Adhesion activity assay of *Lactobacillus plantarum* AKK30 combined with oligosaccharides

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Abstract. This objective of study was to measure the activity of lactic acid bacteria (LAB) adhesions in the intestinal mucosa. Those of lactic acid bacteria used were *L. plantarum* AKK30 and were combined either with inulin oligosaccharides (OS) or mannan oligosaccharides (MOS). The oligosaccharides concentration used in this experiment were, 0%, 0.5%, 1%, 1.5% and 2%, respectively. Data collected were total plate count (TPC) before and after adhesion assay. The microstructure of *L. plantarum* AKK30 was observed by scanning electron microscope (SEM). The TPC results of the combination of *L. plantarum* and inulin (OS) were 18 x 10^7 (0%), 48 x 10^7 (0.5%), 109 x 10^7 (1.0%), 129 x 10^7 (1.5%), and 99 x 10^7 (2.0%), respectively, while the combination of *L. plantarum* and mannan were 18 x 10^7 (0%), 53 x 10^7 (0.5%), 125 x 10^7 (1.0%), 210 x 10^7 (1.5%), and 66 x 10^7 (2.0%), respectively. The highest growth average of LAB were shown by the combination *L. plantarum* and 1.5% of MOS. In addition, the TPC results of assay adhesion showed that the combination of *L. plantarum* and inulin oligosaccharides were: 3.89 x 10^8 (0.5%), 4.39 x 10^8 (1.0%), 3.19 x 10^8 (1.5%), and 5.09 x 10^8 (2.0%), respectively, whereas the combination of *L. plantarum* and mannan were: 7.29 x 10^8 (0.5%), 15.49 x 10^8 (1.0%), 2.89 x 10^8 (1.5%), and 4.49 x 10^8 (2.0%), respectively. There were significant differences in the TPC results of assay adhesion among the treatment groups and control (1.16 x 10^8). The highest activity of LAB adhesion was shown by the combination of *L. plantarum* and 1% of mannan. The TPC result was supported by the SEM observation. The combination of *L. plantarum* with oligosaccharides affects the growth and the adhesion length of lactic acid bacteria in the intestine. Further, the optimum concentration was shown by 1.0% of mannann combined with *L. plantarum.*

1. Introduction
Lactic acid bacteria live and develop in the small intestine in chickens that work by supplying protease and amylase enzymes to help digestion [1]. Besides lactic acid bacteria can hydrolyze glucose to lactic acid and volatile fatty acids also produces bacteriocin pathogenic anti-microbes which can eliminate pathogenic bacteria. The antimicrobial activity of Lactobacillus is mainly caused by the production of lactic acid, acetate, formate, caproic, propionate, butyrate and valeric acid [2]. Lactobacillus also produces other inhibitors such as H₂O₂ [3]. 2003) and bacteriocin, a protein compound produced by bacteria that has bactericidal and bacteriostatic activity [4] and then Ammor et al. [5] added that
bacteriocin is a low molecular weight thermostable compound and has the ability to inhibit Gram positive or Gram negative bacteria and has a therapeutic effect.

*Lactobacillus plantarum* is one of the types of lactic acid bacteria that has the characteristics of a straight rod shape with a width range 0.9 - 1.2 μm and a length of 3 μm, single size or form a short chain, and is a gram-positive [6]. *Lactobacillus plantarum* colonies are white or yellow, and some strains are motile. This bacterial colony in the media has characteristics of round, smooth, solid, white, sometimes bright or dark yellow, 3 mm in diameter, facultative anaerobic. These bacteria can grow at a temperature of 15°C in general and cannot grow at a temperature of 45°C, with optimal temperatures ranging from 30-35°C [7]. According to Fraizer and Dennis [8], *Lactobacillus plantarum* is rod-shaped and immovable (non-motile), this bacterium has negative catalase, aerobic or facultative anaerobic properties, is able to melt gelatin, digest protein quickly, does not reduce nitrate, is acid tolerant, and is capable of produce lactic acid.

Oligosaccharides are one of the prebiotics that has various types [9]. Oligosaccharides are referred to as prebiotics because they are not degraded by endogenous enzymes produced by the host, so they are not digested and not absorbed [10]. For the *Lactobacillus plantarum*, adhesion test can use ingredients that are "non-digestible oligosaccharide" including mannan oligosaccharides (MOS) and inulin. Mannan and inulin play a role in improving health by modifying the balance of the intestinal microflora [11] and selectively stimulating the growth of beneficial bacteria such as lactic acid bacteria[12]. These specific carbohydrates serve as food for beneficial bacteria [13]. Mannan in feed is used as a prebiotic substitute for antibiotics because it can reduce mortality and increase antibodies [14].

MOS supplemented into poultry feed gives some influence. These effects include increasing production, in this case, body weight gain (UN) and feed conversion due to the utilization of nutrients in the gastrointestinal tract [15]. This effect is in line with the use of MOS as reported by Shashidhara [16] which explains that the use of MOS increases the antibody titer to IBD in boiler breeding chickens.

This study aims to measure the activity of lactic acid bacteria adhesions in the intestinal mucosa. The lactic acid bacteria used were *L. plantarum* AKK 30 which was combined with oligosaccharides (OS) inulin and mannan oligosaccharides (MOS) and with different levels of OS administration.

2. Materials and Methods

2.1. Preparation of suspension of bacterial isolates and chicken intestines
Preparation of *L. plantarum* + inulin and *L. plantarum* + MOS suspensions in MRSB incubated for 24 hours at 37 °C. Biomass cells were harvested by centrifugation at a speed of 5000 rpm for 15 minutes. Pellets which had been separated from the supernatant were washed with PBS 2 times then suspended in the same solution (OD culture suspension around 0.9 to 1.0). The suspension is used as an inoculum. 30-day old chicken intestine is taken 10 cm from the duodenum. The small intestine is cut longitudinally to facilitate cleaning of dirt and put into sterile Petri dish then washed with PBS solution 3 times and cut into 1 cm² piece. The intestine is used as a binding medium for bacteria.

2.2 Analysis of the number of initial bacteria on the intestinal surface
Intestinal pieces are inserted into a test tube containing 10 ml of 0.85% NaCl and vortexed at high speed for 60-90 seconds to release bacteria that attached to the intestine. Suspension of intestinal bacteria was diluted in 0.85% NaCl solution, and serial dilution was performed (10⁻¹-10⁻⁴). The result of dilution was plated in MRSB, and after 24 h of incubation the colony number was calculated using the Total Plate Count (TPC) method. The TPC enumeration results in the number of initial bacteria in the intestine (control).
2.3 Calculation of the total number of bacteria on the intestinal surface
The attachment test was done by incubating the intestinal sample into a petri dish containing a solution of bacterial suspension in PBS at 37 °C for 60 minutes then rinsed with PBS. The rinsed intestine is then put into 10 ml of physiological solution and distorted at high speed for 60-90 seconds. The bacterial suspension obtained was then diluted in stages (10^-1-10^-7) and the results of dilution of the plate in MRSA were carried out by calculating the colony using the Total Plate Count (TPC) method. The number of test bacteria attached to the intestine is calculated as the difference in the number of bacteria in the control and the amount in the sample.

2.4 Qualitative analysis of adhesion with SEM
LAB colonies in each treatment and control were taken using an ose needle and placed on the carbon tape and coated using ion sputter with a 10 mA setting for 60 seconds. Coated samples were included in batches for SEM analysis. The images taken are magnification of 5,000, 10,000 and 20,000 x

3. Results and Discussion
The TPC results of the combination of L. plantarum and inulin (OS) were 18 x 10^7(0%), 48 x 10^7 (0.5%), 109 x 10^7 (1.0%), 129 x 10^7 (1.5%), and 99 x 10^7 (2.0%), respectively, while the combination of L. plantarum and mannan were 18 x 10^7 (0%), 53 x 10^7 (0.5%), 125 x 10^7 (1.0%), 210 x 10^7 (1.5%), and 66 x 10^7 (2.0%), respectively. The highest growth average of LAB were shown by the combination L. plantarum and 1.5% of MOS. In addition, the TPC results of assay adhesion showed that the combination of L. plantarum and inulin oligosaccharides were: 3.89 x 10^8 (0.5%), 4.39 x 10^8 (1.0%), 3.19 x 10^8 (1.5%), and 5.09 x 10^8 (2.0%), respectively, whereas the combination of L. plantarum and mannan were: 7.29 x 10^8 (0.5%), 15.49 x 10^8 (1.0%), 2.89 x 10^8 (1.5%), and 4.49 x 10^8 (2.0%), respectively. There were significant different in the TPC results of assay adhesion among the treatment groups and control (1.16 x 10^8). The highest activity of LAB adhesion was shown by the combination of L. plantarum and 1% of mannan.

| Oligosaccharide | Control chicken intestine without treatment(A) 30 x 10^5 | treatment with chicken intestines (B) (..x 10^7) | B-A (..x 10^8) |
|-----------------|------------------------------------------------------|-----------------------------------------------|---------------|
| I0              | 30 x 10^5                                           | 11.6                                         | 1.157         |
| Ia              | 30 x 10^5                                           | 39                                           | 3.897         |
| Ib              | 30 x 10^5                                           | 44                                           | 4.397         |
| Ic              | 30 x 10^5                                           | 32                                           | 3.197         |
| Id              | 30 x 10^5                                           | 51                                           | 5.097         |
| M0              | 30 x 10^5                                           | 0.3                                          | 0.03          |
| Ma              | 30 x 10^5                                           | 73                                           | 7.297         |
| Mb              | 30 x 10^5                                           | 155                                          | 15.497        |
| Mc              | 30 x 10^5                                           | 29                                           | 2.897         |
| Md              | 30 x 10^5                                           | 45                                           | 4.497         |
Figure 1. Scanning electron microscope (SEM) HITACHI SU 3500, 5K magnification. (A) Colonies of lactic acid bacteria from chicken intestinal mucosa that have not been given treatment (B) LAB-combined L. plantarum + Inulin colonies. (C) LAB-colony colonization of L. plantarum + Mannan oligosaccharides.

The TPC result was supported by the SEM observation. The combination of L. plantarum with oligosaccharides affects the growth and the adhesion length of lactic acid bacteria in the intestine. Further, the optimum concentration was shown by 1.0% of mannan combined with L. plantarum.

The attachment of probiotic bacteria (adhesion) on the surface of the intestinal wall has a beneficial effect on the health and condition of the intestinal microflora [17], attachment of Lactobacillus sp on the surface of the intestinal wall is influenced by the hydrophobicity of cell surfaces and bile salts [18]. Mannan in feed is used as a prebiotic substitute for antibiotics because it can reduce mortality and increase antibodies [17]. Mannan oligosaccharides are capable of binding to gram-negative bacteria, where the bacteria will come out of the intestine so that colonization does not occur [19].

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5. References
[1] Ray R 1996 Fundamental Food Microbiology (New York: CRC Press Boca Raton Inc.)
[2] Corsetti A, Gobetti M, Balestrieri F, Paoletti F, Russi L and Rossi J 1998 Sourdough lactic acid bacteria effects on bread firmness and staling. J. Food Sci. 63 347–351
[3] Ito A, Sato Y, Kudo S, Sato S, Nakajima H and Toba T 2003 The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-borne pathogens Curr. Microbiol. 47 231-236
[4] Ogunbanwo S Sanni A and Onilude A 2003 Influence of cultural conditions on the production of bacteriocins by Lactobacillus brevis OG1 Afr. J. Biotechnol. 2 (7) 179-184
[5] Ammor S, Tauveron G, Dufour E and Chevallier I 2006 Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 1-screening and characterization of the antibacterial compounds Food Control 17 454-461
[6] Salminen S and Wright A V 1998 Lactic Acid Bacteria 2nd Ed (New York: Marcell Dekker Inc.)
[7] Haryati T 2011 Probiotics and Prebiotics as Nonruminansia Feed Feed Livestock Research Center Wartaza 21 3
[8] Frazier W B and Dennis W 1998 Food Microbiology 3rd ed. (New York: McGraw-Hill, Inc.)
[9] Durst L 1996 Inclusion of fructooligosaccharides in broiler diets Archiv. Geflugelkunde 60 160 – 164
[10] Gilliland S E 1986 Role of starter culture bacteria in food preservation in Bacterial Starter Cultures for Food. Gilliland, S.E. (ed.). (Florida: CRC Press, Inc.)

[11] Cumming J H, Macfarlane G T and Englyst H N 2001 Prebiotic digestion and fermentation Am. J. Clin. Nutr. 73 415-420

[12] Crittenden R G and Playne M J 1996 Production, Properties and Applications of Food-grade Oligosaccharides Trends in Food Science and Technology 7 353-361

[13] Patterson J A and Burkholder K M 2003 Application of prebiotics and probiotics in poultry production Poult. Sci. 82 627-631

[14] Murwani R 2008 Antibiotic Replacement Natural Additives (Semarang : Unnes Press)

[15] Ferket P L, Parks C W and Grimes J L 2002 Benefits of dietary antibiotics and mannan oligosaccharides supplementation for poultry Proc. of Poultry state meeting

[16] Shashidhara R G and Devegowda G 2003 Effect of Dietary Mannan Oligosaccharide on Broiler Breeder Production Traits and Immunity Poultry Science Association Inc. 82 1319–1325

[17] Thompson K L and Applegate T J 2006 Feed withdrawal alter small-intestinal morphology and mucus of broilers Poultry Science Association 85 1535–1540.

[18] Muñoz-Provencio D, Llopis M, Antolín M, de Torres I, Guarner F, Pérez-Martínez G and Monedero V 2009 Adhesion properties of Lactobacillus casei strain to resected intestinal fragments and components of the extracellular matrix Arch. Microbiol. 191 153-161

[19] Dimitroglou ADL, Merrifield R, Moate SJ, Davies P, Spring J, Sweetman and Bradley G 2009 Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, Oncorhynchus mykiss (Walbaum) J. Anim. Sci. 87 3226-3234