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Small intestinal mucosal cells in piglets fed with probiotic and zinc: a qualitative and quantitative microanatomical study

Running head: Mucosal cells of small intestine in piglets

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ABSTRACT

Background: Probiotics and zinc are commonly used and beneficial in pig production. This work aimed to assess the effects of probiotic and zinc on the mucosal cells of the small intestine in respect to digestive capacity and immunity in pre and post-weaned piglets.

Materials and methods: Eighteen LWY piglets were divided equally into control and treatment groups. The piglets were maintained in standard management conditions and were weaned at 28 days of age. The treatment group of piglets fed a mixture of probiotics orally @ 1.25×10^9 CFU/day and zinc @ 2000 ppm/day from birth to 10 days of age. At three different age-groups viz. day 20 (pre-weaning) and, day 30 and day 60 (post-weaning), the animals were sacrificed. For histomorphology, the tissue samples were processed and stained

with Mayer's hematoxylin and eosin for routine study, combined PAS-Alcian blue for mucopolysaccharides and Masson-Hamperl argentaffin technique for argentaffin cells. The stained slides were observed under the microscope. The samples were processed as per the standard procedure for scanning and transmission electron microscopy. The statistical analysis of the data using the appropriate statistical tests was also conducted.

Results: The mucosal epithelium of villi and crypts were lined by enterocytes, goblet cells, argentaffin cells, microfold (M-cell) cells, tuft cells and intraepithelial lymphocytes (IEL). The multipotent stem cells were located at the crypt base. The length of the enterocyte microvilli was significantly longer ($P < 0.05$) in the treatment group of piglets. The number of different types of goblet cells and argentaffin cells was more in treated piglets irrespective of segments of intestine and age. The IEL was located in apical, nuclear and basal positions in the lining epithelium of both villus tip and base with their significant increased in the treatment group of piglets. The TEM revealed the frequent occurrence of tuft cells in the lining mucosa of the small intestine in treated piglets.

Conclusions: Dietary supplementation of probiotic and zinc induced the number of different mucosal cells of villi and crypts in the small intestine that might suggest the greater absorptive capacity of nutrients and effective immunity in critical pre and post-weaned piglets.

Key words: probiotic, zinc, lining cells, small intestine, piglets

INTRODUCTION

Piglets during the suckling period, exposed to a variety of stresses. Weaning stress in piglets is the major cause for economic loss to pig farmers [52]. The weaned piglets have limited digestive capacity that might trigger fermentation of undigested protein by opportunistic pathogens residing in the GI tract results in diarrhea [20, 30]. In pig production, diarrhea has been one of the most frequently encountered clinical signs of disease in neonatal pigs [1]. Enteric diseases in newborn piglets are estimated to account for 5-24% of the overall pre-weaning mortality [51]. The economic impact of such high death rates is huge. The immunology of the porcine intestinal tract is important to resist the piglets from disease, which may lead to retarded growth and death.

There has been considerable interest in using some probiotic microorganisms and antioxidants in feeds. Probiotics are viable microorganisms and supportive substances that, once ingested by animals, produce beneficial physiology effects by assisting in the

establishment of an intestinal population, which is beneficial to the host entity and antagonistic to harmful bacteria.

Zinc is an important trace element that is naturally present in the feed and involved in various physiological functions. Feeding supplemental zinc in the form of zinc oxide (ZnO) to nursery pigs has decreased the incidence of nonspecific post-weaning diarrhea [40]. Zinc is virtually present in all body tissues, but only a small amount is stored in the body. Zinc can immediately be mobilized if the intake of the element is reduced or too low. Therefore, daily consumption of sufficient zinc is necessary, as the body can only compensate for a minimal extent by the use of internal zinc pools for even a short temporary deficiency [6].

There is a paucity of available literature regarding the effects of probiotic and zinc in the cellular structure of intestinal epithelium in pre and post-weaned piglets. Therefore, the present study was undertaken to evaluate and compare the combined effects of probiotic and zinc on different mucosal cells of villi and crypts of the small intestine in the control and treatment group of piglets that are responsible for digestive capacity and immunity in critical pre and post-weaned periods.

MATERIALS AND METHODS

Animals

Eighteen healthy Large White Yorkshire (LWY) piglets, irrespective of sex obtained from three sows, were utilized for the study. Care and management of the animals were provided in Instructional Pig Farm, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl, Mizoram, India. The Institutional Animal Ethics Committee (IAEC) ethically approved the animals used for the experiment vide Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/17-18/490 dated 09.08.2017.

Selection, dose and period of treatment

A mixture of probiotic consisted of *Lactobacillus acidophilus* (650 million), *Lactobacillus rhamnosus* (400 million) and *Bifidobacterium longum* (200 million) was orally administered to the treatment group of piglets @ 1.25×10^9 CFU/day from birth to 10 days of age [35]. The ZnO was given orally to the treatment group of piglets @ 2000 ppm/day from birth to 10 days of age [8]. The piglets of the control group were given the same volume of sterilized saline solution.

Experimental design

Each of 6 (six) numbers of piglets was selected from 3 (three) sows at different stages of development as age-group of 20, 30 and 60 days. Out of the 6 piglets, 3 piglets from each litter were used as the control group (C) with basal diet and the other 3 piglets were fed orally with combined probiotic and zinc supplement along with the basal diet and used as treatment group (T). The basal diet used in this experiment was in pellet form and was formulated to provide the nutrient requirements [38]. The piglets were weaned at 28 days of age.

Sample preparation

The experimental animals were first anesthetized using diazepam @ 2mg/kg body weight followed by ketamine @ 10 mg/kg body weight intravenously and then exsanguinated the animals. The animals were sacrificed at day 20, 30 and 60 from both the groups. After sacrifice, the abdominal cavity of the animal was opened and parts of the small intestine were observed [24]. Tissue samples were taken immediately after sacrifice from the duodenum (5 cm caudal to the pylorus), jejunum (In the middle of the jejunum) and ileum (5 cm cranial to the ileocaecal valve).

Preparation for light microscopic examination

For histomorphology, the tissue samples (0.5 cm) from each location were fixed in 10 percent neutral buffered formalin for 24 to 48 hours. All the tissues were dehydrated, cleared and embedded in paraffin wax as per Luna [36]. The paraffin blocks were sectioned at 5 μ m thicknesses, dried in room temperature overnight and stained with Mayer's hematoxylin and eosin for routine study [36], combined PAS-Alcian blue for mucopolysaccharides [37] and Masson-Hamperl argentaffin technique for argentaffin cells [47]. The stained slides were visualized in Olympus BX 51 microscope and the images were captured with a ProgRes C5 Cool CCD camera.

Preparation for electron microscopic examination

For Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), the tissue samples were cut into small pieces of 1-2 mm size and were fixed in Karnovsky's fixative (2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2) for 4 hours at 4°C. After washing in 0.1M buffer (3 changes of 15 minutes each), the samples were fixed in 0.2M sodium cacodylate buffer till further use. The processing of samples for SEM was done as per Skrzypek et al. [48]. The viewing of the samples was carried out with a Zeiss Scanning Electron Microscope operated at 20 kV at the Institute of Advanced Study in Science and Technology (IASST), Guwahati, Assam. The processing of samples for TEM

was done at Sophisticated Analytical Instrument Facility (SAIF), North-Eastern Hill University (NEHU), Shillong as per the standard method [39]. The semi-thin sections were cut with an ultramicrotome at 400 nm thicknesses and stained with toluidine blue before making the ultra-thin sections. Ultra-thin sections were made at 50 nm thicknesses with an ultramicrotome, mounted on copper grids and contrasted with uranyl acetate and lead citrate. The viewing of the sections was carried out with Joel (JEM-2100) Transmission Electron Microscope operated at 120 kV at SAIF, NEHU, Shillong, Meghalaya.

Statistical analysis

The data obtained were analyzed using statistical package SPSS version 20. General Linear Model of two way ANOVA based on Fisher's Least Significant Difference method was used to determine the significant difference among days (20, 30 and 60 days) for control and treatment groups. The significant values in the ANOVA were further tested through the Duncan multiple range test. The obtained results were presented as mean \pm SEM and differences were considered significant when $P < 0.05$. An independent sample t-test has been applied between groups (Control and treatment) on different days to see the significant changes.

RESULTS

The mucosal epithelium of villi and crypts of the small intestine was covered by lining cells consisting of enterocytes, goblet cells, enteroendocrine (argentaffin) cells, microfold (M-cell) cells, tuft cells and intraepithelial lymphocytes (IEL). In addition, multipotent stem cells were located at the crypt base irrespective of the group of piglets and age.

The enterocytes were simple columnar cells with basally located nuclei in villi (Fig. 1a) and crypts (Fig. 1b). The enterocyte microvilli was significantly higher ($P < 0.05$) in the treated piglets (Fig. 1c) than the control animals (Fig. 1d) in jejunum and ileum at day 30 and day 60. An abundance of mitochondria, Golgi bodies, rough ER, and lysosomes were observed in the enterocyte cytoplasm in treated piglets (Fig. 1e) than the control group of animals (Fig. 1f).

The goblet cells were located in the epithelial layer of villi (Fig. 2a) and crypts (Fig. 2b). The apical portion was distended by abundant mucus laden granules (Fig. 2c) and the basal portion shaped like a stem (Fig. 2d). Numerous rough ER, mitochondria and secretory vesicles were recorded in the goblet cells of treated piglets (Fig. 2e). PAS-AB sequential staining showed neutral, acidic and mixed neutral-acidic mucin goblet cells (Fig. 2f),

irrespective of group and age. In most segments of the small intestine in treated piglets, the mixed, neutral and acidic mucin goblet cells were significantly higher both in the villi and crypts at different age-groups than the control group of animals (Table 1).

The argentaffin cells were scattered singly in villi (Fig. 3a) and crypts (Fig. 3b) among other cells within the lining epithelium. Their populations were more in the crypts than villi (Fig. 3c) in both the groups of all ages. They had a narrow apex, wide base and contained many small, spheroidal, electron-dense granules (Fig. 3d). In duodenum at day 60, the number of argentaffin cells was significantly higher in villi ($P < 0.05$) and crypts ($P < 0.01$) in the treatment group of piglets (Table 2).

The M-cells were mostly found in the follicle associated epithelium (FAE) of Peyer's patches (PP) in jejunum and ileum. They had less developed brush border with irregular microvilli (Fig. 4a) and basolateral indentations or pockets for transportation of antigens and microbes across intestinal epithelium (Fig. 4b). The cytoplasm of villus M-cells was less electron-dense (Fig. 4c) that contained few secretory granules and lysosomes, rich in mitochondria and numerous small vesicles (Fig. 4d).

Under TEM, the mucosal epithelium of the small intestine showed tuft cell in between the enterocytes (Fig. 5). These pear-shaped cells had a broad base, narrow apex, and a "tuft" of microvilli projecting into the lumen. In this study, these cells were encountered more in the duodenum than jejunum and ileum. The frequent occurrences of tuft cells were noted in the treatment group of piglets in comparison to the control group of animals.

The intraepithelial lymphocytes (IELs) were unevenly distributed at apical, nuclear and basal positions in the lining mucosa of the small intestine (Fig. 6a) in both the groups. In most of the segments of the intestine, the number of IEL population was significantly higher in the treatment group of piglets both in the villus tip and base (Table 3). The treatment group of piglets had a significantly higher number of basally located IEL (Fig. 6b) followed by nuclear level both in villus tip and base at different age-groups.

At the bases of the crypts, TEM analysis revealed the presence of crypt base columnar stem (CBC) cells, goblet cells, enteroendocrine cells and absorptive enterocytes in all segments of the small intestine (Fig. 7). The stem cells were irregularly shaped, small, columnar cells with basally located nuclei and scarce cytoplasm. They were found in between the goblet cells, enterocytes or enteroendocrine cells in piglets. The CBC stem cells could be differentiated from the mature absorptive enterocytes with their irregular columnar cells containing uneven elongated nuclei in between the goblet cells (Fig. 7).

DISCUSSION

The results of the current study revealed that treatment with probiotic and zinc significantly increased ($P < 0.05$) the length of microvilli in jejunum and ileum at day 30 and day 60 in post-weaned piglets. This might be showing the greater surface area, which could attribute more absorption of nutrients by the small intestine in piglets fed with probiotic and zinc. The comparison of the present finding could not be discussed with the available literature due to the dearth of information in this regard. In the current study, the electron microscopy studies confirmed the high activation of enterocytes after dietary inclusion of probiotic and zinc in pre and post-weaned piglets as compared to the control group. This finding might indicate that probiotic and zinc interacted and activated the intestinal epithelial cells (IECs), which could improve their functions and activate the immune cells present in the small intestine. This fact is in line with previous reports where probiotic strains interact and activate the intestinal epithelial cells [21, 32].

Goblet cells containing different mucins acted as an innate defense mechanism, diffusion barrier and providing a microecological barrier in the gut [33]. Acid glycoconjugates especially helped the intestinal mucosa to counteract microorganisms and resisted bacterial enzymes [16]. The secretion of mucous from goblet cells was also affected by weaning and age of the animals [11], the composition of intestinal microbiota [12], and dietary treatment [27]. In our study, the goblet cells were observed both in the villi and crypts of the small intestine as reported by Liu et al. [34] in piglet. These cells appeared as an accumulation of mucous granules with a stem-like basal portion. Similar type of observation was also recorded by Hodges and Dartt [28] in the conjunctiva of eye. In the current study, the number of goblet cells with different mucins increased significantly in most of the segments of the small intestine at different age-groups of treated piglets as compared to the control group of animals. Many researchers studied goblet cells in piglets after feeding with probiotic and zinc [5, 7, 14, 17, 34, 42]. Most of the results obtained from these researchers were in agreement with the present findings. In the present study, the higher number of different types of goblet cells recorded in the treatment group piglets might be concluded with the better enhancement of epithelial barrier and defense mechanism. This might result in effective immunity and digestibility in this group of piglets.

The distribution of argentaffin cells in the small intestine of control and treated piglets was studied to know the effects of probiotic and zinc on these cells. These cells were concentrated more in the jejunum, followed by duodenum and ileum. However, Sadeghi et al. [45] reported more number of argentaffin cells in the first part of the duodenum in rats. The reports of the present study was not consistent with the above findings, which might be due to variation in species. These argentaffin cells were located as a single cell within the lining

epithelium of both villus and crypt in a large population on non-endocrine cells as previously reported by Sadeghi et al. [45] in rats. Under the TEM of this study, these cells had narrow apex, wide base with many small, spheroidal, electron-dense granules in the cytoplasm. The present findings were similar to the findings of Gonzalez et al. [23] in pigs. The mean number of argentaffin cells in the current study was significantly higher in villi ($P < 0.05$) and crypts ($P < 0.01$) of the duodenum at day 60 in the treatment group of piglets. More number of these cells revealed in the treatment group of the present study might be correlated with more production of gastrointestinal hormones for better digestion of food [41, 49]. The increase of serotonin secretion by the argentaffin cells created a greater peristaltic movement of the small intestine resulting in effective digestion of food particles [25], which supported the current observation.

The M-cells were specialized epithelial cells of the mucosa-associated lymphoid tissue. They were mostly dispersed in the Peyer's patches (PP) of jejunum and ileum. This finding was in consonance to the finding of Hsieh and Lo [29] in mice. In our study, the M-cells showed less developed brush border with irregular microvilli. They transport antigens from the lumen of the intestine to the dome epithelium and neutralize them with a variety of mechanisms. This finding was in agreement with the report of Gebert et al. [22] in pig. The transmission electron microscopy revealed less electron-dense cytoplasm containing abundant mitochondria, lysosomes and small vesicles with few secretory granules. The present findings had a close resemblance to the findings of Renfeng et al. [43] in piglets. In the present study, the differences in M-cells between control and treatment group piglets could not be made due to constraints in enough exposure availability of transmission electron microscopy.

In the present investigation, the mucosal epithelium of the small intestine showed a tuft cell in between the enterocytes. This pear-shaped cell had a broad base, narrow apex, and a "tuft" of microvilli projecting into the lumen as also reported by Ethan [19] in humans. In this study, these cells were encountered more in the duodenum than jejunum and ileum as revealed by Cheng et al. [10] in mouse intestine. The role of tuft cells in epithelial cell survival/self-renewal was reported by Chandrakesan et al. [9], in mucosal healing by Banerjee et al. [3] and possible contacts with nerve fibers relating to endocrine cells by Cheng et al. [10] in mouse intestine. In the present study, the counting of tuft cells in the small intestine of control and treatment group of piglets could not be made as these cells could be identified only with transmission electron microscopy and some special staining. However, frequent occurrences of these cells were noted in the treatment group of piglets in comparison with the control group of animals. This finding might be suggestive of better

survival/self-renewal, mucosal healing and digestive ability in the gut of piglets fed with probiotic and zinc compared to control animals.

The intraepithelial lymphocytes were unevenly distributed in the apical, nuclear and basal positions of the lining mucosa of the small intestine in both the groups, as also opined by Deng et al. [15] in hen. In the villus tip of the treatment group of piglets, the number of these IEL was significantly increased ($P < 0.01$) at day 30 in the duodenum and, day 20 and day 30 in the jejunum. Similarly, in the villus base of the treatment group, the total IEL population was significantly higher ($P < 0.01$) at day 20 in jejunum and day 60 in the ileum. In agreement with the present result, several authors had reported an increased number of IEL after probiotic treatments in pig [46] and in chicken [2, 13, 15, 31]. The slight but significant increase in the number of IEL in the treatment group of the present study could be the result of a nonspecific stimulation of the local immune system, possibly by certain antigens of probiotic bacteria. In the present study, most of the IELs were localized at the basement membrane of the epithelium, numerous at the enterocyte nuclear level and relatively few apically in the epithelium. The present findings were in support of the findings of Rieger et al. [44] and Vega-Lopez et al. [50] in the porcine small intestine. In most of the segments of intestine, the number of basally located IEL was significantly increased in the treatment group of piglets as compared to the control group of animals both in villus tip and base. The basal IEL belongs to the “conventional type”, i.e., antigen-experienced cells originated from peripheral T cells and homed the gut mucosa, which had immunologic memory function and mounted an adaptive response as reported by Hayday et al. [26] in human. In the present study, the treatment group of piglets had more basally located IEL that might be correlated with effective adaptive immune response in this group of piglets. A significantly higher population of IEL was also recorded at the nuclear level of villus tip and base in the jejunum of treated piglets. The more number of IEL present in the nuclear level of the epithelium was explained by Hayday et al. [26]. According to them, the IEL found at the nuclear level belongs to the “unconventional type”. They had functions in between adaptive and innate responses and responsible for the protection of epithelial integrity. Further, Edelblum et al. [18] demonstrated the ability of an IEL population fitting to the “unconventional type” to contact multiple epithelial cells over a short time and thus provide a potential mechanism by which they could prevent epithelial injury and infection. The significantly higher numerical values for nuclear IEL recorded in the present study might conclude with better epithelial cell integrity against injury and infection in the piglets fed with probiotic and zinc compared to control piglets.

The Transmission electron microscopy revealed the presence of crypt base columnar stem (CBC) cells along with goblet cells, enteroendocrine cells and absorptive enterocytes, irrespective of segments of intestine and group. These stem cells were irregularly shaped, small, columnar cells with basally located nuclei and scarce cytoplasm. They were found in between the goblet cells, enterocytes or enteroendocrine cells in piglets. The present findings were in agreement with the findings of Gonzalez et al. [21] in pigs. However, Barker et al. [4] reported the presence of stem cells in between the paneth cells on the crypt base in mice. This finding slightly deviated from the present investigation might be due to the absence of paneth cells in the crypt base of piglets. In the current study, the CBC stem cells could be differentiated from the mature absorptive enterocytes with their irregular columnar cells containing uneven elongated nuclei in between the goblet cells, as described earlier by Gonzalez et al. [23] in pigs. The alterations of CBC stem cells in the present study in between control and treatment group piglets could not be made due to a lack of sufficient view under transmission electron microscopy.

CONCLUSIONS

From the present investigation, it can be concluded that dietary supplementation of probiotic and zinc induced the length of enterocyte microvilli, increased the number of different goblet cells, argentaffin cells, tuft cells and intraepithelial lymphocytes in pre and post-weaned piglets. These alterations might provide better absorption of available nutrients and stimulation of local and adaptive immune responses that resulted in effective digestibility and immunity in the treatment group of piglets as compared to the control group of animals.

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Table 1. Numbers of Alcian blue-Periodic Acid-Schiff (AB-PAS) positive goblet cells in the small intestine of piglets fed with probiotic and zinc

Parameter	Intestinal segment	Pre-weaning		Post-weaning				p-value	
		Day 20		Day 30		Day 60			
		Control	Treatment	Control	Treatment	Control	Treatment	Cont	Treat
Villus neutral per 200 µm	Duodenum	0.33±0.11 ^{ap}	0.87±0.13 ^a	0.73±0.19 ^{ab}	1.03±0.29	0.87±0.21 ^b	1.20±0.19	0.08	0.55
	Jejunum	0.57±0.16 ^a	0.97±0.15 ^d	0.93±0.16 ^{ab}	1.33±0.13 ^{de}	1.10±0.15 ^b	1.60±0.28 ^e	0.05	0.08
	Ileum	0.67±0.12 ^a	1.07±0.18 ^d	1.03±0.20 ^{ab}	1.50±0.18 ^{de}	1.23±0.23 ^{br}	1.93±0.26 ^{es}	0.10	0.02
Villus acidic per 200 µm	Duodenum	0.23±0.09 ^{ar}	0.73±0.19 ^s	0.70±0.19 ^b	0.93±0.21	0.80±0.17 ^b	1.07±0.25	0.03	0.55
	Jejunum	0.47±0.12	0.87±0.18	0.80±0.24	1.20±0.22	0.93±0.21	1.50±0.26	0.22	0.14
	Ileum	0.47±0.12 ^{ar}	0.87±0.15 ^s	0.93±0.17 ^{ab}	1.43±0.29	1.10±0.27 ^b	1.80±0.46	0.07	0.13
Villus mixed per 200 µm	Duodenum	2.83±0.29	2.90±0.28 ^D	2.90±0.27	3.03±0.34 ^D	3.40±0.43 ^P	4.97±0.38 ^{Eq}	0.44	0.001
	Jejunum	2.97±0.36	3.57±0.34 ^D	3.57±0.42 ^r	4.77±0.30 ^{Es}	3.73±0.34 ^P	5.17±0.31 ^{Eq}	0.32	0.002
	Ileum	3.0±0.24	3.70±0.32 ^D	3.77±0.42 ^r	5.03±0.25 ^{Es}	3.93±0.34 ^P	5.57±0.27 ^{Eq}	0.12	0.001
Villus Total per 200 µm	Duodenum	3.40±0.29 ^{Ar}	4.50±0.37 ^{Ds}	4.33±0.35 ^{AB}	5.0±0.36 ^D	5.07±0.45 ^{Bp}	7.23±0.49 ^{Eq}	0.01	0.001
	Jejunum	4.0±0.45 ^{ar}	5.40±0.41 ^{Ds}	5.30±0.47 ^{bp}	7.30±0.36 ^{Eq}	5.77±0.39 ^{bp}	8.27±0.47 ^{Eq}	0.02	0.001
	Ileum	4.13±0.32 ^P	5.63±0.34 ^{Dq}	5.73±0.54 ^P	7.97±0.39 ^{Eq}	6.27±0.46 ^P	9.30±0.53 ^{Eq}	0.003	0.001
Crypt neutral per 200 µm	Duodenum	0.50±0.15 ^a	0.93±0.17 ^D	0.83±0.19 ^{ab}	1.23±0.22 ^D	1.30±0.25 ^b	2.0±0.32 ^E	0.02	0.009
	Jejunum	0.77±0.17 ^{ap}	1.43±0.18 ^{dq}	1.13±0.21 ^{abr}	1.77±0.15 ^{des}	1.60±0.28 ^b	2.13±0.32 ^e	0.04	0.11
	Ileum	0.80±0.24 ^r	1.47±0.23 ^{ds}	1.23±0.20	1.83±0.27 ^{de}	1.40±0.24 ^r	2.33±0.29 ^{es}	0.16	0.07
Crypt acidic per 200 µm	Duodenum	0.20±0.12 ^{Ap}	0.67±0.12 ^q	0.63±0.14 ^B	0.87±0.15	0.77±0.12 ^B	0.93±0.18	0.01	0.44
	Jejunum	0.30±0.09 ^{Ap}	0.77±0.15 ^{dq}	0.73±0.14 ^B	1.07±0.17 ^{de}	0.87±0.12 ^{Br}	1.47±0.19 ^{es}	0.003	0.02
	Ileum	0.47±0.10 ^{ar}	0.83±0.16 ^{Ds}	0.83±0.20 ^{ab}	1.37±0.19 ^E	1.0±0.21 ^{br}	1.63±0.19 ^{Es}	0.10	0.006
Crypt mixed per 200 µm	Duodenum	4.30±0.34	4.63±0.37 ^d	4.40±0.28	4.77±0.42 ^{de}	4.73±0.36 ^r	5.90±0.42 ^{es}	0.62	0.06
	Jejunum	4.43±0.37	4.73±0.34 ^d	4.50±0.48	5.40±0.39 ^{de}	4.83±0.40 ^r	6.0±0.42 ^{es}	0.77	0.07
	Ileum	4.73±0.46	5.0±0.38	4.80±0.32	5.60±0.52	5.10±0.44	6.20±0.38	0.80	0.15
Crypt Total per 200 µm	Duodenum	5.0±0.37 ^{Ar}	6.23±0.44 ^{Ds}	5.87±0.38 ^{AB}	6.87±0.46 ^D	6.80±0.43 ^{Bp}	8.83±0.60 ^{Eq}	0.01	0.001
	Jejunum	5.50±0.32 ^{ap}	6.93±0.41 ^{Dq}	6.37±0.57 ^{abr}	8.23±0.47 ^{DEs}	7.30±0.57 ^{bp}	9.60±0.59 ^{Eq}	0.04	0.001
	Ileum	6.0±0.54	7.30±0.51 ^D	6.87±0.46 ^r	8.80±0.60 ^{DEs}	7.50±0.56 ^P	10.17±0.49 ^{Eq}	0.13	0.001

Data are presented as goblet cells/200µm (Mean ± SEM) in different age-groups. ^{A, B}Means with different superscripts between control groups significantly differ (P<0.01); ^{D, E, F}Means with different superscripts between treatment groups significantly differ (P<0.01); ^{a, b}Means with different superscripts between control groups significantly differ (P<0.05); ^{d, e}Means with different superscripts between treatment groups significantly differ (P<0.05); ^{P, q} Means with different superscripts within groups significantly differ (P<0.01); ^{r, s}Means with different superscripts within groups significantly differ (P<0.05).

Table 2. Numbers of argentaffin cells in the small intestine of piglets fed with probiotic and zinc

Parameter	Intestinal segment	Pre-weaning		Post-weaning				p-value	
		Day 20		Day 30		Day 60			
		Control	Treatment	Control	Treatment	Control	Treatment	Cont	Treat
Villus argentaffin cell	Duodenum	0.40 ± 0.10	0.47 ± 0.10 ^A	0.43 ± 0.09	0.60 ± 0.10 ^A	0.53 ± 0.13 ^r	1.0 ± 0.16 ^{Bs}	0.68	0.009
	Jejunum	0.50 ± 0.13	0.77 ± 0.18	0.50 ± 0.12	0.67 ± 0.11	0.53 ± 0.10	0.60 ± 0.14	0.97	0.72
	Ileum	0.33 ± 0.13	0.43 ± 0.09	0.50 ± 0.09	0.63 ± 0.11	0.57 ± 0.11	0.70 ± 0.12	0.33	0.20
Crypt argentaffin cell	Duodenum	1.27 ± 0.10	1.40 ± 0.11 ^A	1.33 ± 0.10	1.53 ± 0.09 ^A	1.43 ± 0.11 ^P	2.03 ± 0.16 ^{Bq}	0.52	0.002
	Jejunum	1.40 ± 0.13	1.53 ± 0.14	1.40 ± 0.12	1.60 ± 0.17	1.43 ± 0.16	1.67 ± 0.15	0.98	0.83
	Ileum	0.80 ± 0.12	1.07 ± 0.13	0.90 ± 0.12	1.17 ± 0.14	1.0 ± 0.15	1.27 ± 0.14	0.57	0.57

Data are presented as the argentaffin cells/0.24mm² area (Mean ± SEM) in different age-groups. ^{A, B}Means with different superscripts between treatment groups significantly differ (P<0.01); ^{Bq} Means with different superscripts within groups significantly differ (P<0.01); ^{r, s}Means with different superscripts within groups significantly differ (P<0.05).

Table 3. Numbers of intraepithelial lymphocytes (IEL) in the small intestine in piglets fed with probiotic and zinc

Parameter	Intestinal segment	Pre-weaning		Post-weaning				p-value	
		Day 20		Day 30		Day 60			
		Control	Treatment	Control	Treatment	Control	Treatment	Cont	Treat
Apical IEL villus tip per 100 µm	Duodenum	1.57 ± 0.21	1.63 ± 0.21 ^D	1.90 ± 0.26 ^P	3.17 ± 0.25 ^{Eq}	2.13 ± 0.26	2.30 ± 0.19 ^F	0.26	0.001
	Jejunum	1.47 ± 0.29 ^a	1.27 ± 0.24 ^{de}	0.87 ± 0.17 ^{abp}	1.60 ± 0.19 ^{dq}	0.73 ± 0.16 ^b	0.90 ± 0.12 ^e	0.04	0.04
	Ileum	0.97 ± 0.19 ^a	0.87 ± 0.17 ^d	0.60 ± 0.13 ^{ab}	0.73 ± 0.14 ^{de}	0.47 ± 0.12 ^b	0.37 ± 0.11 ^e	0.05	0.04
Nuclear IEL villus tip per 100	Duodenum	1.27 ± 0.16 ^A	1.37 ± 0.17 ^d	2.30 ± 0.26 ^B	2.07 ± 0.17 ^e	2.17 ± 0.17 ^B	1.90 ± 0.20 ^e	0.001	0.02
	Jejunum	0.97 ± 0.19 ^{Ap}	3.13 ± 0.26 ^{dq}	2.10 ± 0.24 ^B	2.07 ± 0.30 ^e	1.17 ± 0.19 ^{Ap}	2.80 ± 0.34 ^{deq}	0.001	0.05
	Ileum	1.67 ± 0.21 ^{ar}	1.0 ± 0.15 ^{Ds}	1.73 ± 0.21 ^a	1.80 ± 0.20 ^E	1.07 ± 0.17 ^b	1.40 ± 0.19 ^{DE}	0.04	0.01

μm									
Basal IEL villus tip per 100 μm	Duodenum	2.20 \pm 0.19 ^A	2.47 \pm 0.18 ^D	2.77 \pm 0.18 ^{AB}	3.30 \pm 0.32 ^E	3.40 \pm 0.29 ^{Br}	4.30 \pm 0.25 ^{Fs}	0.001	0.001
per 100 μm	Jejunum	1.10 \pm 0.18 ^A	1.67 \pm 0.23	1.60 \pm 0.20 ^{Ar}	2.30 \pm 0.23 ^s	2.73 \pm 0.29 ^{Bp}	1.73 \pm 0.19 ^q	0.001	0.08
	Ileum	2.07 \pm 0.27 ^{Ar}	3.27 \pm 0.43 ^{DEs}	2.50 \pm 0.19 ^A	2.63 \pm 0.18 ^D	3.77 \pm 0.24 ^B	3.93 \pm 0.21 ^E	0.001	0.01
Total IEL villus tip per 100 μm	Duodenum	5.03 \pm 0.32 ^A	5.47 \pm 0.29 ^D	6.97 \pm 0.40 ^{Bp}	8.53 \pm 0.33 ^{Eq}	7.70 \pm 0.33 ^B	8.50 \pm 0.31 ^E	0.001	0.001
per 100 μm	Jejunum	3.53 \pm 0.23 ^{ap}	6.07 \pm 0.40 ^q	4.57 \pm 0.35 ^{bp}	5.97 \pm 0.33 ^q	4.63 \pm 0.36 ^b	5.43 \pm 0.27	0.03	0.36
	Ileum	4.70 \pm 0.25	5.13 \pm 0.49	4.83 \pm 0.32	5.23 \pm 0.28	5.30 \pm 0.34	5.70 \pm 0.25	0.35	0.49
Apical IEL villus base per 100 μm	Duodenum	1.63 \pm 0.15	1.70 \pm 0.15 ^d	2.10 \pm 0.28 ^r	1.27 \pm 0.28 ^{des}	2.0 \pm 0.21 ^p	1.07 \pm 0.17 ^{eq}	0.30	0.09
per 100 μm	Jejunum	1.10 \pm 0.19	0.97 \pm 0.20 ^d	0.87 \pm 0.16 ^p	1.73 \pm 0.20 ^{eq}	0.73 \pm 0.12	1.13 \pm 0.17 ^d	0.25	0.014
	Ileum	1.13 \pm 0.16 ^A	0.90 \pm 0.17 ^D	1.27 \pm 0.14 ^A	1.27 \pm 0.14 ^D	0.50 \pm 0.10 ^B	0.37 \pm 0.13 ^E	0.001	0.001
Nuclear IEL villus base per 100 μm	Duodenum	1.37 \pm 0.18 ^A	1.47 \pm 0.18 ^D	2.43 \pm 0.31 ^B	2.60 \pm 0.22 ^E	2.40 \pm 0.25 ^B	2.73 \pm 0.19 ^E	0.004	0.001
base per 100 μm	Jejunum	0.90 \pm 0.15 ^{Ap}	1.83 \pm 0.14 ^q	1.93 \pm 0.20 ^B	2.0 \pm 0.19	1.07 \pm 0.17 ^{Ap}	2.23 \pm 0.21 ^q	0.001	0.30
	Ileum	0.93 \pm 0.14 ^a	0.60 \pm 0.14 ^D	1.53 \pm 0.15 ^b	1.60 \pm 0.15 ^E	1.17 \pm 0.15 ^{abr}	0.73 \pm 0.15 ^{Ds}	0.014	0.001
Basal IEL villus base per 100 μm	Duodenum	1.87 \pm 0.20 ^A	2.10 \pm 0.18 ^D	2.80 \pm 0.23 ^{Bp}	4.60 \pm 0.34 ^{Eq}	3.70 \pm 0.24 ^C	4.40 \pm 0.27 ^E	0.001	0.001
per 100 μm	Jejunum	1.47 \pm 0.17 ^A	1.97 \pm 0.26	2.10 \pm 0.22 ^A	2.20 \pm 0.26	3.03 \pm 0.30 ^{Bp}	2.03 \pm 0.20 ^q	0.001	0.78
	Ileum	1.77 \pm 0.20 ^{ar}	2.60 \pm 0.35 ^{Ds}	2.50 \pm 0.24 ^b	2.63 \pm 0.23 ^D	2.03 \pm 0.24 ^{abp}	4.10 \pm 0.22 ^{Eq}	0.07	0.001
Total IEL villus base per 100 μm	Duodenum	4.87 \pm 0.41 ^A	5.27 \pm 0.37 ^D	7.33 \pm 0.62 ^B	8.47 \pm 0.35 ^E	8.10 \pm 0.31 ^B	8.20 \pm 0.34 ^E	0.001	0.001
per 100 μm	Jejunum	3.43 \pm 0.28 ^{Ap}	4.77 \pm 0.38 ^q	4.90 \pm 0.33 ^B	5.83 \pm 0.35	4.83 \pm 0.35 ^B	5.40 \pm 0.39	0.002	0.13
	Ileum	3.83 \pm 0.20 ^A	4.10 \pm 0.40 ^D	5.30 \pm 0.23 ^B	5.50 \pm 0.21 ^E	3.70 \pm 0.27 ^{Ap}	5.20 \pm 0.27 ^{Eq}	0.001	0.004

Data are presented as IEL/100 μm (Mean \pm SEM) in different age-groups. ^{A,B,C}Means with different superscripts between control groups significantly differ (P<0.01); ^{D,E,F}Means with different superscripts between treatment groups significantly differ (P<0.01); ^{a,b}Means with different superscripts between control groups significantly differ (P<0.05); ^{d,e}Means with different superscripts between treatment groups significantly differ (P<0.05); ^{p,q}Means with different superscripts within groups significantly differ (P<0.01); ^{r,s}Means with different superscripts within groups significantly differ (P<0.05).

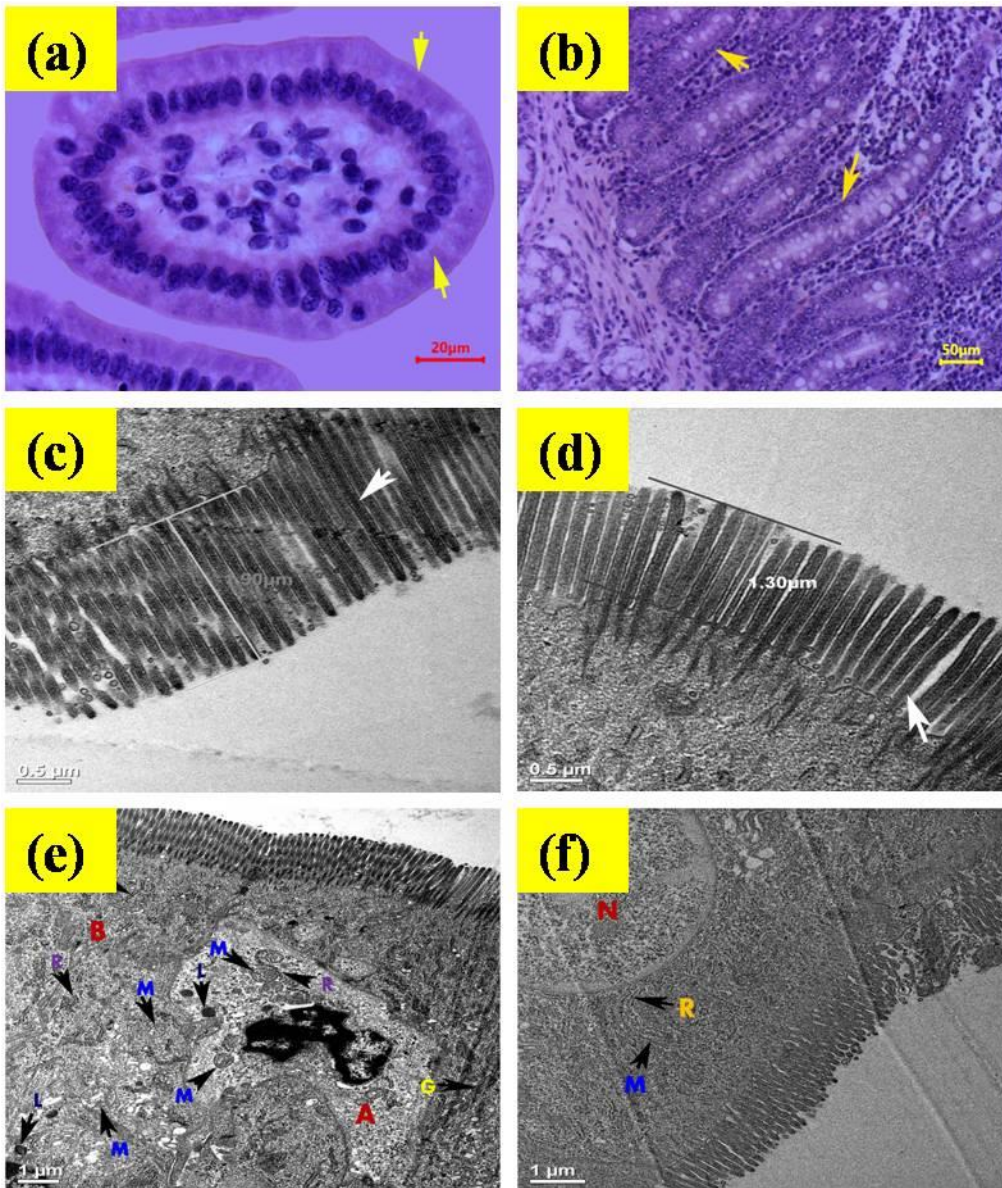


Figure 1. Enterocytes (a) Photomicrograph showing basally located nuclei (arrow) in duodenal villi of 20 days old treated piglet (H&E, X400). (b) Photomicrograph showing basally located nuclei (arrow) in jejunal crypts of 60 days old treated piglet (H&E, X100). (c) TEM micrograph showing longer microvilli (arrow) in the jejunum of 60 days old treated piglet. (d) TEM micrograph showing shorter microvilli (arrow) in the jejunum of 60 days old control piglet. (e) TEM micrograph showing Macrophage (A) and enterocyte (B) with an abundance of mitochondria (M), rough ER (R), lysosomes (L) and Golgi bodies (G) in the jejunum of 60 days old treated piglet. (f) TEM micrograph showing nucleus (N) and inadequacy of mitochondria (M) and rough ER (R) in the jejunum of 60 days old control piglet.

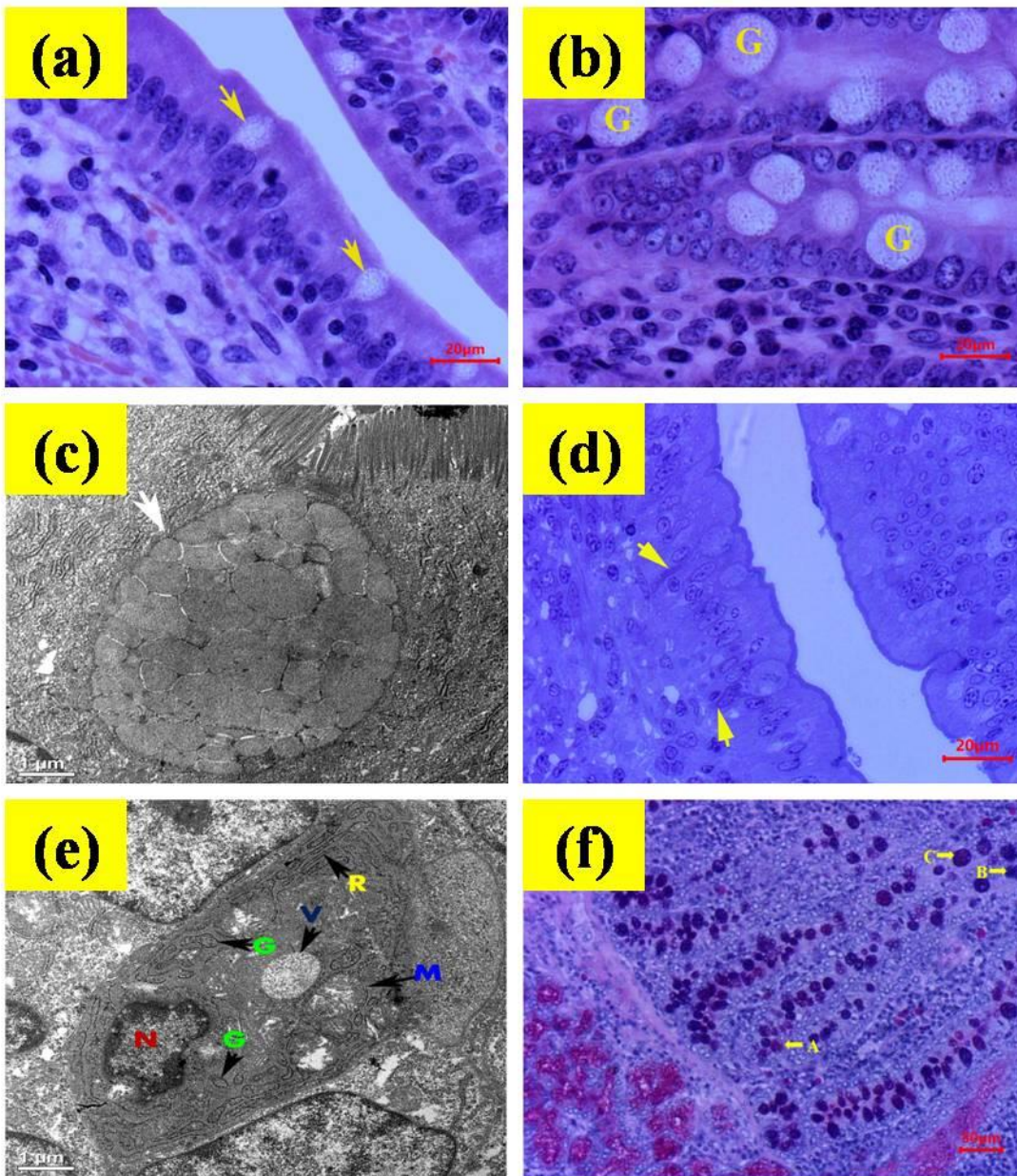


Figure 2. Goblet cells (a, b) Photomicrographs showing goblet cells in villus epithelium (arrow) and crypt epithelium (G) in the duodenum of 60 days old treated piglet (H&E, X400). (c) TEM micrograph of goblet cell showing abundant mucous laden granules in the ileum of 60 days old treated piglet. (d) Photomicrograph of the semi-thin section showing goblet cells with distended apical portion and stem-like basal portion (arrow) in the jejunum of 60 days old treated piglet (Toluidine-blue, X400). (e) TEM micrograph of goblet cell showing mitochondria (M), Golgi bodies (G), rough ER (R), nucleus (N) and secretory vesicles (V) in the ileum of 60 days old treated piglet. (f) Photomicrograph showing Neutral (A), acidic (B) and mixed (C) mucins goblet cells in the jejunum of 60 days old treated piglet (AB-PAS, X100).

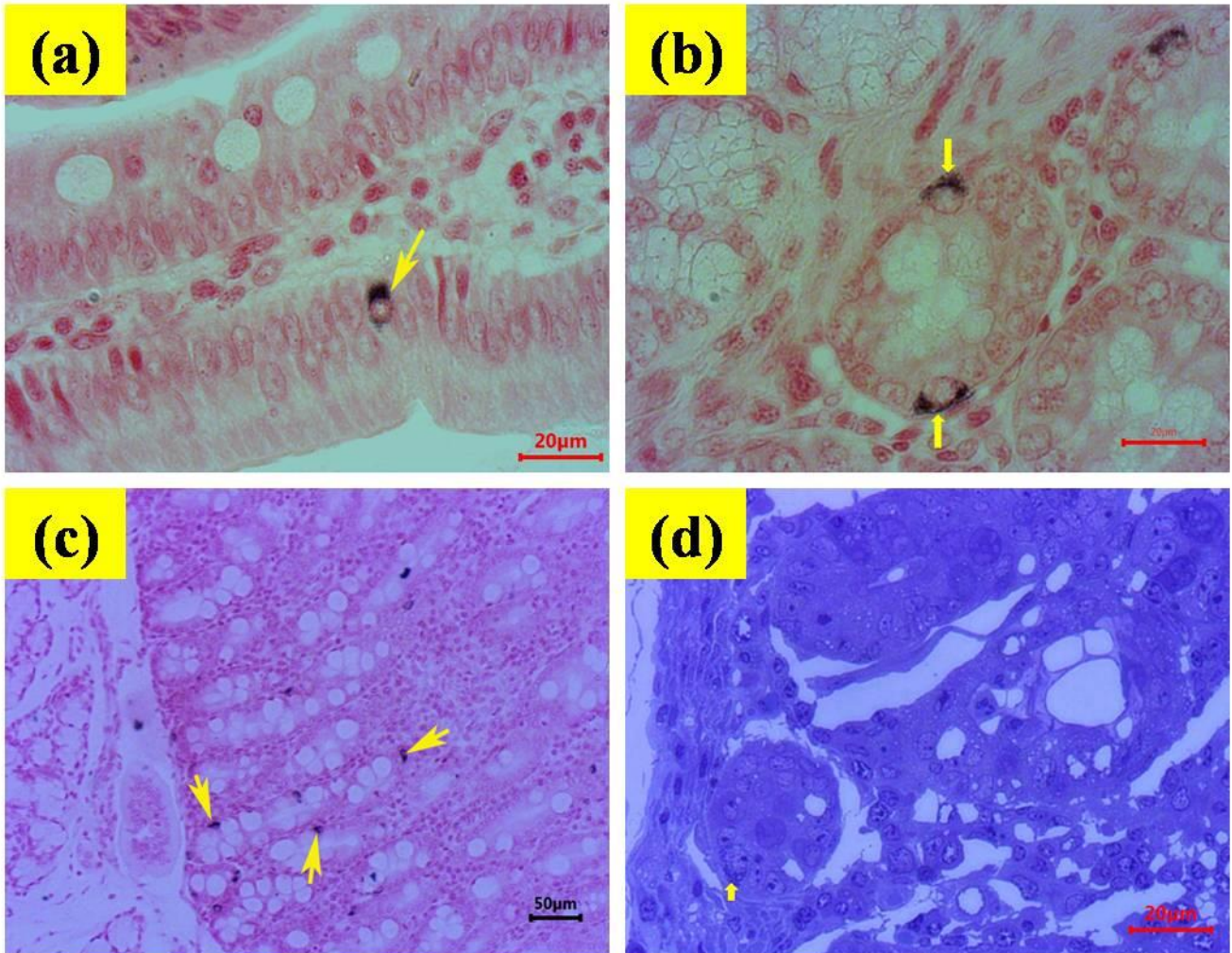


Figure 3. Photomicrographs of Argentaffin cells (a, b) Argentaffin cells (arrow) in the villus and crypt epithelium of duodenum in 20 days old treated piglet (H&E, X100). (c) Abundant numbers of argentaffin cells (arrow) in the crypts of the duodenum of 30 days old treated piglet (H&E, X100). (d) Semi-thin section showing argentaffin cells (arrow) in the crypts of jejunum in 30 days old treated piglet (Toluidine blue, X400).

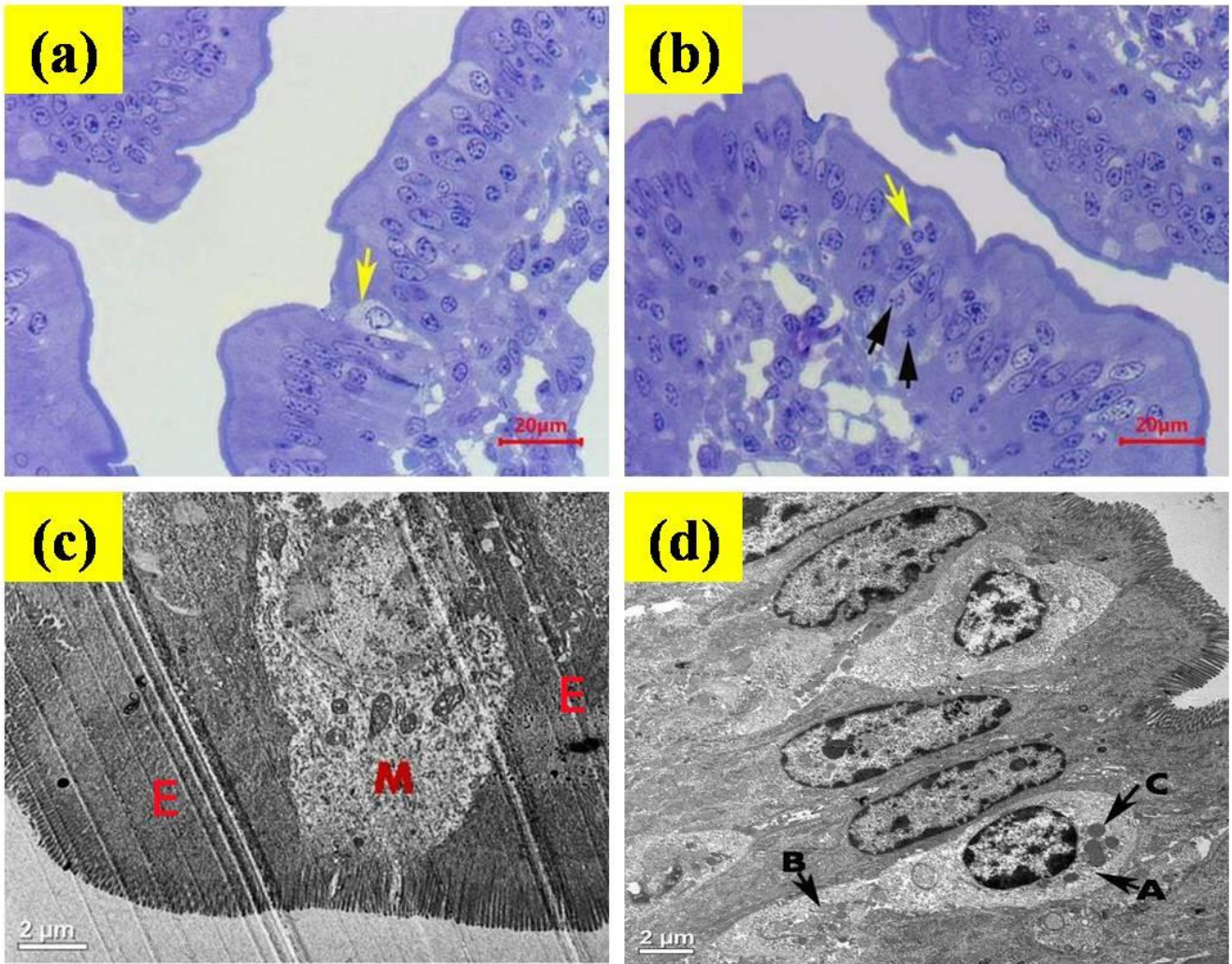


Figure 4. Photomicrographs of M-cells (a) Semi-thin section showing M-cell with irregular microvilli (arrow) in 60 days old treated piglet (Toluidine-blue, X400). (b) Semi-thin section showing M-cell with basolateral pockets (yellow arrow) and trapped antigens (black arrow) in 30 days old treated piglet (Toluidine-blue, X400). (c) TEM micrograph showing less electron-dense M-cell (M) adjacent to enterocytes (E) in 30 days old treated piglet. (d) TEM micrograph of M-cell containing small vesicles (A), mitochondria (B) and lysosomes (C) in 30 days old treated piglet.

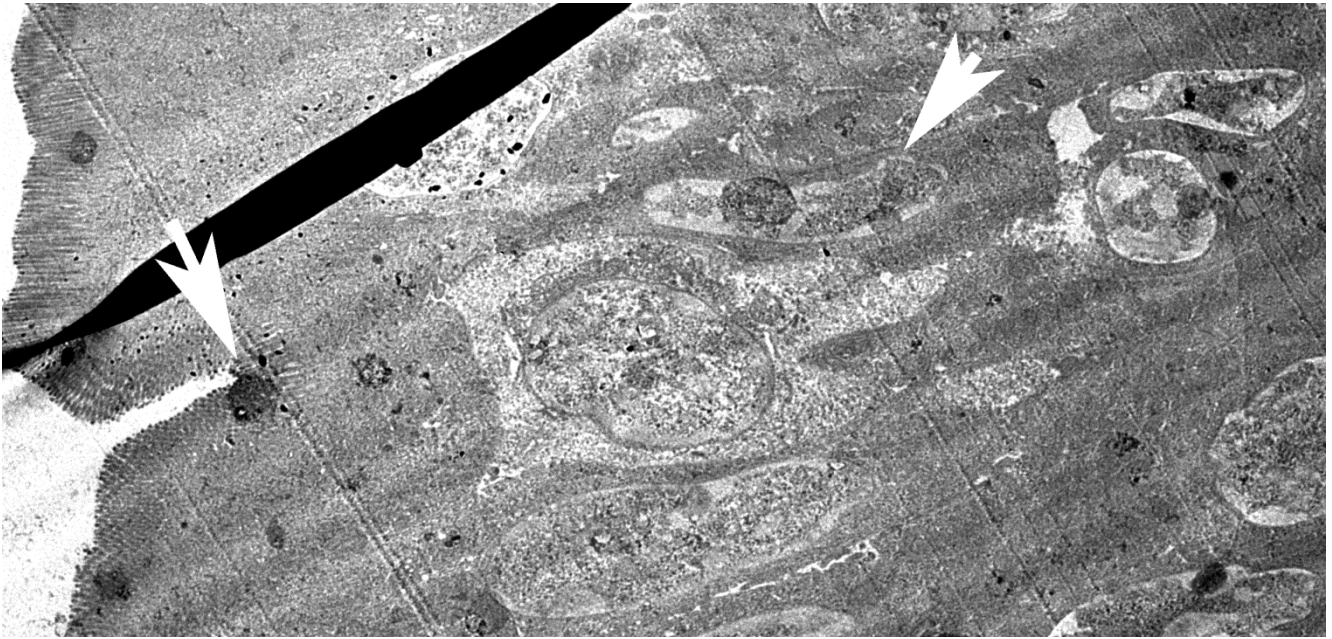


Figure 5. TEM micrograph showing pear-shaped, wide base and narrow apex tuft cell containing tuft (arrow) in the jejunum of 30 days old treated piglet.

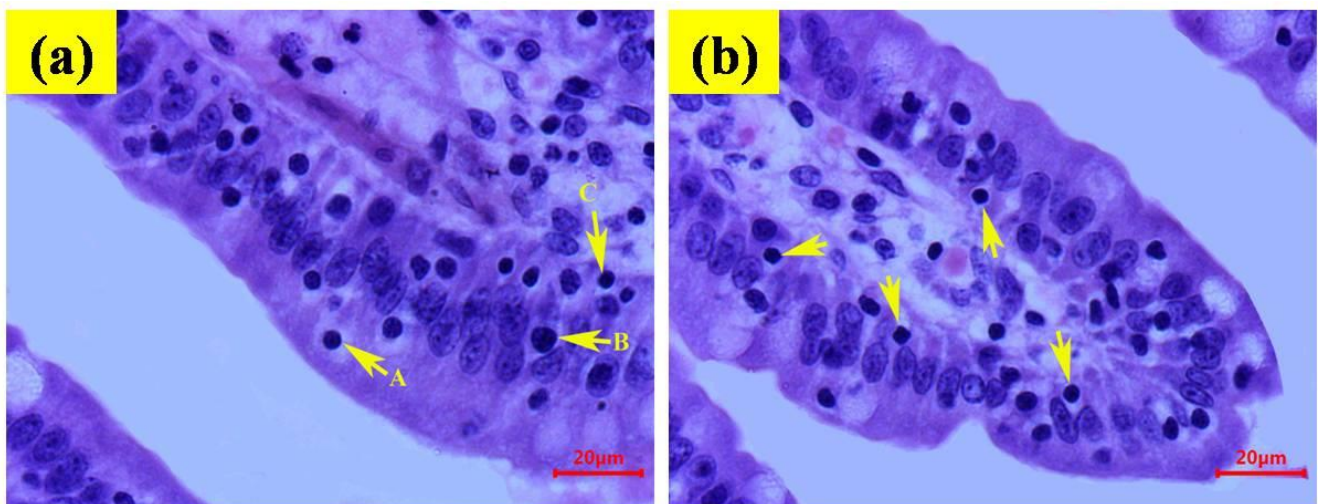


Figure 6. Photomicrographs of jejunum in 30 days old treated piglet (H&E, X400). (a) IEL in apical (A), nuclear (B) and basal (C) positions (b) IEL located in basal positions (arrow).

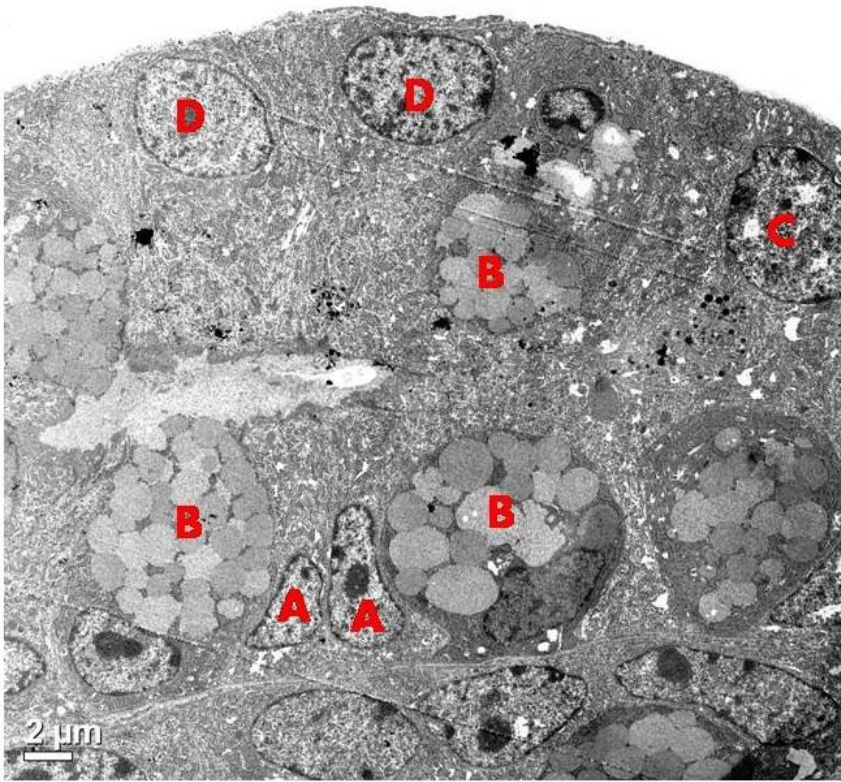


Figure 7. TEM micrograph of jejunal crypt showing the presence of stem cells (A), goblet cells (B), argentaffin cell (C) and enterocytes (D) in 60 days old treated piglet.