The effects of single lactic acid bacteria probiotic supplementation on intestinal mucosa profile and immune response in broilers

Bambang Ariyadi and Sri Harimurti

Faculty of Animal Science Gadjah Mada University Jl.Fauna 3 Bulaksumur Yogyakarta Indonesia 55281

ABSTRACT: The aim of this study was to examine the effects of single lactic acid bacteria probiotic supplementation on intestinal mucosa profile and immune response in broiler chicks. In randomized design, sixty 7-days-old broiler chicks were assigned to 4 treatments with 3 replicates and 5 chicks in experimental unit with water and allow to feed *ad libitum*. The experimental treatments were added to basal (starter and finisher) diets (without added antibiotics, coccidiostats or growth promoter) as follow: T(0): control group (C) that received starter and finisher diets, T(1): C added daily *Lactobacillus murinus* for 21 days, T(2): C added daily *Streptococcus thermophilus* for 21 days and T(3): C added daily *Pediococcus acidilactiti* for 21 days. All chicks were given the commercial Avian influenza and Newcastle disease vaccines, the former was given intra muscularly and the latter was given ocularly. Result showed that additive on all single lactic acid bacteria (*Lactobacillus murinus, Streptococcus thermophilus,* and *Pediococcus acidilactiti*) significantly increased villi high of duodenum, jejunum, and ileum ($P<0.05$) as well as villi width of duodenum, jejunum, and ileum ($P<0.05$) compared with the control group. Additives all of the single lactic acid bacteria significantly ($P<0.05$) increased blood Avian influenza antibody titer compared with the control group. Result indicated that, supplementation of single lactic acid bacteria had positive effect on intestinal mucosa profile and blood antibody titers.

Key words: broilers, probiotics, intestinal mucosa Profile, immune response

INTRODUCTION

There are two populations of microorganisms that are found within the gastrointestinal tract of poultry. The first, the *autochthonous* bacteria, colonize the gut by inoculation resulting from environmental exposure and normal feeding activities of the bird (Gusils *et al*., 1999). The second, *allochthonous* bacteria, are exogenous in nature and are introduce as a dietary supplement into the gastrointestinal tract through the feed or drinking water as direct fed microbial or probiotics (Petterson and Burkholder, 2003). They may contain only one, or several (a consortium) different bacterial species. The mechanisms of action of different bacterial strains in a probiotic consortium may differ (Bomba *et al*., 2002).

The intestinal micro flora of an animal is the first barrier in protecting the host from disease caused by colonization of pathogens in the gastrointestinal tract. Probiotics, defined as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989) or "a live microbial feed that is beneficial to health" (Fooks and Gibson, 2002), have been administered to farm animals to enhance production performance and immune responses. Probiotics are as a source of live micro-organism that includes bacteria, fungi and yeasts (Miles and Bootwalla, 1991). Lactic acid bacteria such as *Lactobacilli streptococci* and *Bifidobacteria* are the most common organisms used in probiotics preparations. The mechanism of action of probiotics has not been fully explained although there are several hypotheses (Ahmad, 2006). Its inhibitory action against pathogens may be mediated by competition for receptors on the gut mucosa, competition for nutrients, the production of antibacterial substances and the stimulation of immunity (Bal *et al*., 2004).

As feed additive, probiotics has a good impact on the poultry performance (Stavric and Kornegay, 1995). These live organisms after residing intestinal tract and their metabolites can act as immunomodulatory agent by activating specific and non-specific host immune responses in chicks, which in turn help in prevention and control of various infectious diseases (Koenen *et al*., 2004). The

most important advantage of probiotic is that doesn't have any residues in animal production and in contrast to antibiotics which could have serious consequences such as drug resistance and harmful alternation of bacterial population in the intestine (Abe *et al*., 1995), probiotics are not made any resistance by consumption. Therefore, some researchers have replaced antibiotics with probiotics as therapeutic and growth promoting agent (Martins *et al*., 2005). Probiotics and organic acids are the most promising alternative to antibiotics. Probiotics are viable microbial additives which assist in the establishment of an intestinal population which is beneficial to the animal and antagonistic to harmful microbes (Green and Sainsbury, 2001).

Successful probiotic colonization depends on the survival and stability of the probiotic strain, specificity of the strain relative to host, dose and frequency of administration, health and nutritional status of the host, effect of age, stress and genetics of the host (Mason *et al*., 2005). The crop, proventriculus and gizzard have very low anaerobic bacteria numbers due to the presence of the oxygen consumed with the feed as well as the low luminal pH, primarily associated with the hydrochloric acid within the proventriculus (Rastall, 2004). The small intestine has large bacterial numbers consisting of facultative anaerobes such as *Lactobacilli, Streptococci* and *Enterobacteria* as well as anaerobes such as *Bifidobacterium* spp., *Bacteroides* spp. and *Clostridia* spp. at levels ranging from 10^4 to 10^8 CFU/ml (Gaskins, 2003). The most heavily colonized regions of the gastrointestinal tract are the colon and cecum with colonization of 10^{10} to 10^{13} CFU/ml (Heczko *et al*., 2000).

Probiotic bacteria colonize three different areas within the gastrointestinal tract, the enterocyte surface, the cecal epithelia surface and the colonic epithelia surfaces (Yamauchi and Snel, 2000). Each of these areas generally includes three microenvironment components. The digesta, the surface of the enterocytes and the cecum and colon and the mucous blanked covering the epithelial surface as well as the epithelial cells of the cecum and colon (Chichlowski *et al*., 2007). The digesta, which is created by the consumption of a rich milieu of feed nutrients and water, is an ideal environmental niche within which many bacterial species flourish. Probiotic bacteria can be found attached to individual feed particles such as starch granules. Other bacteria are not associated with the feed particles, but simply exist within the aqueous matrix of the digesta. The second microenvironment of the gastrointestinal tract where microbes are found is within the mucous blanked that covers the epithelial lining of the gastrointestinal tract including the intestinal villi and cecal and colonic surfaces. The mucous not only serves as an environment within which these microbes exist, but also serves as a source of nutrients for bacteria (Jacobsen *et al*., 1999). Finally, bacteria can also exist associated with or attached to the surface of apical plasmalemma of the epithelial cells lining these areas (Marteau *et al*., 2004). The functional relationship of bacteria associated with the three gastrointestinal micro environment described above and its biological significance has not been established (Kankaanpaa *et al*., 2004). The ability of many strains of probiotic bacteria to physically adhere to portions of the gastrointestinal micro environments may speak to their ability to effect changes in enteric health (Sarem-Damerdji *et al*., 1995).

The dietary supplementation of probiotic benefit the host animal by stimulate the immune system (Koenen *et al*., 2004) and have beneficial effect on the health of the host (Soomro *et al*., 2002). The strain of selected microorganisms in probiotics, method of preparation, the dosage and condition of animals could be partially responsible for such description (Huang *et al*., 2004). The intestinal epithelial layer constitutes a barrier that protects the host against luminal pathogens (Deitch *et al*., 1995). Reduced epithelial cell proliferation and mucosal atrophy of the intestine allow various pathogens in the intestinal lumen to invade. Feed additives such as probiotic, antibiotic or organic acids can help intestinal tissue, since supplementation of their to diets decrease pathogens (Gunal *et al*., 2006). This study was conducted to investigate the effects of single lactic acid bacteria probiotic supplementation on intestinal mucosa profile and immune response in broilers.

MATERIALS AND METHODS

Experimental Materials

A total of sixty 3-days-old broiler chicks (Lohman strain) vaccinated with Newcastle Disease were obtained from the local market. Birds were raised in the cages of slat type in order to avoid

contamination between feces and birds. All birds were fed a standard diet as recommendation of NRC (1994) based on corn and soybean meal (without antibiotics, coccidiostats or growth promoter). A composition of basal diets consisted of crude protein of 22.81%, metabolizable energy (ME) of 3053.45 kkal/kg, Calcium of 0.26%, Phosphor of 0.21%, Lysine of 1.23%, Methionine of 0.52% and Tryptophan of 0.24%. Probiotics used in this study were a pure culture of *Lactobacillus muricus acidilactici* isolated from cecum of the Indonesian village chickens (Harimurti, *et al*., 2007).

Experimental Design

Chicks were individually weighed and given a wing web then randomly assigned into four treatments which each treatment consist of fifteen chicks. The treatments were: T(0): control group (C) that received starter and finisher diets, T(1): C added daily *Lactobacillus murinus* per oral for 21 days, T(2): C added daily *Streptococcus thermophilus* per oral for 21 days and T(3): C added daily *Pediococcus acidilactiti* per oral for 21 days. The experimental design is shown in Table1. Probiotic concentration used in this study was 10^8 CFU/ml. Probiotic supplementation was individually administered per oral for 21 days.

Table 1. Experimental design

Treatment	n	Additive	Concentration
Control (T0)	15	none	none
T(1)	15	added daily *Lactobacillus murinus* per oral for 21 days	10^8 CFU/ml
T(2)	15	added daily *Streptococcus thermophilus* per oral for 21 days	10^8 CFU/ml
T(3)	15	added daily *Pediococcus acidilactiti* per oral for 21 days	10^8 CFU/ml

Feed and water were provided *ad-libitum* until the chick's age was 35 days. On the end of experiment, 5 randomly chicks from each treatment were killed to collect the histological tissue. Small intestine (duodenum, jejunum and ileum) were collected for histological examination including length and width of duodenum, jejunum and ileum.

Antibody Titers

All chicks were administered Avian Influenza vaccination at day 30 and Newcastle Disease vaccination at day 15. A commercial killed vaccines of Avian Influenza was given intramuscularly and a commercial live vaccines Newcastle disease was given intraocular. One cc of blood from each chick was collected from brachial vein to examine blood titer antibody against vaccination. Collection of blood was conducted 2 weeks after vaccination, 5 randomly chicks from each treatment were bled using 3 cc spuit. One cc of blood collected from brachial vein was placed into the Effendorf's tubes, serum obtained from this blood was immediately sent to laboratory for further examination. All serum samples were tested using hemaglutinin inhibition (HI) test (for titer antibody of Newcastle Disease vaccination) according to Xu *et al*., (1997) and indirect antibody enzyme-linked immunosorbent assay (ELISA) kit (for titer antibody of Avian Influenza vaccination) according to the manufacturer's instruction (Looraine and Clarke, 1982).

Histological Preparation

The intestinal tract was removed immediately after killing and severed from the gizzard. Small intestine prepared for histology was duodenum, jejunum and ileum. Small sections of duodenum were taken from the proximal side of the duodenal loop. Jejunum was defined as midway between the end of duodenum and Meckel's diverticulum. Ileum was defined as extending from Meckel's diverticulum to a point 4 cm to distal. For histological analysis, 2-cm tissue samples from the duodenum, jejunum and ileum were obtained and fixed in 10 % buffered formalin (100 mL of 40 % formaldehyde, 4 g phosphate, 6.5 g dibasic sodium phosphate and 900 mL of distilled water) for 24-48 h. Tissues were dehydrated by transferring through a series of alcohols with increasing concentrations, placed into xylol and embedded in paraffin. A microtome was used to make 5 cuts that were 5 µm. The cuts were

stained with hematoxylin-eosin. The values were measured using a light microscope (Olympus BX 51 attached with Olympus DP 12 projector). Measurements of villus height and villus width were determined at magnification of 10X. A minimum 3 measurements per slide were made for each parameter and averaged into one value.

Statistical Analyses

The results were evaluated using SPSS® (1999) program. Statistical differences among treatments means were separated using the Duncan's Multiple Range Test with a percentage 5 probability (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of single lactic acid bacteria probiotic supplementation on some intestinal mucosa parameters is available at Table 2.

Table 2. The effects of a *Lactobacillus murinus* (T1), *Streptococcus thermophillus* (T2), *Pediococcus acidilactici* (T3) probiotic supplementation on some intestinal mucosa parameters, (n=15).

		Single Probiotic Supplementation		
Criteria	Control (T0)	T1	T2	T3
Duodenum				
Villus height, µm	497.33[a]	627.67[b]	697.00[c]	708.00[c]
Villus width, µm	73.33[a]	105.33[c]	115.67[c]	111.00[c]
Jejunum				
Villus height, µm	558.33[a]	786.00[c]	766.67[c]	789.00[c]
Villus width, µm	75.33[a]	103.33[b]	142.00[c]	119.67[b]
Ileum				
Villus height, µm	516.67[a]	694.67[b]	750.00[c]	730.67[b,c]
Villus width, µm	69.67[a]	142.67[c]	124.33[b]	126.67[b]

[a – c] Different superscript on the same row differ significantly (P< 0.05).

Additives all of the single lactic acid bacteria (*Lactobacillus murinus, Streptococcus thermophilus,* and *Pediococcus acidilactiti*) significantly increased villi height of duodenum, jejunum, and ileum (P<0.05) as well as villi width of duodenum, jejunum, and ileum (P<0.05) compared with the control group. A similar result was reported by Gunal *et al*., (2006). They reported that villus height in jejunum and ileum significantly increased in 21-day and 42-day old chick fed probiotics. Samanya and Yamauchi (2002) also reported that villus height in duodenum and ileum significantly increased in 28-day old chick fed *Bacillus subtilis.* Santin *et al.* (2001) recorded that fed *Saccharomyces cerevisiae* were higher villus height than that of control group during the first 7th day in broilers. These results were most probably due to enhanced short chain fatty acids formation induced by probiotics. It has been reported that under in vitro, probiotics increased the levels of the short chain fatty acids while decreasing the production of ammonium (Sakata *et al*., 1999). The short chain fatty acids which are by product of bacterial fermentation stimulate the proliferation of epithelial cells of the bowel Ichikawa *et al*., 1999). Sakata *et al.* (1999) obtained that *Lactobacillus casei* increased the crypt cell production rate of the ileum by 40% in rats. Moreover, the short chain fatty acids produced by fermentation process of probiotic bacteria strain have a role to stimulate in proliferation of intestinal epithelia cells. It can be known that the short chain fatty acids were component of epithelial membrane phospholipids. In the fermentation of homo fermentative lactic acid bacteria, the piruvat will not be changed entirely to be the acid lactic. A part of piruvat will come into the dehydrogenization produced acetyl-coA and then will go through the biochemistry reactions to become the short chain fatty acids (Greulach, 1976; Atlas, 1996).

Effects of single lactic acid bacteria probiotic supplementation on blood antibody titers are available at Table 3.

Blood antibody titers of Newcastle Diseases were not affected by treatments (P>0.005). However, additives all of the single lactic acid bacteria (*Lactobacillus murinus, Streptococcus thermophilus,* and *Pediococcus acidilactiti*) significantly increased blood antibody titers of Avian Influenza (P<0.05). The positive effect of feeding probiotics on immune response is in agreement with the finding of Huang *et al*., 2004, Koenen *et al*., 2004, and Rowghani *et al*., 2007. Probiotics after residing intestinal tract and their metabolites can act as immunomodulatory agent by activating specific and non-specific host immune responses in chicks, which in turn help in prevention and control of various infectious diseases (Koenen *et al*., 2004).

Table 3. The effects of a *Lactobacillus murinus* (T1), *Streptococcus thermophillus* (T2), *Pediococcus acidilactici* (T3) probiotic supplementation on blood antibody titers, (n=15)

Antibody titers	Control (T0)	Probiotic Supplementation		
		T1	T2	T3
Avian Influenza	0.6067^{a}	0.7933^{b}	0.7933^{b}	0.7000^{b}
Newcastle Disease	16.00^{ns}	32.00^{ns}	42.67^{ns}	58.67^{ns}

^{a,b,}Different superscript on the same row differ significantly (P< 0.05).
^{ns} Non significant.

CONCLUSIONS

The results of the present study showed that additives all single lactic acid bacteria (*Lactobacillus murinus, Streptococcus thermophilus,* and *Pediococcus acidilactiti*) increased villi height of duodenum, jejunum, and ileum as well as villi width of duodenum, jejunum, and ileum and also increased blood antibody titers.

LITERATURE CITED

Abe F., N. Ishibashi and I. Shimamura, 1995. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy sci., 78: 2838-2846.

Ahmad, I. 2006. Effect of probiotics on broiler performance. International Journal of Poultry Science 5: 593 - 597.

Atlas, R.M., 1996. Principles of Microbiology. Wm. C. Brown Publishers. London. Second Edition.

Bomba, A. R. Nemcova, S. Gancarcikova, R. Herich, P. Guba and D. Mudronova, 2002. Improvement of the probiotic effect of microorganisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. Br. J. Nutr., Suppl. 1: S95-99.

Chichlowski, M., J. Croom, B.W. Mcbride, G.B. Havenstein and M.D. Koci, 2007. Metabolic and physiological impact of probiotics on poultry: A brief review of current knowledge. International Journal of Poultry Science 6 (10): 694-704.

Deitch, E., D. Xu, M.Naruhn, D. deitch, Q. Lu, and A. Marino, 1995. Elemental diet and IV-TPN-induced bacterial translocation is associated with loss of intestinal mucosa barrier function against bacteria. Ann. Surg., 221: 299-307.

Duncan, D.B., 1955. Multiple range test and multiple F tests. Biometrics, 11: 1-42.

Fooks, L. and G. Gibson, 2002. Probiotics as modulators of the gut flora. Br. J. Nutr., 88: S39-S49.

Fuller, R. 1989. probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378.

Gaskins, H., 2003. The commensal microbiota and developments of enterocytes defense in the mammalian intestine. 9th Int. Symp. Dig. Physiol. Pigs, 1: 57-71.

Green, A.A. and D.W.B. Sainsbury, 2001. The role of probiotic in producing quality poultry products. XV European Symposium on the Quality of Poultry Meat. September 2001. Kusadasi, Turkey, 245-251.

Greulach, V.A., 1976. Plant Function and Structure. Collier Macmillan Publishers. London.

Gunal, M., Yayli, G., Kaya, O., Karahan, N. and Sulak, O., 2006. The effect of antibiotics growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of Broilers. International Journal of Poultry Science 5: 149-155

Gusils, C. S. Gonzalez and G. Oliver, 1999. Some probiotic properties of chicken lactobacilli. Can. J. Microbiol., 45: 981-987.

Harimurti, S., Rahayu, E.S., Nasroedin and Kurniasih, 2007. Lactic Acid Bacteria from chicken intestines as probiotics agents (*Bakteri asam laktat dari usus ayam sebagai agensia probiotik, Jurnal Produksi Ternak*) 9: 82-91. (In Indonesian with abstract in English).

Heczko, U., A. Abe and B.B. Finlay, 2000. Segmented filamentous bacteria prevent colonization of enteropathogenic Escherichia coli O103 in rabbits. J. Infect. Dis. 181: 1027-1033.

Huang, M.K., Choi Y.J., Houde, R., Lee, J.W., Lee, B. and Zhao, X., 2004. Effect of Lactobacilli and an Acidophilic Fungus on the production performance and immune responses in broiler chickens. Journal of Poultry Science 83: 788-795.

Ichikawa, H., T. Kuroiwa, A. Inagaki, R. Shineha, T. Nishihira, S. Satomi and T. Sakata, 1999. Probiotic bacteria stimulate gut epithelial cell proliferation in rat. Digestive Diseases and Sci., 44: 2119-2123.

Jacobsen, C.N., V.R. Nielsen, A.E. hayford, P.L. Moller, K.F. Michaelsen, A. Paerregaad, B. Sandstrom, M. Tvede and M. Jacobsen, 1999. Screening of probiotic activities of forty-seven strains of Lactobacillus spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Appl. Environ. Microbiol., 65: 4949-4956.

Kankaanpaa, P., B. Yang, H. Kalilo, E. Isolauri and S. Salminen, 2004. Effects of polyunsaturated fatty acids in growth medium on lipid composition and on physicochemical surface properties of Lactobacilli. Appl. Environ. Microbiol., 70: 129-136.

Koenen, M.E., J. Karmer, R. Van der Hulst, L. Heres, S.H. Jeurissen and W.J. Boersma. 2004. Immunomodulation by probiotic lactobacilli in layer and meat-type chickens. Br. Poult. Sci., 45: 355-366.

Looraine, M. and R.T.J. Clarke, 1982. Clinical microbiology procedures. Hand book. Volume 2. Section 8: Viruses, Rickettsiae, Chlamydiae and Mycoplasma. American Society of Microbiology, Washington, D.C. USA.

Marteau, P., P. Seksik, P. Lepage and J.Dore, 2004. Cellular and physiological effects of probiotics and prebiotics. Mini-reviews in Medical Chemistry, 4: 889-896.

Martins, F.S., R.M. Nardi. R.M. Arantes, C.A. Rosa, M.J. Neves and J.R. Nicoli. 2005. Screening of yeasts as probiotic based on capacities to colonize the gastrointestinal tract and to protect against enteropathogen challenge in mice. J. Gen. Appl. Microbiol., 51: 83-92.

Mason, C.K. M.A. Collins and K. Thompson, 2005. Modified electroporation protocol for Lactobacilli isolated from the chicken crop facilitates transformation and the use of genetic tool. J. Microbiol. Methods, 60: 353-363.

Miles, R,D, Butcher, G.D., Henry, P.R. and Littell, R.C., 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. Journal of Poultry Science 85: 476- 485.

NRC (National Research Council), 1994. Nutrient Requirements of Poullry. National Academy Press, Washington, D. C., USA.

Petterson, J.A. and K. Burkholder, 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82: 627-631.

Rastall, R., 2004. Bacteria in the gut: friends and foes and how to alter the balance. J. Nutr., 134: 2022S-2026S.

Rowghani, E. Arab, N. and Akbarian, A. 2007. Effects of a probiotic and other feed additives on performance and immune response of broiler chicks. International Journal of poultry science 6 (4): 261-265.

Sakata, T., T. Kojima, M. Fujieda, M. Miyakozawa, M. Takahashi and K. Ushida, 1999. Probiotic preparation dose-dependenly increase net production rates of organic acids and decrease that of ammonia by pig caecal bacteria in batch culture. Digestive Diseases and Sci., 44: 1485-1493.

Santin, E., A. Maiorka, M. Macari, M. Grecco, J.C. Sanchez, T.M. Okada and A.M. Myasaka, 2001. Performance and intestinal mucosa development of broiler chickens fed diets containing Saccharomyces cerevisiae cell wall. J. Appl. Poult. Res., 10: 236-244.

Sarem-Damerdji, L., F. Sarem, L. Marchal and J.P. Nicolas,1995. In vitro colonization ability of human colon enterocytes by exogenous Lactobacillus strains. FEMS Microbiol. Lett., 131: 133-137.

Soomro, A.H., T. Masud and H.A. Rathore, 2002. Application of probiotic culture. J. Am. Vet. Adv., 1: 4-42.

SPSS® 10.0 Computer Software, 1999. SPSS Inc., Headquarters, 233 p., Wacker Drive, Chicago, Illinois. 60606, USA.

Stavric, S and E.T. Kornegay, 1995. Microbial probiotic for pigs and poultry biotechnology in animal feeds and animals feeding. R.J Wallace and A. Cheesen, Eds. V.C.H., Weinheim, Germany, pp: 205-231.

Xu, H., J. Lohr and M. Greiner, 1997. The selection of Elisa cut-off points for testing Ab to ND by two-graph receiver operating characteristics analysis. J. Immunol. Methods, pp: 61-64.

Yamauchi, K. and J. Snel, 2000. Transmission electron microscopic demonstration of phagocytosis and intracellular processing of segmented filamentous bacteria by intestinal epithelial cells of the chick ileum. Infect. Immun., 68: 6496-6504.