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Alterations of Small Intestinal Morphology on Villi and Crypts after Feeding Probiotic and Zinc in Pre and Post-weaned Piglets

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ABSTRACT

Background: Neonatal and early post-weaned piglet mortality causes huge economic losses to the farmers. The beneficial effect of probiotic and zinc in respect to the gut health in piglets has already been established. Therefore, this study was aimed to evaluate age-related changes and the effect of dietary inclusion of probiotic and zinc on morphological and morphometrical characteristics on villi and crypts of the small intestine in pre and post-weaned piglets.

Methods: A total of 18 Large White Yorkshire piglets, irrespective of sex were divided into three different age-groups [pre-weaning (20 days old, n=3) and, post-weaning (30 days old, n=3) and (60 days old, n=3)]. The piglets were weaned at 28 days of age. They were divided into control group (C) fed with basal diet and treatment group (T) fed with combined probiotic and zinc oral supplement along with the basal diet. After sacrifice the animals, tissue samples were collected and processed for routine stain and SEM. The alterations on morphology and morphometry of villi and crypts were recorded both in the control and treatment group of piglets.

Result: The villus height and width and crypt depth and width were increased, however, the villus crypt ratio was found to be lower in the treatment group of piglets. The villus and crypt enlargement factor showing higher numerical values in the piglets fed with probiotic and zinc than the control group of piglets. Scanning electron microscopy revealed a clear extrusion zone with prominent shedding of cells in the villi of jejunum and ileum in the treatment group of piglets at day 30 and 60.

Key words: Crypts, Piglets, Probiotic, Villi, Zinc.

INTRODUCTION

As the small intestinal mucosa absorbs most of the nutrients, maintaining of villi and crypts are essential for optimum absorption. Weaning piglets have to undergo many challenges such as low feed intake, acute diarrhoea and body weight loss, which are caused by nutritional, immunological and psychological disruptions (Lalles *et al*., 2007). To prevent the weaning stress, nutritional strategies such as dietary supplementation of probiotic as well as zinc are used for improving intestinal development and immune function (Shen *et al*., 2009). Dietary supplementation with probiotic had a positive effect on performance and health in weaned piglets through stimulating the immune system and maintaining a favorable intestinal environment (Gogineni *et al*., 2013). Similarly, Hu *et al*. (2013) and Song *et al*. (2014) reported improved daily gain, feed intake and improved intestinal villus and crypt morphology in early-weaned pigs after feeding with zinc oxide supplementation. High dietary zinc intakes in pigs improve performance, change the composition of the intestinal microbial communities and reduce the incidence of intestinal disorders (Li *et al*., 2001; Hojberg *et al*., 2005).

Therefore, the present investigation was undertaken to study the combined effect of probiotic and zinc on gut morphology in pre-weaned piglets in general and critical post-weaned piglets in particular.

MATERIALS AND METHODS

A total of 18 healthy Large White Yorkshire piglets, irrespective of sex from 3 litters, were utilized at different stages of development as age-group of 20, 30 and 60 days for the present study. The investigation was conducted in the pig farm, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University (I), Selesih, Aizawl, Mizoram, India. The piglets were reared under standard management conditions. The animal used for the experiment was ethically approved by the Institutional Animal Ethics Committee vide

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From each litter, six numbers of piglets were selected and were divided into two groups *viz*., control group (C) and treatment group (T) with combined probiotic and zinc supplement, consisting of nine animals in each group. The control group of animals fed with basal diet and the treatment group of animals fed with combined probiotic and zinc supplement orally along with the basal diet. The basal diet used in this experiment was in pellet form and were formulated to provide the nutrient requirements recommended by the NRC (1998). A mixture of probiotic consisted with *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 (1 gm powder dissolved in 3 ml of sterilized saline solution) once a day from birth to 10 days of age (Liu *et al*., 2014). The ZnO was given orally to the treatment group of piglets @ 2000 ppm once a day from birth to 10 days of age (Case and Carlson, 2002). The piglets of the control group were given the same volume of sterilized saline solution. The piglets were weaned at 28 days of age.

The experimental animals were sacrificed 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 opening the abdominal cavity, the parts of the small intestine were dissected out as per the method of Habel (1964). Tissue samples were taken immediately after the sacrifice from the duodenum, jejunum and ileum and were fixed in 10% neutral buffered formalin for 24 to 48 hours. All the tissues were dehydrated, cleared and embedded in paraffin wax as per Luna (1968). The paraffin blocks were sectioned at 5 µm thicknesses, dried in room temperature overnight and stained with Mayer's hematoxylin and eosin for routine study (Luna, 1968). The stained slides were visualized in Microscope (Olympus BX 51, Japan) and the images were captured with a ProgRes C5 Cool CCD camera (D-07739 Jena, Jenoptik, Germany).

For scanning electron microscopy, the tissue samples were fixed in Karnovsky's fixative (2.5% glutaraldehyde in 0.1M sodium cacodylate or phosphate 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 scanning electron microscopy was done in the Department of Veterinary Anatomy and Histology, College of Veterinary Sciences and Animal Husbandry, Aizawl, Mizoram as per the procedure followed by Skrzypek *et al*. (2005). 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 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 results were presented as mean ± SE and differences were considered significant when $P<0.05$. An independent sample t-test has been applied between groups (Control and treatment) at different days to see the significant changes.

RESULTS AND DISCUSSION

In the present study, the architecture of villi and crypts were studied by histomorphological observations and scanning electron microscopy.

Histomorphological characteristics

The villus and crypt morphometry of different segments of the small intestine in the control and treatment group of piglets are presented in Table 1 and Fig 1 to Fig 8.

Villus height and width, crypt depth and width and villus: crypt ratio

The mean villus height of the study showed a fluctuating pattern with the advancement of age being lowest at day 30 in both the groups (Table 1). The villus height was affected by dietary treatment with probiotic and zinc. It was increased in the treated piglets than the control animals irrespective of segments of small intestine and age (Fig 1). In the duodenum, the mean height was significantly increased in the treatment group at day 20 ($P<0.05$) and day 30 ($P<0.01$). The mean villus height of duodenum at day 60 was non-significantly higher in the treatment group. In jejunum and ileum, the piglets that had received probiotic and zinc revealed longer villi than the control animals. These results were not statistically significant. However, at day 20, the mean villus height of ileum was significantly higher in the treatment group ($P<0.01$). Increasing the villus height suggested an increased number of enterocytes in the small intestinal mucosa capable of greater absorption of available nutrients (Hampson, 1986). Bontempo *et al*. (2006) and Di Giancamillo *et al*. (2008) also found higher villi length in the small intestine of piglets fed probiotic than controls. In the current investigation, within 48 hours of weaning (at day 30), there were greater decreases in villus height of the three small intestinal regions. Similar reports were made by Hampson (1986) and Budiño *et al*. (2005) in pigs. Kenworthy (1976) and Hampson (1986) believed that the shortening of villus height was due to an interaction between introduced creep feed and the intestinal microflora within 24 hours of weaning. The cell column counts indicated that this was caused by a reduction in the number of villus cells and not to villus contraction. The reduction in enterocyte numbers could have either been the result of an increased rate of cell loss or a brief reduction in crypt cell production rate. Hampson (1986) also observed that the reduction of villus height was continued until five days after weaning in the pig.

Table 1: Villus and crypt morphometry of 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
Villus height (µm)	Duodenum	863.56±64.91^{Ar}	1063.05±75.33^{Ds}	538.70±27.55^{Bp}	724.39±28.94^{Eq}	696.82±43.24^{C}	842.72±68.48^{E}	0.001	0.001
	Jejunum	645.81±59.16	812.96±68.09^{d}	594.56±30.15	630.35±20.68^{ef}	618.51±45.23	734.50±41.11^{df}	0.74	0.03
	Ileum	509.79±22.09^{p}	622.23±34.85^{q}	498.92±43.15	594.65±45.44	562.39±32.54	601.36±51.39	0.37	0.90
Villus width (µm)	Duodenum	255.10±15.93	286.30±19.37	257.48±15.44	284.56±16.86	267.37±19.14	292.62±16.38	0.86	0.94
	Jejunum	190.12±11.62^{r}	219.16±8.80^{Ds}	208.86±11.19^{r}	260.70±16.64^{Es}	232.03±17.95^{p}	302.94±16.43^{Fq}	0.11	0.001
	Ileum	171.32±7.97^{Ar}	209.46±13.89^{ds}	201.26±10.41^{Br}	247.16±18.03^{des}	216.89±10.54^{Bp}	272.22±12.75^{efq}	0.005	0.02
Crypt depth (µm)	Duodenum	233.55±17.79^{r}	295.66±23.82^{s}	246.33±21.56^{p}	349.29±24.71^{q}	273.45±20.66	343.20±31.91	0.36	0.31
	Jejunum	249.86±18.61^{p}	343.20±16.55^{q}	282.99± 23.66	340.49±21.12	319.45±31.55^{r}	403.73±28.46^{s}	0.15	0.09
	Ileum	266.64±20.22^{ap}	367.29±14.26^{q}	288.38±17.15^{abp}	359.41±16.58^{q}	340.90±20.00^{bcr}	398.97±15.30^{s}	0.02	0.16
Crypt width (µm)	Duodenum	86.49±3.37^{B}	97.90±5.63	68.99±3.47^{Ap}	86.90±4.04^{q}	92.99±4.11^{B}	99.04±5.51	0.001	0.19
	Jejunum	89.25±5.79^{a}	105.84±6.66^{D}	83.76±3.07^{ap}	99.67±5.03^{Dq}	102.34±4.34^{bp}	134.39±7.80^{Eq}	0.02	0.001
	Ileum	114.16±5.95	123.38±5.78^{A}	104.39±5.47	111.77±5.13^{B}	121.40±5.93	130.48±6.84^{AC}	0.12	0.09
Villus height: Crypt depth (V:C)	Duodenum	4.31±0.46^{A}	4.25±0.38^{d}	2.54±0.18^{B}	2.53±0.34^{ef}	2.90±0.29^{B}	3.09±0.51^{df}	0.001	0.014
	Jejunum	2.92±0.30	2.55±0.25	2.42±0.20	2.07±0.16	2.59±0.33	2.17±0.29	0.45	0.34
	Ileum	2.26±0.21^{r}	1.74±0.11^{s}	1.97±0.22	1.81±0.19	1.85±0.16	1.58±0.15	0.33	0.57
Villus enlargement factor (VEF)	Duodenum	3.92±0.31	4.14±0.34	3.54±0.24	4.03±0.20	4.30±0.22	4.63±0.25	0.14	0.27
	Jejunum	4.10±0.37	4.23±0.30	3.87±0.30	4.17±0.23	4.35±0.31	4.69±0.36	0.59	0.43
	Ileum	3.67±0.18	4.17±0.29^{d}	3.12±0.35	3.41±0.19^{e}	3.79±0.26	4.03±0.21^{de}	0.20	0.05
Crypt enlargement factor (CEF)	Duodenum	4.05±0.28	4.43±0.41	3.66±0.23	4.15±0.20	4.46±0.27	4.77±0.26	0.12	0.37
	Jejunum	4.23±0.38	4.44±0.41	3.98±0.30	4.31±0.25	4.49±0.31	4.83±0.40	0.56	0.56
	Ileum	3.80±0.20	4.35±0.36	3.24±0.36	3.57±0.20	3.94±0.28	4.18±0.24	0.22	0.13

Data are presented as Mean ± 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,c}Means with different superscripts between control groups significantly differ (P<0.05);

^{d,e,f}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).

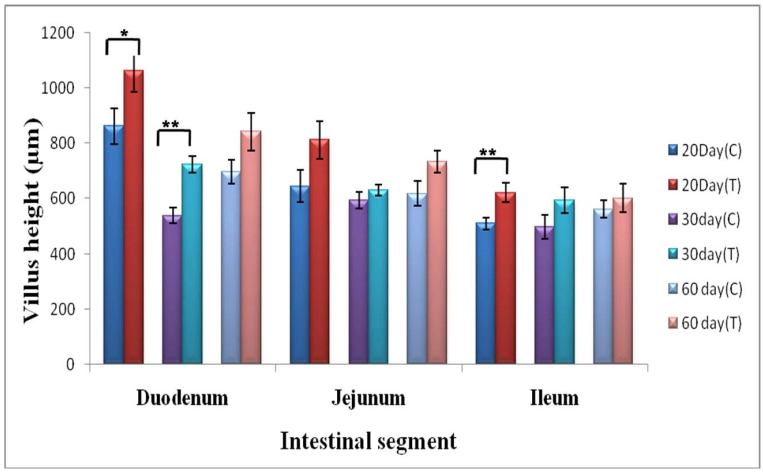

Fig 1: Comparative villus height of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets (*P<0.05, **P<0.01).

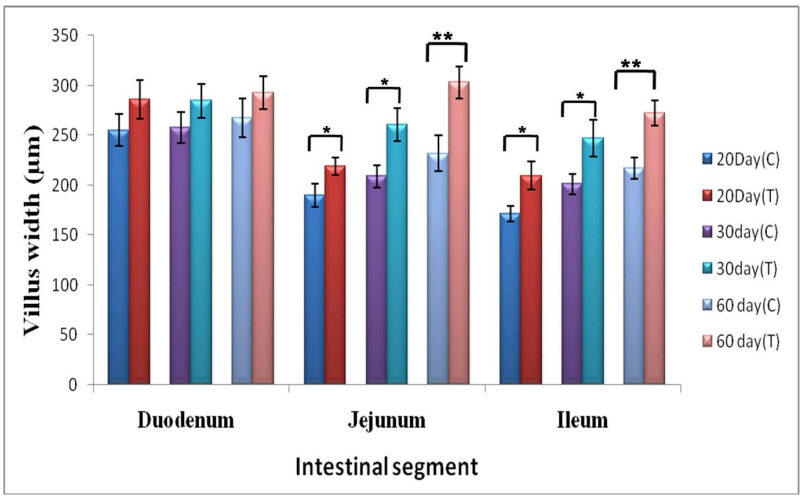

Fig 2: Comparative villus width of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets. (*P<0.05, **P<0.01).

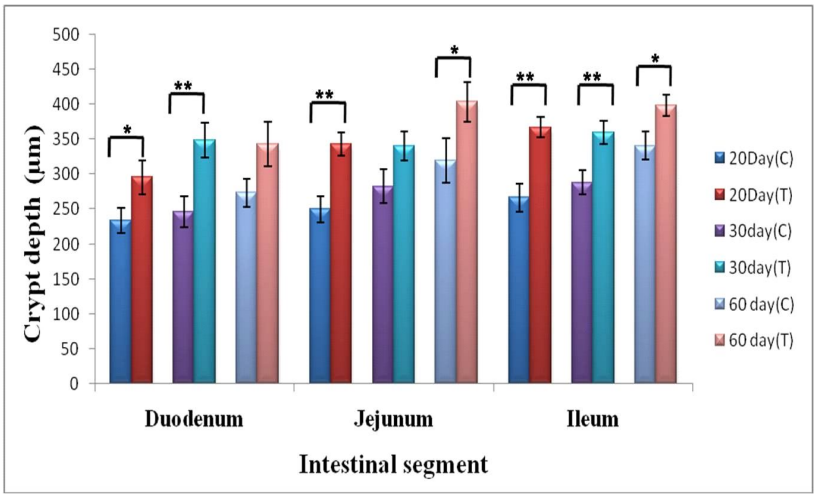

Fig 3: Comparative crypt depth of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets. (*P<0.05, **P<0.01).

In the current study, the mean villus width was found to be increased in most cases as the age advanced (Table 1). It was decreased towards the caudal parts of the small intestine in both the groups, as also reported by Galeano *et al*. (2016) in probiotic fed piglets. Dietary inclusion of probiotic and zinc revealed higher villus width in all segments of the small intestine in different age-groups (Fig 2). In jejunum and ileum, the mean villus width was significantly higher (P<0.05) in the treated animal at day 20 and 30. At day 60, the mean villus width was highly significant (P<0.01) in treated piglets compared with the control piglets. Similar types of reports were made by Li *et al*. (2001) after zinc oxide supplementation to weaned pigs. Increased villus width could be correlated with the more surface area present in the small intestine of treated piglets, which could absorb more nutrients (Caspary, 1992) for better growth and development.

During the study period, piglets fed with probiotic and zinc showed an increasing trend in crypt depth of all the age-groups (Table 1). In all the segments of the small intestine, the crypt depth was higher in the treated piglets compared to control animals (Fig 3). It was significantly increased in the duodenum (P<0.05), jejunum (P<0.01) and ileum (P<0.01) at day 20. The mean crypt depth at day 30 of treated piglets was significantly more (P<0.01) in duodenum and ileum. A significant increase (P<0.05) in crypt depth was also observed in jejunum and ileum at day 60 in treated animals. The higher crypt depth in the treatment group of all small intestinal segments might be indicative of higher cell proliferative activity for allowing adequate epithelial turn-over rate and compensate losses in the height of villi as reported by Pluske *et al*. (1997) in pig.

In the present investigation, the mean crypt width recorded an undulating trend as per the advancement of age being lowest at day 30 in all the parts of the small intestine of both the groups (Table 1). It was found to be numerically higher in treated piglets than the control group of animals (Fig 4). There was significantly increased (P<0.01) in the mean crypt width of duodenum and jejunum at day 30 and in jejunum at day 60 in the treatment group of piglets. The increased crypt width in the treatment group might suggest the more number of proliferating cells in the crypt and migrated to the villi to maintain villi length. The increased

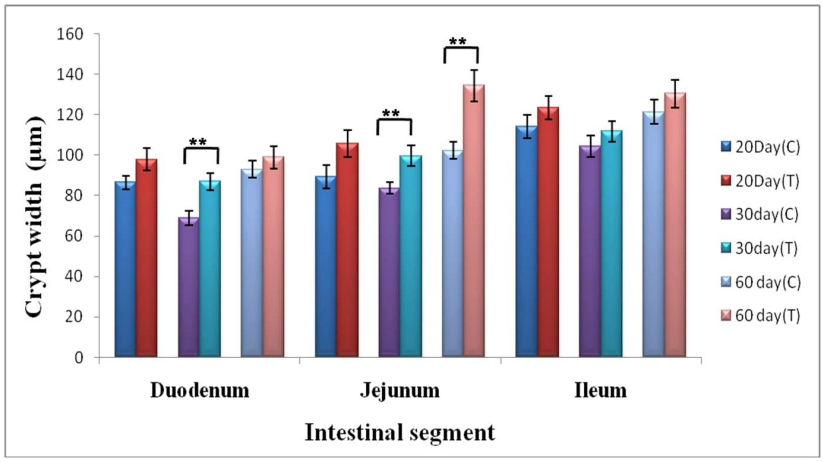

Fig 4: Comparative crypt width of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets (**P<0.01).

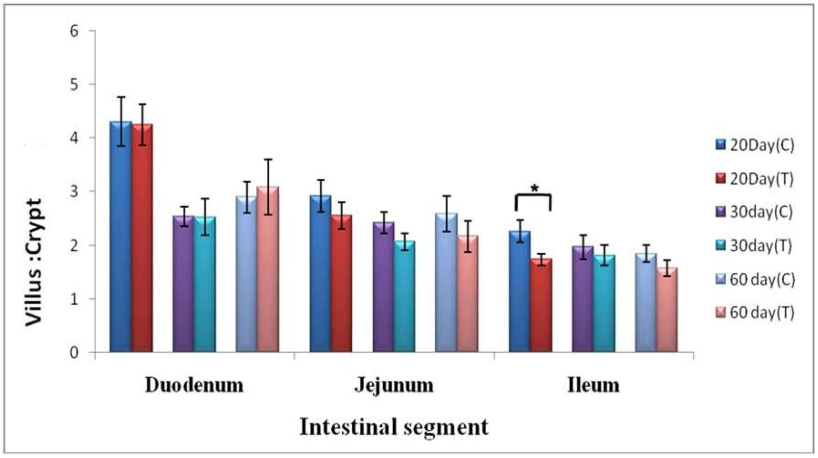

Fig 5: Comparative villus crypt ratio of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets (*P<0.05).

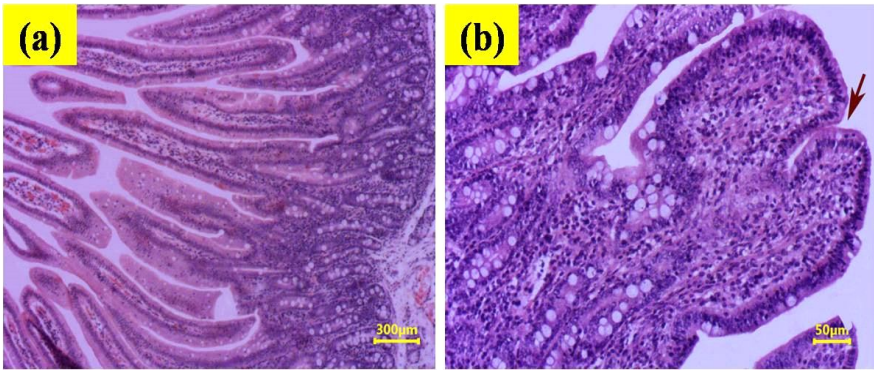

Fig 6: Photomicrographs showing villi of small intestine. (a) Leaf-like duodenal villi in 20 days old treated piglet (H&E, ×40). (b) Incompletely divided ileal villi (arrow) in 60 days old treated piglet (H&E, ×100).

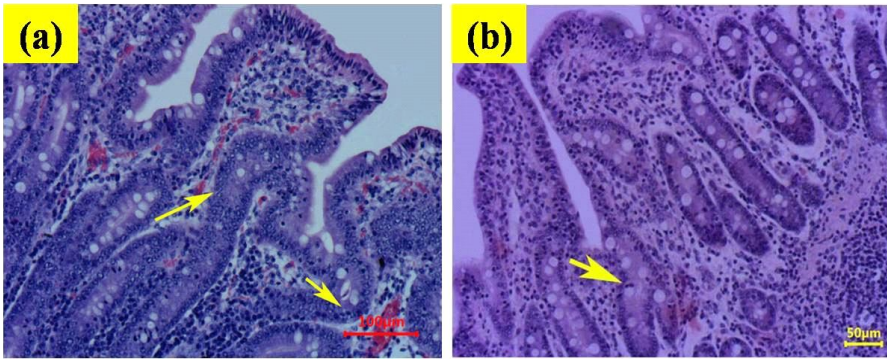

Fig 7: Photomicrographs showing villus and crypt (H&E, ×100). (a) Continuation of crypt and villus epithelial cells (arrow) in jejunum of 30 days old treated piglet. (b) Bifurcated ileal crypt (arrow) in 60 days old treated piglet.

crypt width in the treatment group under study was corroborated by Rieger *et al*. (2015) in piglets.

The micrometric analysis revealed that villus height and crypt depth ratio (V: C) varied in different parts of the small intestine with the advancement of age (Table 1). It was observed lower in treatment group piglets compared to control animals except in the duodenum at day 60 (Fig 5). A significantly ($P<0.05$) decreased villus crypt ratio was recorded in the ileum of treated animals at day 20. Bontempo *et al*. (2006) and Dowarah *et al*. (2017) also reported a lower V: C ratio in probiotic fed pigs.

In the present study, probiotic and zinc supplementation showed longer villus and the deeper crypt with lower V: C ratio compared to control animals, which might be indicative of improved intestinal morphology for better growth and development, especially during early post-weaned piglets. The post-weaning piglets often exhibited villus atrophy and crypt hyperplasia (Loh *et al*., 2002). According to Rodrigues *et al*. (2007), such villus atrophy in early weaning piglets might be due to sudden changes in the diet and low feed intake which was corrected by feeding *L. acidophilus* (2.5×10^8 CFU/g), as the animals had longer villi and deeper crypts compared to animals without probiotic. Similarly, greater villus height and crypt depth and lower V: C ratio was recorded in probiotic supplemented piglets than the control animals (Mair *et al*., 2010).

The mucosa of the entire small intestine showed numerous villi of variable shapes and sizes depending upon the age of piglets and segments of the intestine. In the duodenum, the villi were leaf-like and more numerous (Fig 6). In the jejunum, the villi were finger-like but not uniform in length. In the ileum, numerous flat finger-like villi were observed, which seem to be not completely divided (Fig 6). Many crypts of Lieberkühn were located ventral to each villus, which was the nest for the population of stem and progenitor cells. They differentiated into various functional cells on the villi to replace the epithelial cells being lost via anoikis at the villus tip (Fig 7) as described by Barker (2014). The crypts showed some bifurcations and fissions (Fig 7), particularly in ileum as reported by Wiese *et al*. (2003) in piglets.

Villus and crypt enlargement factor

The mean villus enlargement factor observed higher numerical values in the piglets fed with probiotic and zinc than the control group (Table 1). However, this higher numerical value was not statistically significant. This finding was agreed with the finding of Reiter *et al*. (2006) in piglets. The villus enlargement factor was found maximum in the jejunum, followed by duodenum and ileum in both the control and treatment groups. It was also maximum in day 60 followed by day 20 and day 30. As the shape of the villi varied depending on parts of the small intestine and age, the enlargement factor for the villus could be regarded as a

parameter representing the absorptive capacity of the small intestine (Wiese *et al*., 2003). In the present study, the higher villus enlargement factor reported in the treatment group might be contributed to a higher absorptive capacity of available nutrients by the small intestine.

In the present study, the mean crypt enlargement factor showed higher numerical values in the treatment groups (Table 1). A similar finding was also recorded by Reiter *et al*. (2006) in probiotic fed piglets. The higher crypt enlargement factor might suggest an increased number of crypt base columnar (CBC) cells in treated piglets (Fig 8), which resulted in much wider and deeper crypts covering more area in the lamina propria. The mean crypt enlargement factor was maximum in jejunum, followed by duodenum and ileum in both the groups. The values of this enlargement factor were also maximum in day 60 followed by day 20 and day 30.

Ultrastructural characteristics

In the present investigation, the architecture of villi and crypts of duodenum, jejunum and ileum were studied by scanning electron microscopy and are presented in Fig 9 to Fig 15.

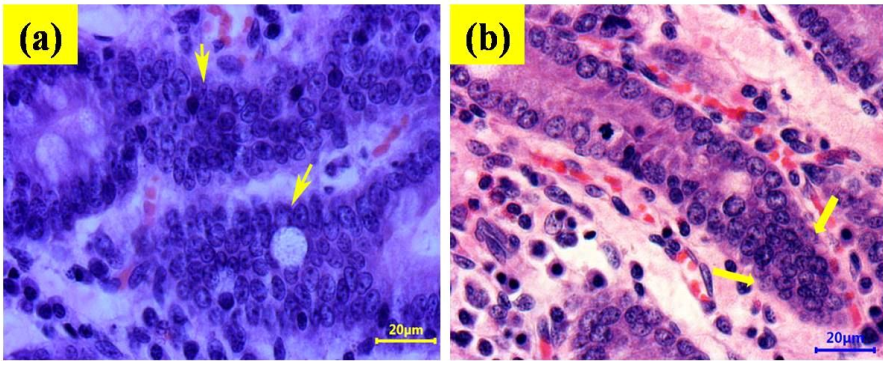

Fig 8: Photomicrographs showing jejunal crypts (H&E, ×400). (a) More number of crypt base cells (arrow) in 30 days old treated piglet. (b) Less number of crypt base cells (arrow) in 30 days old control piglet.

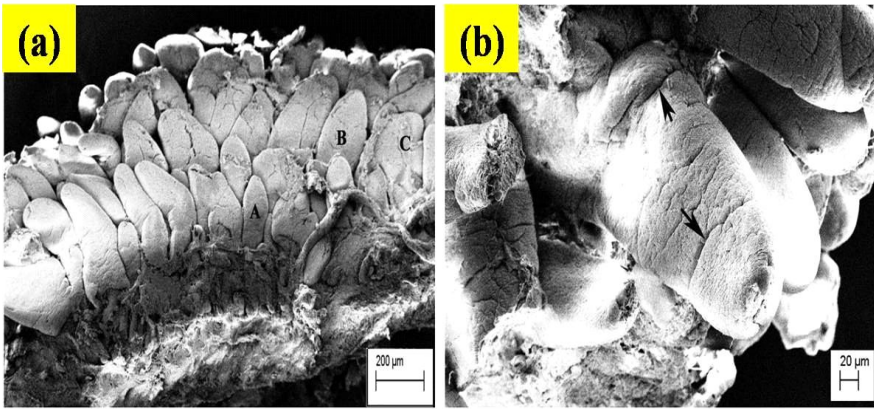

Fig 9: Scanning electron micrographs showing duodenal villi. (a) Finger-like (A), tongue-like (B) and twin-like (C) villi in 30 days old treated piglet. (b) Prominent transverse furrows (arrow) at various lengths in 20 days old control piglet.

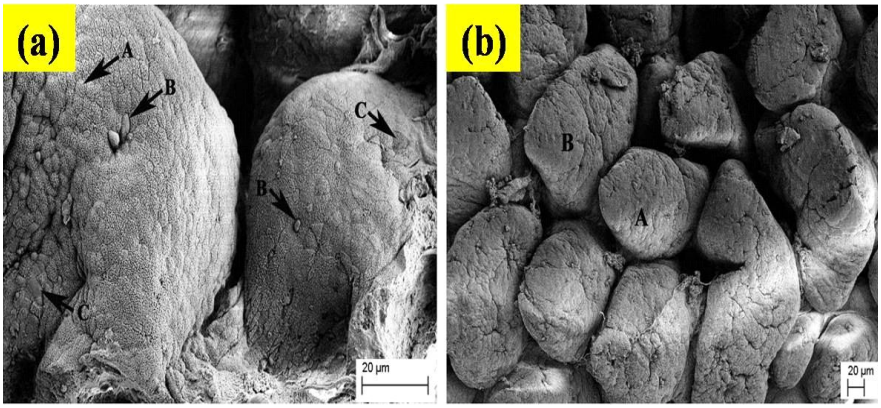

Fig 10: Scanning electron micrographs showing duodenal and jejunal villi. (a) Enterocyte (A), goblet cell (B) and M-cell (C) in duodenum of 30 days old treated piglet. (b) Finger-like (A) and Tongue-like (B) villi in jejunum of 20 days old treated piglet.

Duodenum

The mucosal surface of the duodenum was folded and covered by villi of regular shape and size; most of the villi were finger-like at day 20. Though the majority of villi were finger-shaped at day 30 and 60, there were a few tongue-shaped and twin-shaped villi with longitudinal indention (Fig 9) were observed. The transverse furrows were prominent at day 20 (Fig 9), followed by day 30 and day 60. The status of transverse furrows might be indicative of hemodynamic indexing conditions of the neonatal gut (Skrzypek *et al*., 2005). The pentagonal and hexagonal outlines of single enterocytes were seen. The goblet cells (Fig 10) were visible like a droplet of mucus on the surface of the villi. These goblet cells were found to be increased in the treatment group of piglets and with the advancement of age. The M-cells were present on the surface of the villi (Fig 10). These findings were in consonance to the findings of Wiese *et al*. (2003), Skrzypek *et al*. (2005) and Reiter *et al*. (2006) in pigs.

Jejunum

In the present study, the shape of the villi was finger-like to flat and tongue-like at day 20 and day 30 (Fig 10) and flat wide tongue-like at day 60 (Fig 11) in both control and treatment groups. The transverse furrows were in decreasing pattern as per the advancement of age. The tip of the villi showed clear extrusion zones with numerous goblet cells, deep incisions and distorted continuity of epithelial cells at day 20 and day 60. This finding was also supported by Skrzypek *et al*. (2005) in pigs at three weeks after birth. The shape of the epithelial cells in the extrusion zone was changed to irregular and elongated type from its regular hexagonal shape (Fig 11). The extrusion zone was more prominent in the treated piglets than the control animal. The acceleration of extrusion zone activity observed in the treated piglets of the present study might be linked to the intensive rebuilding of the population of enterocytes and enhanced enterocyte turn-over (Trahair and Sangild, 2004). The crypt was found to be a long cylindrical tube-like structure penetrating ventrally in the lamina propria (Fig 12). Most of them were extended up to the lamina muscularis mucosae.

The villus M-cells were observed between the enterocytes (Fig 12) and had irregular microfolds on their luminal surface (Fig 13). The shape of M-cells varied from round to polygonal and was found extensive (Fig 13) in the jejunum at day 30 in both the groups of piglets.

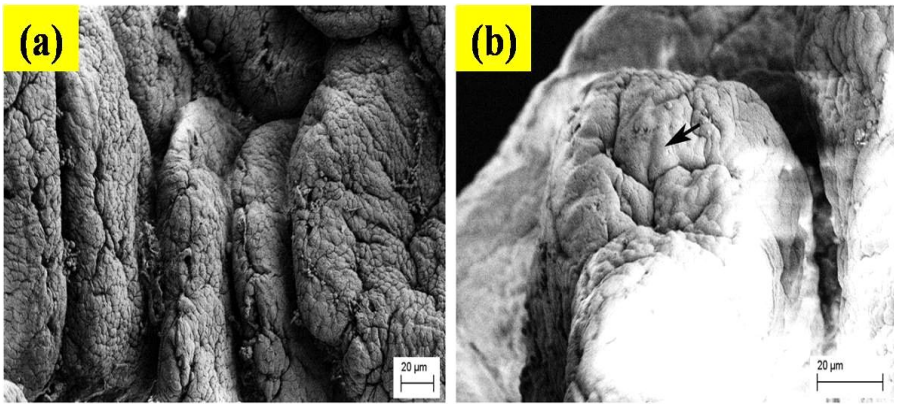

Fig 11: Scanning electron micrographs showing jejunal villi in 60 days old treated piglet. (a) Flat wide tongue-shaped villi. (b) Well developed extrusion zone containing irregular shaped epithelial cells (arrow).

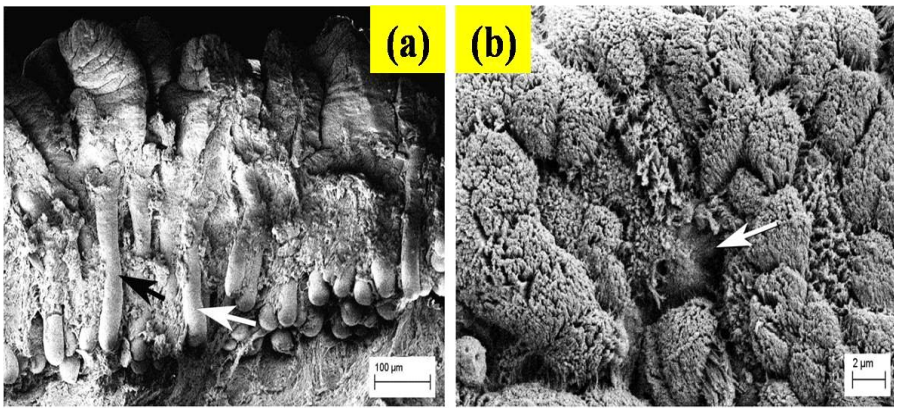

Fig 12: Scanning electron micrographs showing jejunum of 60 days old treated piglet. (a) Long cylindrical crypts (arrow). (b) Villus M-cell (arrow) in between the enterocytes.

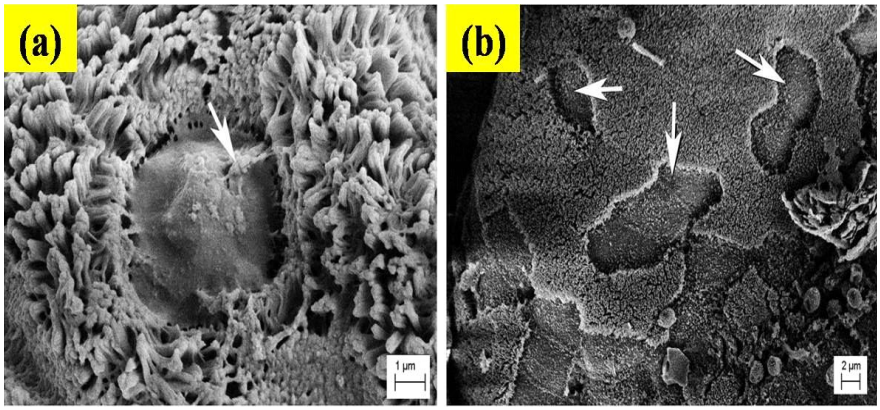

Fig 13: Scanning electron micrographs showing jejunum. (a) Irregular microfolds on the luminal surface of M-cell (arrow) in 20 days old treated piglet. (b) Large round to polygonal shapd M-cells (arrow) in 30 days old treated piglet.

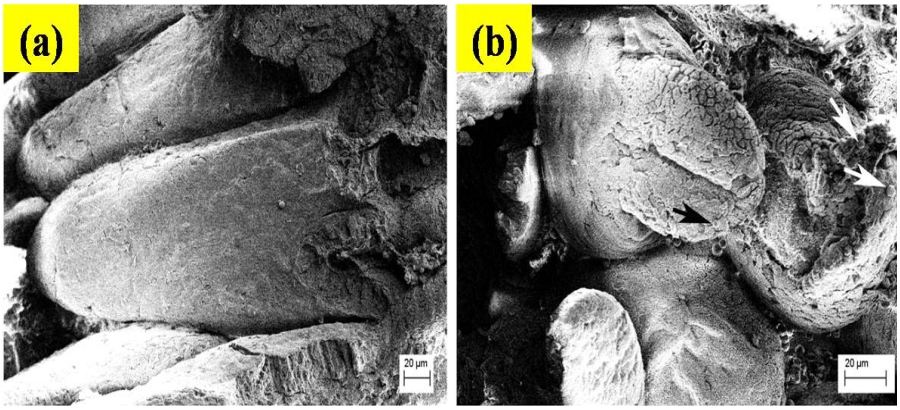

Fig 14: Scanning electron micrographs showing ileum. (a) Smooth tongue-shaped villi in 60 days old treated piglet. (b) Incompletely divided villi with a number of shedding cells (arrow) in 30 days old treated piglet.

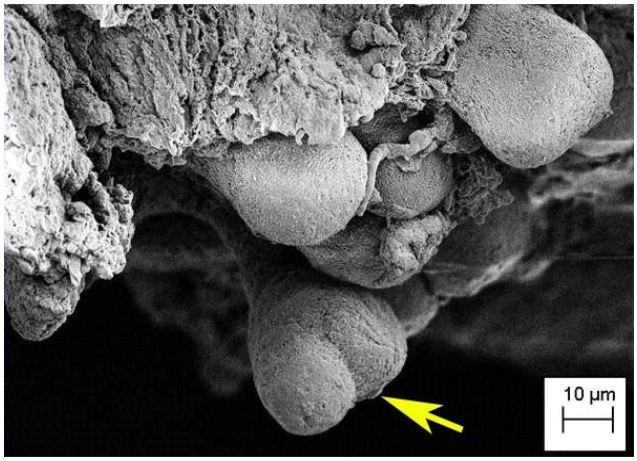

Fig 15: Scanning electron micrograph showing bifurcation of ileal crypt (arrow) in 60 days old treated piglet.

Ileum

The shape of the villi of ileum was mostly tongue-shaped (Fig 14); however, finger or leaf-like and incompletely divided villi (Fig 14) were also not uncommon in both the group of piglets. Despite the presence of enterocytes, the surface of the villi was relatively smooth (Fig 14). The goblet cells were predominant irrespective of group and age. The transverse furrows were almost absent in all the age-groups. In the apical region of the villi, a clear extrusion zone was observed with a number of shedding cells (Fig 14) at day 30 and day 60 in both the groups. Moreover, prominent shedding of cells seen in the treatment group might suggest a quick rebuilding of enterocytes to compensate losses in the villi (Trahair and Sangild, 2004). In the current study, the crypt showed some bifurcations at their bases (Fig 15), which was in close agreement with the finding of Wiese *et al*. (2003) in piglets.

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