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MORPHOLOGICAL CHARACTERIZATION OF TUNICA MUCOSA OF JEJUNUM AND CECUM OF SWINE GIVEN PROBIOTICS (*Lactobacillus casei KE-99*) IN DRINKING WATER

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ABSTRACT

This study was conducted to describe the morphology of tunica mucosa of jejunum and cecum of swine given probiotics (Lactobacillus casei KE-99) in the drinking water. A total of 27 pigs weaned at 21 days of age crossbred pigs [Landrace × (Large White × Duroc)] of both sex and of the same age (~30 days) with initial weight of 7 to 10 kg were allocated in 3 treatment groups and reared for 3 months. The control group (T1) followed the conventional or the recommended farm medication program which included antibiotic (tylosin and zinc bacitracin) in the feeds and without probiotics in drinking water. Treatment 2 (T2) was given 12 g of probiotics mixed with drinking water every other day, while Treatment 3 (T3) was given 12 g of probiotics mixed with drinking water every day. Both T2 and T3 consumed non-medicated feeds. After 3 months, pigs were slaughtered in Animal Science abattoir, College of Agriculture, University of the Philippines Los Baños. After which, the middle part of jejunum and cecum that were about 5 cm long were collected. The results showed that the various morphometric observations (mucosal thickness (µm) and crypt depth (µm) of jejunum and cecum; and villus height (µm), villus width (µm) and villus volume (µm³) of jejunum) on the tunica mucosa of pigs given probiotics supplementation in drinking water (T2 and T3) did not differ significantly from those antibiotic supplemented pigs (T1).

Key Words: pigs, probiotics, *Lactobacillus casei* KE-99, jejunum, cecum

INTRODUCTION

Probiotics are live microorganisms (e.g. *Lactobacillus* sp.) which improve balance in the intestinal microflora in a way that can also improve the health and production performance of the animal (Ng *et al*., 2009). Bacterial populations colonizing the gastrointestinal tract modify the intestinal microstructure (Vitini *et al.,* 2000) and induce functional changes in the intestinal mucosa (Babinska *et al.,* 2005). Shirkey *et al.* (2006) and Peric *et al*. (2010) reported that probiotics had a beneficial influence on the epithelial structure, villus height and villus surface area of pig's jejunum supplemented with *Lactobacillus fermentum*.

Maintaining the number of beneficial bacteria in the gut is necessary, but this is not always the case. Some factors like stress could cause imbalance in the intestinal microflora that would cause damage in the intestine, inadequate absorption of nutrients and will eventually lead to disease and poor production performance (Collins *et al*., 2009).

Antibiotics have been used to control many bacterial and parasitic diseases, and adding small amount of antibiotic in animal feed has been shown to correlate with

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improved production performance in terms of feed conversion or weight gain (Collins *et al*., 2009). However, bacterial resistance and drug residues in animal tissue caused by antibiotics in feeds are an issue of public health concern worldwide. Resistance of the bacteria to the drug could be due to the dose, duration and over-used of antibiotic in animal husbandry (JETACAR, 1999). In addition, resistant bacteria like *Salmonella* sp. and *E. coli* can be transferred from animals to human indirectly via food (JETACAR, 1999).

Probiotics can be used as an alternative to antibiotics in swine rations though they have different action on the intestinal microorganisms (Patience *et al*., 1995). Antibiotics suppress the growth and multiplication of microorganisms in the gut, whereas probiotics promote live microorganisms in the digestive tract (Patience *et al*., 1995).

The specific mechanisms of probiotic efficiency are yet to be fully clarified but it is accepted that the organic acids produced by probiotics can have a strong antimicrobial effect against intestinal pathogens (Collins *et al*., 2009). Probiotics may also lessen pathogens by enhancement of epithelial barrier function, the antagonism of receptor sites on the host epithelium, production antimicrobial peptides and low molecular weight antimicrobials, competition for nutrients, inhibition of quorum sensing systems and production of organic acids (Collins *et al*., 2009).

Lactic acid bacteria (LAB), in particular *Lactobacillus* sp., are ideal probiotic candidates for use in pigs (Collins *et al*., 2009). *Lactobacillus casei* is a harmless, nonpathogenic microorganism that has been well known for the attributes and properties it possesses that have been found to be beneficial in animal gut, therefore it is usually categorized as a probiotic (Riboulet-Bisson *et al*., 2012). The structure and efficiency of intestinal villi were restored at a much faster rate in pigs administered the probiotic (Budiño *et al*., 2005).

MATERIALS AND METHODS

The procedures in this study were approved by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, University of the Philippines Los Baños (UPLB). A total of 27 pigs weaned at 21 days of age crossbred pigs [Landrace × (Large White × Duroc)] of both sex and of the same age (~30 days) with initial weight of 7 to 10 kilograms were used in the study. Pigs were obtained from a commercial farm in Alfonso, Cavite and were kept at the Experimental Animal Farm of the Veterinary Teaching Hospital- Los Baños Station, randomly distributed and reared in a rough concrete floor pens and were subjected to standard husbandry and management procedures of grower-finisher pigs. Fluorescent light was provided between 6 PM to 6 AM. Pens were washed with water everyday and disinfection was also done. Virkon-s Fro aerial disinfection was done inside the building 2 to 3 times a week and Glutaquat was used to disinfect outside the building once a week. Footbath with disinfectant was also provided. Pig starter diet was given at 31 to 60 days of age while pig grower diet was given from day 61 to 90 days of age. Finisher diet was given until the pigs reached the market weight of 90 kg. Feed and water were provided on *ad libitum* basis.

This was an experimental study that used complete randomized design (CRD)

that randomly assigned 27 pigs into 3 different treatment groups (T1, T2 and T3) with 3 replicates of 3 animals per treatment for a total of 9 pigs per treatment group. There were 9 pens used in this study which were equally divided for the 3 treatment groups. Three pens per treatment group with a stocking density of 3 pigs per pen. The control group (T1) follows the conventional or the recommended farm medication program which includes antibiotic (tylosin and zinc bacitracin) in the feeds and without probiotics in drinking water. Treatment 2 and 3 both consumed non-medicated feeds and probiotics (*Lactobacillus casei* KE-99) in powder form which was mixed in drinking water. Treatment 2 (T2) was given 12 grams of probiotics in non-consecutive days or every other day, while Treatment 3 (T3) was given same amount of probiotics every day.

One container contains 300 g probiotic and each gram contains ~400 million CFU of *Lactobacillus casei* KE-99. Probiotic was given once a day every 6 AM. One scoop (12 g with 4,800 million CFU of *Lactobacillus casei* KE-99) of probiotics was mixed in 2.5 L of water placed in a large plastic basin (size L x W x H cm: 40x40x16 cm) for each pen was monitored until completely consumed. Three pigs in each pen can finished 2.5 L of water with probiotics within 5 minutes. Then, water was given *ad libitum* for the rest of the day.

After 3 months, pigs were slaughtered in Animal Science abattoir, College of Agriculture, University of the Philippines Los Baños. Tissue samples from the middle part of jejunum and cecum that were about 5 cm long were collected. Tissue samples were trimmed to 5 x 5 mm and then fixed in a 10% formalin solution for 72 hr. The sample were then paraffin-embedded, sectioned at 5 µm thickness and stained with hematoxylin-eosin. Tissue samples were cut cross-sectionally. Two slides were prepared per tissue sample, with 3-4 tissue sections for each slide. Only one section per slide will be measured but an extra of 3-4 sections were provided just to make sure to find the perfect section (without artifacts and has all the structures needed). The slides were examined using light microscope (NIKON® Eclipse E-200) equipped with NIS-elements imaging software. Tunica mucosa of jejunum and cecum were measured in micrometer (µm) using a calibrated ocular micrometer under the low power objective (LPO).

The villi, lamina propria and muscularis mucosae of tunica mucosa were observed. Parameters that were measured in the study were the mucosal thickness, crypt depth, villus height, villus width and villus volume of jejunal mucosa, and mucosal thickness and crypt depth of cecal mucosa. Mucosal thickness (µm) was measured from the tip of the villus or the highest point in the tunica mucosa to the inner border of the muscularis mucosa. Villous height (µm) was measured by taking the average of the left, middle and right measurements from the tip of the villi to the villous crypt junction. Villous width (µm) was the average of the measured base, middle and top part of the villi. Villous volume (μm^3) was obtained using the formula: villous height (µm) X villous width (µm) X thickness of the section (5 µm). Crypt depth (µm) was the depth of the invagination between adjacent villi.

Analysis of variance (ANOVA) in a complete randomized design (CRD) and means of standard deviation (SD) were used to analyze the data. Duncan's multiple range test (DMRT) at 95% level of confidence was used in comparing the difference among the treatment means.

RESULTS AND DISCUSSION

The jejunal mucosa (figure 1) in all treatment groups were populated by numerous club-shaped villi and a few filiform-shaped villi. Each villus was lined by simple columnar epithelium composed of tall columnar cells or enterocytes with a few goblet cells. Crypts of Lieberkuhn were lined with columnar absorptive cells and goblet cells. Lamina propria in all three treatment groups was composed of loose connective tissues, blood vessels, lymphocytic infiltrates and has thin muscular layer (muscularis mucosae) that serves as border to tunica submucosa as described by Eroschenko (2000) and Eurell and Frappier (2006).

The mean values (± standard error) of morphological measurements in the jejunum of finishing pigs in three treatment groups are shown in Table 1. Statistical analysis at 95% level of confidence showed no significant differences among treatment groups in all parameters. This indicates that probiotics (*Lactobacillus casei* KE-99) supplementation in drinking water (T2 and T3) did not differ significantly from those antibiotic supplemented pigs (T1). The result agreed with the study by Rekiel *et al.,* (2010) on grower-finisher pigs, sows and piglets wherein probiotic supplementation involving *Lactobacillus acidophilus* and *Bifidobacterium* spp., showed no significant changes in the small intestinal morphology.
The positive effect of probiotics on jejunal mucosa has been reinforced by morphological measurements observed in this study that can be due to prevention of harmful effect of pathogens (Jankowski *et al.,* 1994). Probiotics compete with pathogens for binding to intestinal epithelial cells by competitive exclusion (Fioramonti *et al.,* 2003) thus, prevents damages or cell death in the mucosal lining cause by pathogen. Treatment groups given

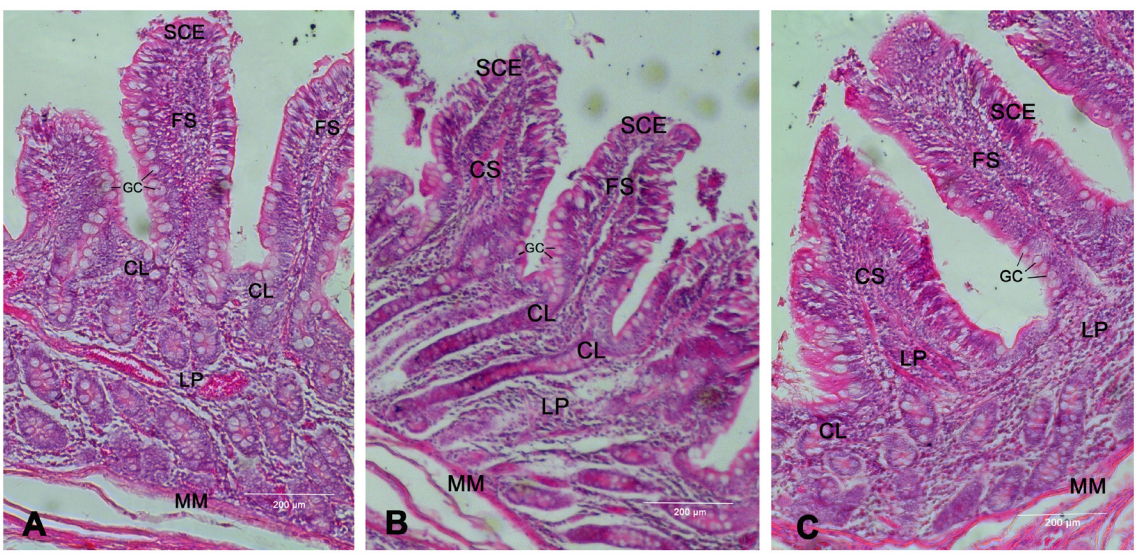

Fig. 1. Histological section of jejunal mucosa of pig in T1 (A), T2 (B) and T3 (C) showing the club-shaped (CS) and filiform-shaped (FS) villi that were lined by simple columnar epithelium (SCE). Crypts of Lieberkuhn (CL) were lined with columnar absorptive cells and goblet cells (GC). Lamina propria (LP) and muscularis mucosae (MM) were also shown. H & E. Bar scale: 200 µm.

Table 1. Mean values (± standard error) of morphological measurements in the jejunum of finishing pigs in three treatment groups.

Parameters (Jejunum)	Treatment			
	T1	T2	T3	P-value
Mucosal Thickness (µm)	19.88 ± 2.64	20.82 ± 3.52	24.24 ± 2.28	0.60
Crypt depth (µm)	4.81 ± 2.67	5.08 ± 4.61	4.49 ± 0.34	0.88
Villus height (µm)	7.29 ± 2.12	7.04 ± 2.53	6.65 ± 4.8	0.78
Villus width (µm)	8.12 ± 2.10	9.26 ± 3.96	10.71 ± 1.65	0.88
Villus volume (μm^3)	209.42 ± 1.23	306.43 ± 3.72	251.21 ± 1.85	0.90

with probiotics (T2 and T3) showed higher morphological measurements compared to group without probiotics (T1), except for villus height and crypt depth. Presence of infection increases the crypt depth due to pathogens that destroy the mucosal lining thus, cause increase in mucosal proliferation that starts in the crypts of Lieberkuhn to replace the dead cells. Highest measurement in crypt depth was shown in T2 may be because of irregular supply of probiotics which gives opportunity for the bad bacteria to inhabit the jejunum resulting in epithelial cell death and increased mucosal proliferation found in crypts. T1 has the highest measurement in villus height because antibiotics in feeds controls the multiplication of bacteria, therefore it also protects the jejunal mucosa against bad bacteria. But unlike antibiotics, probiotics enhance the intestinal epithelial barrier function, prevent apoptosis in intestinal epithelial cells and cytoprotection.

The lamina propria in all treatment groups was composed of loose connective tissue, blood vessels and lymphocytic infiltration. Microscopically, there was no significant difference observed in the structure of the lamina propria or difference in density of lymphocytic infiltrates among treatment groups.

Muscularis mucosae showed no distinct changes in its thickness among treatment groups. There were no reported effects of probiotics in muscularis mucosae but its thickness was correlated to the presence of infection or inflammation (Dunne *et al.*, 2001). Bacteria and enterotoxins signal epithelial secretions that triggers muscularis mucosae contractions. Continuous contractions cause increase in the thickness of the muscularis mucosae (Roselli *et al.*, 2005).

Cecal epithelium (figure 2) in all three treatment groups were lined with simple columnar cells that has abundant goblet cells interspersed with absorptive cells. Intestinal crypts were composed of numerous goblet cells and few columnar cells. Microscopic observation of the lamina propria showed no distinct difference in blood vessels, loose connective tissues and density or distribution of lymphocytic infiltration among treatment groups. Microscopically, there was also no distinct difference observed in the thickness of muscularis mucosae among treatment groups.

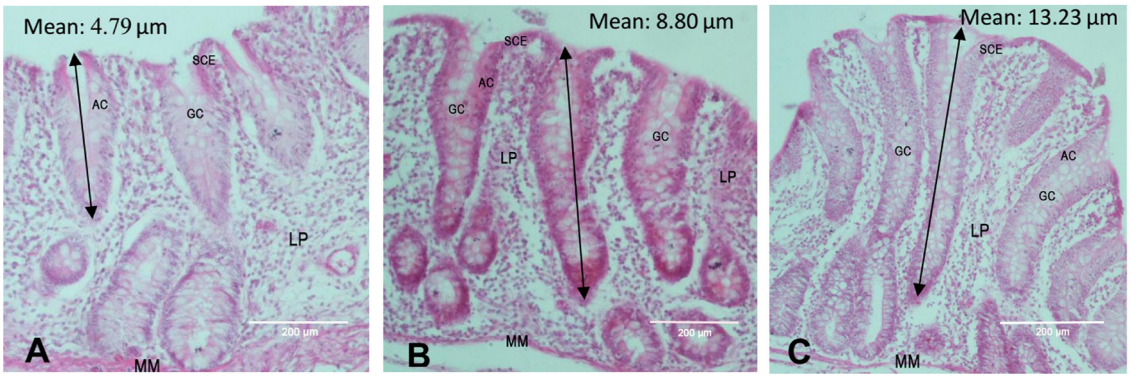

Fig. 2. Histological section of cecal mucosa of pig in T1 (A), T2 (B) and T3 (C) that were lined by simple columnar epithelium (SCE) and have abundant goblet cells (GC) interspersed with absorptive cells (AC). Crypt depth (black double-headed arrow) and the mean values, lamina propria (LP) and muscularis mucosae (MM) were also shown. H & E. Bar scale: 200 µm.

The mean values (± standard error) of morphological measurements in the cecum of finishing pigs in three treatment groups are shown in Table 2. Statistically, mucosal thickness showed no significant difference ($P = 0.43$) among treatment groups at 5% level of significance. Thus, indicates that mucosal thickness of cecum of pigs with probiotics supplement did not differ significantly from those with antibiotic. This histopathological changes was not consistent with the findings of Gargallo and Zimmerman (1980) which showed significant decrease in epithelial cells or smaller mucosal surface due to addition of antibiotic to feeds. For crypt depth, no significant difference between T1 and T2 and between T2 and T3 were observed. However, significant difference ($P = 0.01$) between T1 and T3 was attained. This indicates that probiotics supplementation in drinking water caused significant increased in crypt depth of cecum compared to those group with antibiotic in feeds.

One of the beneficial effects of probiotics on gut function can in part be explained by a trophic action on the colonic mucosa, which was mediated by the short-chain fatty acids produced by microflora during fiber digestion (Jankowski *et al.*, 1994). For pigs, fiber digestion or fermentation mainly happens in the cecum. The mechanism of action of probiotics in the intestine is unclear, however, study about short-chain fatty acids showed how probiotics affect cell division or proliferation of cells. Ichikawa *et al.* (1999)

Table 2. Mean values (± standard error) of morphological measurements in the cecum of finishing pigs in three treatment groups.

Parameters (Cecum)	Treatment			
	T1	T2	T3	*P*-value
Mucosal Thickness (µm)	13.05 ± 2.98 ^{a}	13.59 ± 2.53 ^{a}	15.76 ± 1.27 ^{a}	0.43
Crypt depth (µm)	4.79 ± 0.88 ^{ab}	8.80 ± 3.7 ^{a}	13.23 ± 0.88 ^{ab}	0.01

Means with (^{ab}) superscript within a row are significant from each other at 5% level of significance.

proposed that short-chain fatty acids such as acetate, butyrate and propionate produced by intestinal microflora mediate this effect that are known to stimulate the production of epithelial cells. Therefore, treatment groups given with probiotics (T2 and T3) showed higher measurement of mucosal thickness and crypt depth compared to group without probiotics (T1). T3 has the highest measurement for mucosal thickness and crypt depth, while T1 showed the lowest for both.

CONCLUSION

This study was conducted to determine the effect of *Lactobacillus casei* KE-99 based probiotics added in drinking water on the morphology of jejunal and cecal mucosa of pigs. Based on the statistical data, the results of the study showed that the various morphometric observations on the tunica mucosa of pigs given probiotics supplementation in drinking water (T2 and T3) did not differ significantly from those antibiotic supplemented pigs (T1), except for the measurement of crypt depth in cecum between T1 and T3. Nevertheless, the result showed differences in morphometric measurements of jejunal and cecal mucosa for all the three treatment groups. Those given with antibiotics (T1) showed the lowest morphometric measurements in jejunal and cecal mucosa, except in jejunal villus height. Antibiotics kill the pathogens that damages the intestinal cells, thus it helps to maintain the height of the villi. In comparison to probiotics treated groups (T2 and T3) which showed the highest measurement in morphometric parameters both in jejunal and cecal mucosa because aside from protection of the gut barrier from pathogens, probiotics also have beneficial effects on the intestinal cells like trophic action and stimulation of mucosal cell proliferation.

Further study on the effect of probiotics supplementation on the morphology of other intestinal segment of pig using morphological, histochemical and immunohistochemical examination is highly recommended. Different segments may have different results compared to the present study. Histochemical and immunohistochemical examination of intestine revealed effects, actions, localization and interaction of probiotics to the intestinal mucosa. Also, histochemical and immunohistochemical examination will be more helpful in differentiating or comparing the cellular level of change and action in lamina propria and lining epithelium caused by probiotics (Anwar *et al.*, 2012).

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