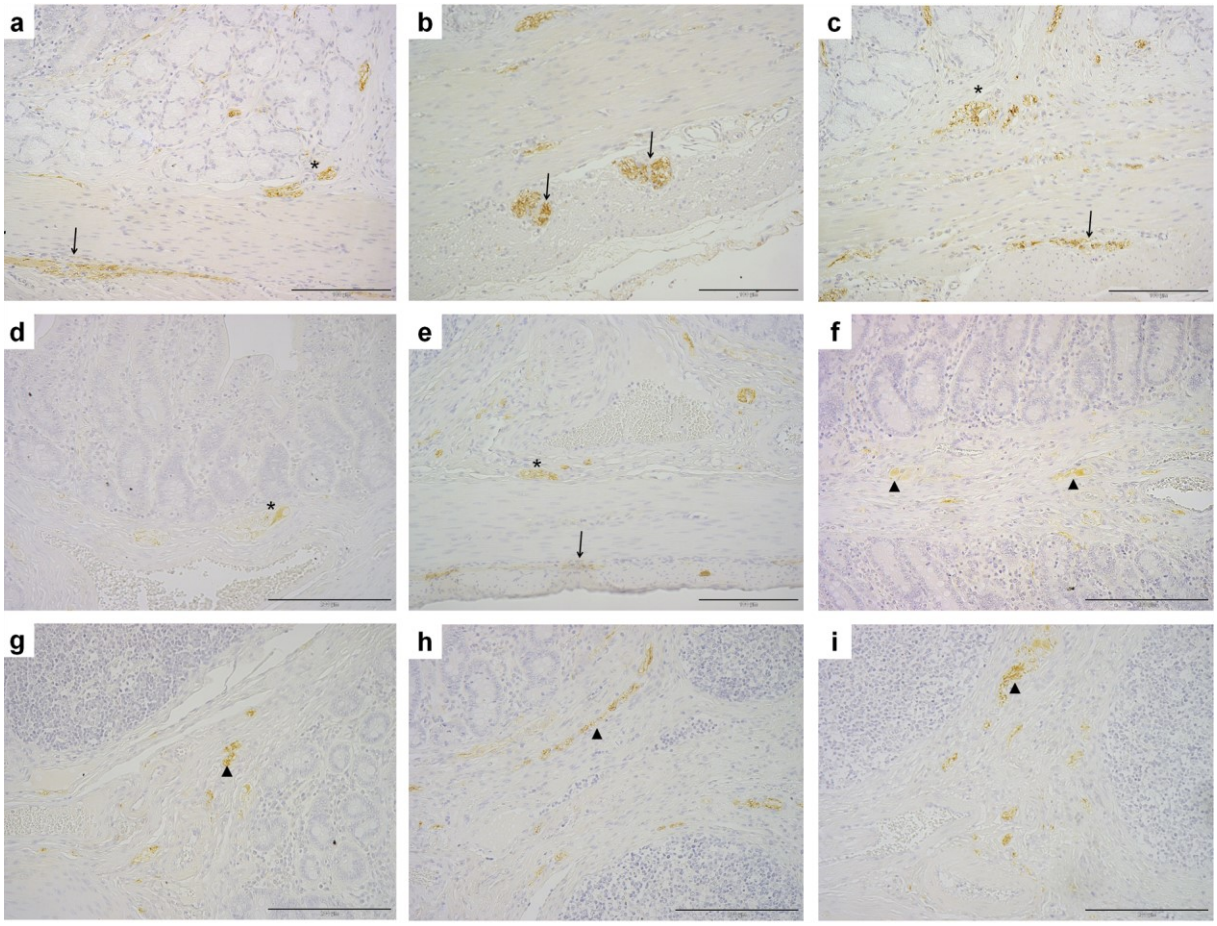
Figure 2. Histochemistry of duodenum, jejunum and ileum, Alcian blue (AB) pH 2.5/periodic acid-Schiff (PAS) or high iron diamine (HID)/AB pH 2.5 sequence.



Histochemistry of duodenum (a, d, g), jejunum (b, e, h) and ileum (c, f, i). All figures have the same scale bar located in (a): scale bar 200 lm. AB pH 2.5/PAS (a,d,g – b,e,h): there is a high prevalence

of mixed (violet color) glycoconjugates in the mucous cells of duodenum and jejunum (arrows); in the duodenum the Brunner glands contain neutral glycoconjugates only (a = CTR, d = 150P, g = 150UP, arrowheads). In ileum (c = CTR; f = 150P, i = 150UP), intestinal gland mucous cells contain prevalently acid (blue) glycoconjugates (asterisks). AB pH 2.5/PAS (j–r): the intestinal mucous cells contained prevalently sulphate (brown-black) glycoconjugates in the villi (arrows) and sialo-glycoconjugates (azure) in the crypts (asterisks) CTR, control; 150P, 150 ppm of CuSO4 in protected form; 150UP, 150 ppm of CuSO4 in unprotected form.

Figure 3. GFAP-immunohistochemical analyses of duodenum, jejunum and ileum sequence.[1]



[1] GFAP-immunohistochemical analyses of duodenum (a, d, g), jejunum (b, e, h) and ileum (c, f, i). Scale bar: 200 µm. A GFAP-positivity was present in small cell bodies and in their cytoplasmic processes in ganglia belonging to the inner (asterisks) and outer (arrowheads), as well as in the myenteric plexuses (arrows).