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Impact of Probiotic and Zinc on Brush-border Enzyme and Histoenzymatic Profile in the Small Intestine of Pre and Post-weaned Piglets

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ABSTRACT

Background: Probiotics and zinc are commonly used and beneficial in pig production. This work aimed to assess the effect of probiotic and zinc on brush-border enzyme activity and histoenzymatic study of the small intestine in pre and post-weaned piglets.

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), day 30 (weaning) and day 60 (post-weaning), the animals were sacrificed. For disaccharidase enzyme estimation, the mucosal brush border of the small intestine was scrapped off and the experiment was conducted. For histoenzymatic assay, the small intestine samples were preserved in liquid nitrogen at -196°C immediately after sacrifice. They were sectioned at 10µm thickness maintained at -20°C and stained for different histochemical staining. The statistical analysis of the data using the appropriate statistical tests was also conducted.

Result: The activity of different brush-border enzymes such as maltase, sucrase and lactase was more in the treatment group of piglets. The activity of different histochemical enzymes such as alkaline phosphatase, acid phosphatase, adenosine tri-phosphatase and non-specific esterase was increased in the treated group of piglets.

Key words: Brush-border enzyme, Histochemistry, Piglets, Probiotic, Zinc.

INTRODUCTION

Neonatal and post-weaning piglet mortality is a major concern in pig husbandry, which causes huge economic losses to pig farmers. Weaning is a major critical period because of increased susceptibility to gut disorders, infections and diarrhoea due to dietary changes. The major effect of weaning is a reduction of feed (energy) intake leading to undernutrition and retarded growth. At this time, there is an alteration of the intestine that includes changes in villus/crypt morphology and in brush-border enzyme activities that further aggravate the conditions. Previous researches illustrated that probiotics enhance the growth performance of swine (Wang *et al*., 2012), modulate the immune system (Gonzalez-Ortiz *et al*., 2013) and promote intestinal health (Tojo *et al*., 2014). Probiotics are the micro-organisms that have potential benefits on the host health (Djurasevic *et al*., 2017). Probiotics are also capable of improving the microecology of the intestine to provide a beneficial effect (Kohler *et al*., 2003). Feeding of zinc is well recognized to have growth-promoting effects in weaned pigs (Poulsen, 1995, Case and Carlson, 2002) and reduce the problems with post-weaning diarrhoea (Poulsen, 1989). Supplementing the zinc in pig diet may promote the processes of tissue repair in the small intestine and stimulate the synthesis of brush-border and other digestive enzymes, resulting in better digestion and absorption of nutrients and potentially improving growth performance.

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Keeping these points in view, the present study was undertaken to investigate the combined effect of probiotic and zinc on brush-border enzyme and histoenzymatic profiles in the small intestine of pre and post-weaned piglets.

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 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.

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 (six) piglets, 3 (three) piglets from each litter were used as the control group (C) with basal diet and the other 3 (three) piglets were fed with combined probiotic and zinc supplement and used as treatment group (T). The combined probiotic and zinc were supplemented orally to the treatment group piglets along with the basal diet. The basal diet used in this experiment was in pellet form and was formulated to provide the nutrient requirements recommended by the (NRC) National Research Council (1998). 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. Subsequent to sacrifice, the abdominal cavity of the animal was exposed by reflecting the skin, fascia, abdominal muscles and peritoneum and parts of the small intestine were observed. The abdominal cavity was after that opened and the parts of the small intestine were then dissected out as per the method of Habel (1964). 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 brush-border (disaccharidase) enzyme activity examination

The activity of disaccharidase enzymes in the small intestine was estimated by the method described by Dahlqvist (1964, 1968 and 1984) with slight modifications. The mucosal brush border of the small intestine was scrapped off using glass slide and stored at -80°C till further use. Mucosal tissue (100 mg) was placed in 5 ml centrifuge tube, homogenized with the help of micro pestle and added 900 µl of chilled normal saline solution (NSS). Tubes were vortexed and the volume of the suspension was increased to three times with chilled NSS. To 0.1 ml of mucosal tissue suspension, 0.1 ml of appropriate substrate solution (maltose/sucrose/lactose) was added. To this mixture, 2 µl of toluene was added and incubated the tubes at 37°C for 1 hr. A sample blank was prepared with the same composition and immersed the tubes in boiling water immediately after mixing of the enzyme and substrate. Estimation of glucose was carried out using glucose (GO) assay kit (Sigma, MI, USA) as per the manufacturer's instruction. Glucose concentration in unknown samples was estimated using the standard curve prepared with glucose standards provided with the kit. The disaccharidase activity of the test samples was obtained by the following formula:

$$\text{Disaccharidase activity (units/ml)} = (a - b) \times d/n \times 540$$

Where,

a = Amount of glucose (µg) found in an aliquot of the incubated sample.

b = Amount of glucose (µg) found in an aliquot of the corresponding blank.

d = Dilution factor of the enzyme solution used for mixing with the substrate.

n= Number of glucose molecules that were liberated per substrate (disaccharide) molecule hydrolyzed.

The maltose was composed of two glucose molecules (n=2).

The sucrose and lactose, which contain one molecule of glucose and one of fructose or galactose, respectively (n=1).

Units of activity = µmoles disaccharide hydrolyzed/ minute

Preparation for the histochemical examination.

For histoenzymatic assay, the small intestine samples were preserved in liquid nitrogen at -196°C immediately after sacrifice. They were sectioned at10µm thickness in cryostat microtome (Shandon Finesse) maintained at -20°C. They were temporarily stored at -22°C and then treated for histochemical staining with the following methods:

1. Gomori's alkaline phosphatase cobalt method (Singh and Sulochana, 1978).
2. Gomori's method for acid phosphatase (Singh and Sulochana, 1978).
3. Lead method for ATPase (Bancroft, 2008).
4. Gomori's method for non-specific esterase (Bancroft, 2008).

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. Results are presented as mean ± 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 AND DISCUSSION

Brush-border (disaccharidase) enzyme profile

The activity of brush border enzymes such as maltase, sucrase and lactase in control (basal diet) and treated (basal diet + probiotic + zinc) piglets were estimated per gm of tissue based on the capacity of these enzymes to convert their respective substrates into glucose and the results are shown in Table 1 and Fig 1 to Fig 3.

Maltase

The activity of maltase increased towards the advancement of age in both the groups, being significant (P<0.05) in the control group and highly significant (P<0.01) in the treatment group (Fig 1a). The increased activity of this disaccharidase was also reported by Hornbuckle *et al*. (2008). In the present study, the maltase activity was lower as compared to the activity of lactase and sucrase. This was due to the procedure of estimation in a one-step method caused by interference between maltose and glucose oxidase, *i.e.*, with the assay system and not with the disaccharidase activity. The lower estimation value of maltase activity was also

Table 1: Brush-border enzyme activity in small intestine of piglets fed with probiotic and zinc.

Enzyme activity	Pre-weaning		Post-weaning				p-value	
	Day 20		Day 30		Day 60			
(µmoles/minute)	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Maltase	0.85 ± 0.18^{a}	1.09 ± 0.10^{D}	1.02 ± 0.07^{ap}	2.77 ± 0.09^{Eq}	2.22 ± 0.45^{br}	6.13 ± 0.82^{Fs}	0.03	0.001
Lactase	58.53 ± 3.14^{A}	61.58 ± 3.72^{D}	42.28 ± 0.12^{B}	45.69 ± 1.42^{E}	36.34 ± 4.76^{B}	45.71 ± 2.44^{E}	0.008	0.009
Sucrase	11.47 ± 3.49^{A}	14.95 ± 4.06^{D}	28.80 ± 0.97^{B}	40.57 ± 6.31^{E}	57.0 ± 2.77^{C}	68.13 ± 5.17^{F}	0.001	0.001

Data are presented as enzyme activity in µmoles/minute/gm of tissue (Mean ± SEM) of 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); ^{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).

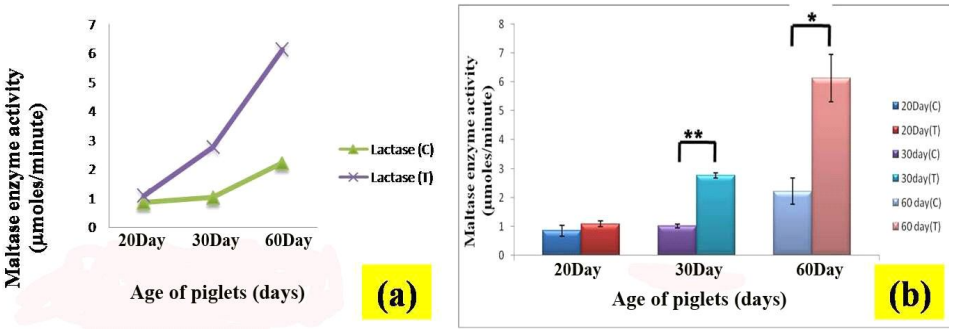

Fig 1: Activity of maltase enzyme (a) Alteration in maltase enzyme activity of small intestine in piglets at different age-groups; (b) Comparative maltase enzyme activity of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets (*P<0.05, **P<0.01).

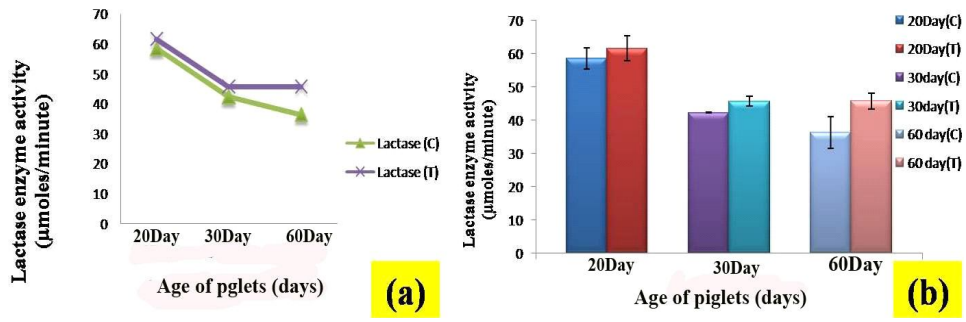

Fig 2: Activity of lactase enzyme (a) Alteration in lactase enzyme activity of small intestine in piglets at different age-groups; (b) Comparative lactase enzyme activity of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets.

recorded by Dahlqvist (1968). In the current study, the maltase activity for the conversion of maltose into two molecules of glucose as end products of starch digestion was higher in the treatment group of piglets than control in all the age-groups (Fig 1b). Moreover, it was significantly increased at day 30 ($P<0.01$) and day 60 ($P<0.05$) in treated piglets. Hedemann *et al.* (2006) and Hu *et al*. (2018) reported significantly higher maltase activity in piglets after feeding 100 ppm Zn ($P<0.01$) and probiotic ($P<0.05$) which were in close agreement with the present findings.

Lactase

In the current investigation, the lactase activity for the conversion of lactose (galactose-glucose) was decreased as per the advancement of age, being highest at day 20 and lowest at day 60 in both the groups (Fig 2a). The present finding was closely similar to the findings of Hornbuckle *et al*. (2008). The relatively high lactase activity in the younger animal might be an advantage in utilizing large quantities of lactose present in their diets. In this present study, the lactase activity was moderately higher ($P>0.05$) in the treatment group of piglets in comparison to the control animal (Fig 2b), which might indicate better conversion of lactose into galactose and glucose. The increased lactase activity was also recorded in piglets after feeding 100 ppm of Zn (Hedemann *et al*., 2006) and probiotics (Hu *et al*., 2018) in the diets. The above findings were in accordance with the present study.

Sucrase

In the present study, the sucrase activity for the conversion of sucrose (fructose-glucose) was increased as the age advanced in both the groups (Fig 3a). This finding was similar to the findings of Hornbuckle *et al*. (2008). In this current study, the activity of sucrase was higher in the treated piglets than that of control animals in all age-groups without any significant difference (Fig 3b). This finding might suggest the effective conversion of sucrose into fructose and glucose in the treatment group of piglets. The enhanced sucrase and lactase activity in intestinal mucosa after dietary inclusion of probiotic were also reported by Southcott *et al*. (2008) and Goyal *et al*. (2013) in rats and Hu *et al*. (2018) in piglets.

Carbohydrates were one of the major components of the diet. In the gastrointestinal tract, carbohydrates were mainly digested by salivary and pancreatic amylases, further broken down into monosaccharides by disaccharidase, such as maltase, lactase and sucrase, which secreted by enterocytes of the brush border and then were absorbed (Zhen *et al*., 2018) in chicken. Thus, higher conversion of disaccharides to monosaccharides in treatment group piglets might be indicative of more absorption of glucose from the available carbohydrate present in the intestine and resulted in better growth and development in this group of piglets.

Histoenzymatic profile

The cryosections from all segments of the small intestine of control and treated piglets were subjected to histochemical staining by respective protocols and the results are illustrated in Table 2 to Table 5 and in Fig 4 to Fig 7. The different histochemical activities were observed in absorptive epithelium, glandular and, follicular and interfollicular areas. The gradation for intensity of histochemical reaction is: NA, Not Available; -, Negative; +, Weak; ++, Moderate; +++, Strong; ++++, Intense.

Alkaline phosphatase

In the present study, the alkaline phosphatase activity in the absorptive epithelium was intense in jejunum and ileum and strong in the duodenum in the treatment group of piglets (Fig 4a). However, this activity was moderate in duodenum and strong in jejunum and ileum of control animals (Table 2). Sprague *et al*. (1963) reported increasing alkaline phosphatase activity from cranial and middle sections of jejunum and ileum in new-born pigs, which was in agreement with the present finding. In the absorptive epithelium of the present study, this enzyme activity was slightly lower at day 30 in the control group of piglets, which might be due to the weaning effect as also revealed by Melo *et al*. (2016). However, in the treatment group piglets, the decreased activity of this enzyme at day 30 was not observed in the current study. The increased alkaline phosphatase activity in the absorptive epithelium recorded in the treated animals might be correlated with more ionic movements across the epithelium and increased number of matured enterocytes in the villi as reported by Kapoor and Singh (2017) in buffalo.

In the interfollicular area of jejunum and ileum, the alkaline phosphatase activity was moderate in control animals, while a strong reaction was observed in the treated piglets (Fig 4b) in all age-groups. The current observation

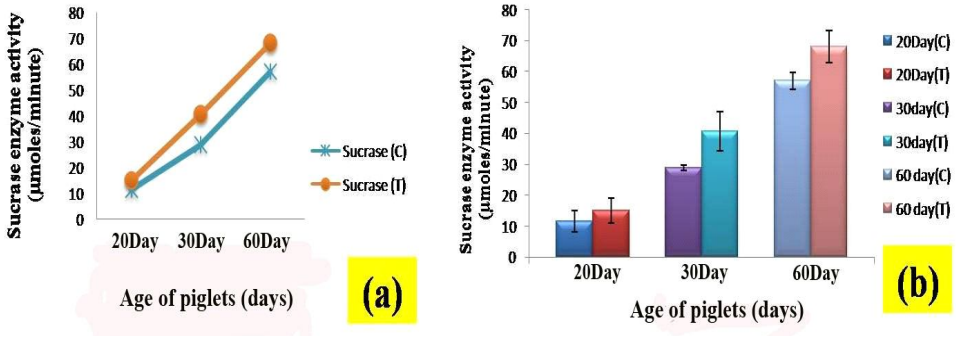

Fig 3: Activity of sucrase enzyme (a) Alteration in sucrase enzyme activity of small intestine in piglets at different age-groups; (b) Comparative sucrase enzyme activity of small intestine in control (basal diet) and treatment (probiotic and zinc) group piglets.

might be an indicator of the presence of more energy-dependent active transport mechanisms at these sites. Gautam (2015) recorded strong alkaline phosphatase reactions in these areas in growing piglets, which was similar to the present study. A weak to negative activity was observed in the glandular epithelium (Fig 4c) throughout all segments of the small intestine in both the groups, as also reported by Gautam (2015) in growing piglets.

Acid phosphatase

The acid phosphatase activity in the current study was found to be moderate in the absorptive epithelium irrespective of segments of intestine and age in both the groups (Table 3). In the glandular epithelium, it was observed intense in the treated piglets (Fig 5a) and strong in the control animal (Fig 5b). The intense reaction of this enzyme in the glandular epithelium of treatment group piglets might indicate the presence of more lysosomal activity in the respective sites as depicted by Gautam (2015) in growing piglets.

In the present study, the activity of acid phosphatase was strong and moderate in the follicular and interfollicular

Table 2: Alkaline phosphatase activity in histocompartments of the small intestine in piglets fed with probiotic and zinc.

Histocompartment	Intestinal segment	Pre-weaning		Post-weaning			
		Day 20		Day 30		Day 60	
		Control	Treatment	Control	Treatment	Control	Treatment
Absorptive	Duodenum	++	+++	+	+++	++	+++
epithelium	Jejunum	+++	++++	++	+++	+++	++++
	Ileum	+++	++++	++	+++	+++	++++
Gland	Duodenum	-/+	-/+	-/+	-/+	-/+	-/+
	Jejunum	-/+	-/+	-/+	-/+	-/+	-/+
	Ileum	-/+	-/+	-/+	-/+	-/+	-/+
Interfollicular	Duodenum	NA	NA	NA	NA	NA	NA
area	Jejunum	++	+++	++	+++	++	+++
	Ileum	++	+++	++	+++	++	+++

Table 3: Acid phosphatase activity in histocompartments of the small intestine in piglets fed with probiotic and zinc.

Histocompartment	Intestinal segment	Pre-weaning		Post-weaning			
		Day 20		Day 30		Day 60	
		Control	Treatment	Control	Treatment	Control	Treatment
Absorptive	Duodenum	++	++	++	++	++	++
epithelium	Jejunum	++	++	++	++	++	++
	Ileum	++	++	++	++	++	++
Gland	Duodenum	+++	++++	+++	++++	+++	++++
	Jejunum	+++	++++	+++	++++	+++	++++
	Ileum	+++	++++	+++	++++	+++	++++
Follicular and	Duodenum	NA	NA	NA	NA	NA	NA
interfollicular	Jejunum	++	+++	++	+++	++	+++
area	Ileum	++	+++	++	+++	++	+++

Table 4: Adenosine tri-phosphatase activity in histocompartments of the small intestine in piglets fed with probiotic and zinc

Histocompartment	Intestinalsegment	Pre-weaning		Post-weaning			
		Day 20		Day 30		Day 60	
		Control	Treatment	Control	Treatment	Control	Treatment
Absorptive	Duodenum	++	+++	++	+++	++	+++
epithelium	Jejunum	++	+++	++	+++	+	+++
	Ileum	++	+++	++	+++	++	+++
Gland	Duodenum	++	++	++	++	++	++
	Jejunum	++	++	++	++	++	++
	Ileum	++	++	++	++	++	++
Follicular and	Duodenum	NA	NA	NA	NA	NA	NA
interfollicular	Jejunum	++	+++	++	+++	++	+++
area	Ileum	++	+++	++	+++	++	+++

areas of jejunum and ileum of treated (Fig 5c) and control group (Fig 5d) of piglets, respectively in all age-groups. A reticular pattern acid phosphatase activity was seen in the interfollicular regions in both groups. This finding was also tuned to the finding of Halleraker *et al*. (1990), who reported reticular pattern acid phosphatase activity in the interfollicular regions of PP in calf, kid and lamb.

Adenosine-tri-phosphatase

In the present investigation, the activity of the adenosine-tri-phosphatase (ATPase) enzyme is presented in Table 4. The follicular and interfollicular areas of PP showed strong reaction in treated piglets (Fig 6a) and moderate reaction in control piglets for adenosine tri-phosphatase activity. This observation was in accordance to the findings of Halleraker

Table 5: Non-specific esterase activity in histocompartments of the small intestine in piglets fed with probiotic and zinc.

Histocompartment	Intestinal segment	Pre-weaning		Post-weaning			
		Day 20		Day 30		Day 60	
		Control	Treatment	Control	Treatment	Control	Treatment
Absorptive	Duodenum	++	+++	++	+++	++	+++
epithelium	Jejunum	++	+++	++	+++	+	+++
	Ileum	++	+++	++	+++	++	+++
Gland	Duodenum	++	++	++	++	++	++
	Jejunum	++	++	++	++	++	++
	Ileum	++	++	++	++	++	++
Follicular and	Duodenum	NA	NA	NA	NA	NA	NA
interfollicular	Jejunum	++	+++	++	+++	++	+++
area	Ileum	++	+++	++	+++	++	+++

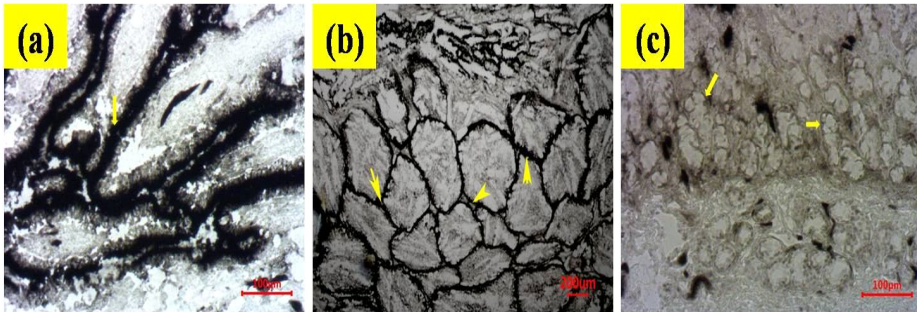

Fig 4: Photomicrographs showing histochemical staining of alkaline phosphatase activity (arrow) in cryosections of 30 days old treated piglet (Gomori's, ×100) (a) Villus epithelium of jejunum; (b) Interfollicular area of PP in ileum; (c) Crypt epithelium of duodenum.

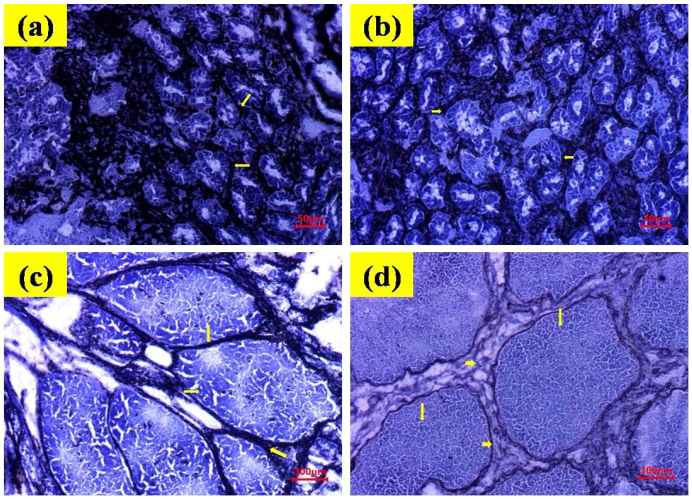

Fig 5: Photomicrographs showing histochemical staining of acid phosphatase activity (arrow) in cryosections. (a) Intense activity in crypt epithelium of duodenum in 30 days old treated piglet (Gomori's, ×100); (b) Strong activity in crypt epithelium of duodenum in 30 days old control piglet (Gomori's, ×100); (c) Strong activity in follicular and interfollicular area of PP in ileum of 30 days old treated piglet (Gomori's, ×40); (d) Moderate activity in follicular and interfollicular area of PP in ileum of 30 days old control piglet (Gomori's, ×40).

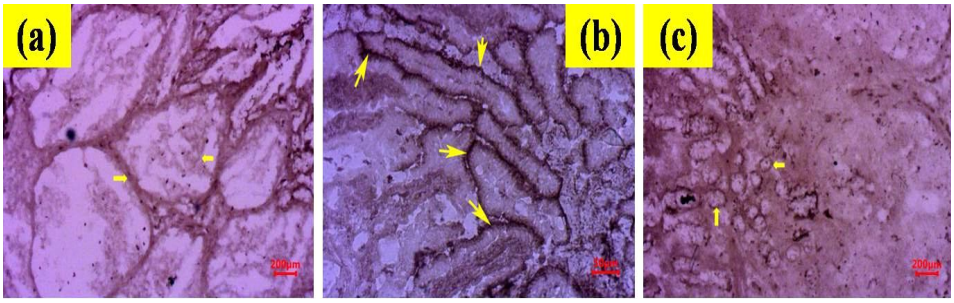

Fig 6: Photomicrographs showing histochemical staining of adenosine-tri-phosphatase activity (arrow) in cryosections of 30 days old treated piglet (a) Activity in follicular and interfollicular area of PP in ileum (Lead method, ×40); (b) Activity in absorptive epithelium of jejunum (Lead method, ×100); (c) Activity in glandular region of jejunum (Lead method, ×40).

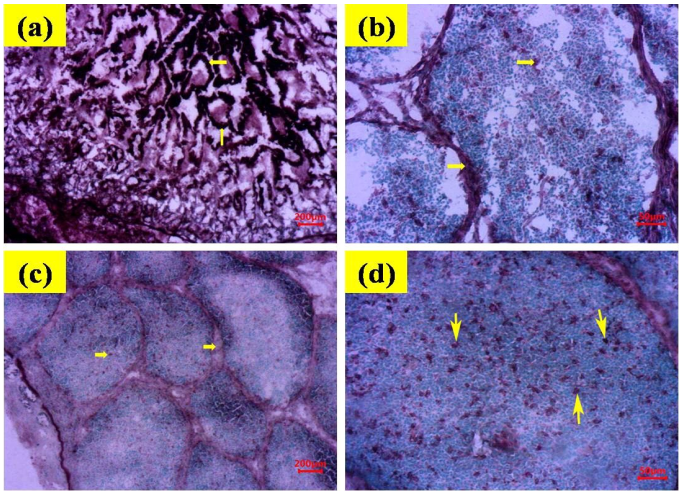

Fig 7: Photomicrographs showing histochemical staining of non-specific esterase activity (arrow) in cryosections (a) Activity in absorptive epithelium of duodenum in 30 days old treated piglet (Gomori's, ×40); (b) Strong activity in follicular and interfollicular area of PP in ileum of 30 days old treated piglet (Gomori's, ×100); (c) Moderate activity in follicular and interfollicular area of PP in ileum of 30 days old control piglet (Gomori's, ×40); (d) PP showing positively stained macrophages (arrow) in follicular area of ileum in 30 days old treated piglet (Gomori's, ×100).

et al. (1990), who opined that follicular dendritic cells and B-cell area were positive for magnesium-dependent ATPase activity. The increased activity of ATPase in the follicular area of treated piglets might suggest induced B-cell area for better immunity in this group of piglets. This finding was also confirmed by immunofluorescence observation in this study.

The ATPase enzyme activity in the present observation was strong in the absorptive epithelium of treated piglets (Fig 6b) and moderate in the control group animals. However, this activity was moderate in the glandular region (Fig 6c) in both the groups irrespective of segments of intestine and age. The present findings were almost similar to the findings of Gautam (2015) in growing piglets.

Non-specific esterase

In the present study, the non-specific esterase enzyme activity has been presented in Table 5. The dietary inclusion of probiotic and zinc revealed strong non-specific esterase activity in the absorptive epithelium (Fig 7a) and, follicular and interfollicular areas of PP (Fig7b). However, this activity was found to be moderate in these areas of control animals (Fig 7c). The follicles of PP showed positively stained macrophages (Fig 7d), which were predominant in the treatment group of piglets. The strong non-specific esterase reaction in PP area was also recorded by Halleraker *et al*. (1990), who described a positive reticular reaction in the T-cell area of the follicles and detected macrophages stained positive with non-specific esterase in ruminants. The sites of non-specific esterase activity were found to be a T-cell rich area, which was demonstrated by the APAP technique by Mishra (1998) and Rajkhowa (2003) in the pig.

The non-specific esterase activity in the glandular area of present observation was found to be moderate in all segments of the small intestine irrespective of treatment and age-groups.

CONCLUSION

In conclusion, the activity of brush border enzymes such as maltase, sucrase and lactase was more in treated piglets that indicate increased capacity of these enzymes to convert their respective substrates to glucose compared to control animals. Thus, higher conversion of disaccharides to monosaccharides in the treatment group of piglets might be indicative of more absorption of glucose from the

available carbohydrate present in the intestine and resulted in better growth and development in this group of piglets. Similarly, the activity of alkaline phosphatase, acid phosphatase, adenosine tri-phosphatase and non-specific esterase was increased in treated group of piglets that might be correlated with increased ionic movements across the epithelium, higher lysosomal enzyme activity, localization of more number of B cells for better immunity and more concentration of T cells, respectively.

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