Molecular diversity of lactic acid bacteria on ileum broiler chicken fed by bran and bran fermentation

Laelatul Baniyah¹, Siti Nur Jannah¹, Isworo Rukmi¹, Sugiharto²

¹Department of Biology, Faculty of Science and Mathematics, Diponegoro University
²Faculty of Agriculture and Animal Husbandry, Diponegoro University

E-mail: Baniyah.2013@gmail.com

Abstract. Lactic Acid Bacteria (LAB) is a digestive tract microflora that have a positive role in poultry health. The number and diversity of LAB in the digestive tract affected by several factors, among them was the kind of feed. The purpose of this research was to know diversity of Lactic Acid Bacteria (LAB) ileum broiler’s after feeding with prebiotic bran and Rhizopus oryzae fermented bran which was added to commercial feed. As much as 15 broilers were used to determine the diversity of LAB. All broilers were fed using commercial feed. The control used commercial feed no addition of bran or fermented bran, and commercial feed with fermented bran and nonfermented bran were as a treatment. To determine the diversity of LAB, T-RFLP method was applied. The Hae III and Msp I were used as restriction enzymes. The number of phylotype, relative abundance, Shannon diversity index (H’), evenness (E), and Dominance (D) were examined. The results indicated that the addition of prebiotic bran on commercial feed showed a higher diversity of lactic acid bacteria on broiler’s ileum, compared with control and addition of Rhizopus oryzae fermented bran. LAB group that dominates in the ileum is Lactobacillus sp. and L. delbruecii subs bulgaricus.

Keywords: Lactic Acid Bacteria, Prebiotics, Bran, T-RFLP

1. Introduction

Lactic acid bacteria (LAB) are part of a healthy microflora in the animal digestive tract [1]. LAB in the gastrointestinal tract have a role to boost immunity, balancing the microflora in the colon and tolerate lactose-containing foods, a maintaining anti-tumor activity, reduce the population of harmful microorganisms, prevent intestinal infections, and possibly prevent cancer [2]. Maintain the intestinal health of broiler chickens. Intestinal health can be improved by giving prebiotics [3].

Prebiotics are defined as feed material that cannot be digested by the human or animal digestive enzymes. That serve to stimulate selectively the growth and activity of beneficial microorganisms in the intestine, such as Bifidobacteria and Lactobacillus, to be beneficial for health [4]. Rice bran is one of the abundance agricultural waste products in Indonesia. The rice bran containing of about 25.3% fiber, 16.5% protein, 21.3% fat, and 49.4% total carbohydrate. it can serve as prebiotics because have oligosaccharide content, such as rafinosa [5]. The fermentation of bran by R. oryzae causes the changes in its physico-chemical composition, making it possible to increase the availability of nutrients in the raw materials [6]. Prebiotic fed bran and R. oryzae fermented bran by to broiler chicken was an effort to maintain the microbial balance of the gut, and increasing the natural defense mechanisms of the host that caused a good effect on the performance and health of the chickens.
Molecular analysis technique whom can be used in the population dynamics study and differences of LAB microbes in the chicken digestive tract, it’s Terminal-Restriction Fragment Length Polymorphism (T-RFLP) [7]. This technique has been effectively used in microbial exploration of complex environments, archae populations, and in natural habitats [8]. Therefore, dynamically require LAB research needs to be done as the development of breeding technology.

2. Materials and Methods

2.1. Sample Preparation
Three samples were the content of broiler’s ileum lumen, from three treatments i.e. commercial feed (P₀), commercial feed with prebiotics bran (P₁), and commercial feed with prebiotics R. oryzae fermented bran (P₂). Samples aseptically taken and keep in eppendorf tube, stored under -20°, until genomic DNA isolation.

2.2. Genomic DNA Isolation
Samples were extracted using the PowerSoilR DNA Isolation Kit in accordance with company instructions (Burbach et.al., 2015). The steps were done as follows, i.e. destruction, RNA and proteins removal, and DNA purification.

2.3. Amplification of 16S gene rRNA
The amplification was conducted using the forward primer 7F (5'-6FAM-AGAGTTGATYMTGGCTCAG-3') labeled and specific reverse primer for lactic acid bacteria-lab SG-0677 (5'CACCGCTACACATGGAG-3') that are not labeled. PCR reaction mixture consisting of 25 μL GoTaq GreenMaster Mix (Promega, USA) 2 μL of each primer (10 pmol), 100 ng of DNA template in a final concentration and destilated water until the end of the 50 μL. PCR conditions was done with an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 40 sec, annealing temperature was 52 °C for 40 seconds and the extension temperature was 72 °C for 1 minute, then the extension end at 72 °C for 7 minutes. Samples were amplified using PCR Thermal Cycler Dice TAKARA R Gradients (TAKARA BIO INC, Japan).

2.4. Electrophoresis of Amplification Results
The PCR products were confirmed by electrophoresis using 1% agarose gel in 1 x TAE buffer 100 ml. Electrophoresis was performed for 30 minutes at 100 volts. After electrophoresis, the DNA was visualized under ultraviolet light in dark spaces and captured the image using Gel Documentation System (Atto Corporation, Japan).

2.5. DNA Purification of Amplification Results
PCR products were purified by GeneJet TM PCR purification kit (Fermentas, USA) in accordance with the instructions of the company. DNA obtained from purification added 40 μL elution buffer and stored at -20 °C until subsequent use.

2.6. Restriction of Purification Products with Restriction Enzymes
DNA cutting were used HaeIII and MspI (Fermentas, USA) restriction enzyme. Reaction consisted of 15 units restriction enzyme (1.5 mL), 10 x restriction buffer (2 mL, R buffer for HaeIII and Tango buffer for MspI) and <100 ng of DNA samples (10 mL), Nuclease-free water were add until the final volume of 20 μL. Samples were then incubated at 37 °C for 16 hours, disabling at 80 ° C for 20 minutes, and immediately cooled in ice bath according to company instructions. The restriction products were precipitated in ethanol and dried.

2.7. T-RFLP analysis
The analysis of PCR fragments products was performed on fragment analysis services 1st base (http: www.base-asia.com/fragment_analysis/). The reaction conditions used to find out the length of the
PCR fragments product labeled with the fluorescence material was a sample of cutting DNA with a purified restriction enzyme (1 μL) plus a mixture of 10 ml of formamide and internal standard (GeneScan-500 ROX, Applied Biosystems) 5, v/v). Pieces T-RF labeled analysed by electrophoresis using automatic sequence analyzer (ABI PRISM 3100. Applied Biosystems) on gene Scan mode, and the length of the T-RF were determined by comparing a standard size using the Peak ScannerTM v1.0 software (Applied Biosystems).

2.8. Data processing
The T-RF values > 50 bp and the percentage of peak area > 1% were used for further analysis. The diversity of bacteria was determined by the richness and abundance of phylotypes from the bacterial community. The value of biodiversity was calculated based on the number of phylotype (S), relative abundance (%) were calculated with divided the peak area by the total of the entire size of T-RF peak area and multiplied by 100%. Shannon-Wiener (H’) index, Pielous Evenness Index (E), and Dominance Index were determined. The identification of T-RF was done using MiCAIII program (Microbial Community Analysis III) based on RDP database (R10, U27) which consisting of 1,519,356 of bacterial 16S rRNA (http://mica.ibest.Uidaho.edu/).

3. Result and Discussion
Analysis of microbial communities in the gastrointestinal tract requires adequate quality and quantity of DNA. The value of good quality DNA that is 1.8 to 2.0 nm [9]. The DNA concentrations of samples ranged from 6.5-11.8 ng / μL, with 260/280 nm ratio value ranging from 1.66 to 2.15 nm. The PCR amplification process using a universal forward 7F primer labeled 6-carboxy fluorescein and a specific reverse primer SG-Lab-0677 is not labeled. Visualization obtained by 650 bp DNA band on each sample (Figure 1). It is informed that the results of the positive control sample in accordance with Lactobacillus salivarius.

![Figure 1. Visualization of the amplified of 16S rRNA](M=Marker 1kb; 1= P₀; 2= P₁; 3= P₂; 4= positive control (Lactobacillus salivarius). P₀ = commercial feed control; P₁ = commercial + prebiotic bran; P₂ = commercial feed + prebiotic bran fermentation of R. Oryzae.)

Purification product be restriction with enzyme HaeIII (Thermo Scientific) and MspI (Thermo Scientific) and then, incubated at 16 hours. Figure 2 showed the population dynamics pattern of LAB. The addition of prebiotics bran tends increased the population of uncultured bacteria (87 bp), Lactobacillus sp (278 bp), L. gallinarum (247 bp), L.delbrueckii subs. Bulgaricus (278 bp), L. intermedius (322 bp) and uncultured bacteria (331 bp) in ileum compared with control, while the addition of prebiotic of bran fermentation tends decreased uncultured bacteria (87 bp), Lactobacillus sp (278 bp), L. delbrueckii subs. bulgaricus (278 bp), and uncultured bacteria (331 bp), and eliminated the population of L. gallinarum (247 bp) and L. intermedius (322 bp).

The tendency enhancement of bacterial in ileum due the addition of prebiotic bran and decrease it due the addition of prebiotic bran fermentation, can be caused by feed quality and viscosity of ileum.
contents of feed. Fermentation will improve the quality of feed, because the complex organic matter is decomposed into simple organic matter, so it is easily absorbed [10]. Fermentation results can increase feed intake, growth rate, and improve feed conversion ratio in chickens [11]. Quality of raw materials, nutritional content, mycotoxin contamination, and physical quality of feed (particle size) can accelerate or slow down the movement rate in the digestive tract (feed passage) [12]. Increased viscosity of intestinal lumen contents can reduce digestibility, bowel performance, and inhibit the movement of particles and solutes in the intestinal lumen, thereby supporting the growth of intestinal bacteria [13]. Factors that affect the presence of bacteria in the intestine such as age, diet, fiber type, horizontal gene transfer, chicken type, geography, climate, and environment [14].

![Figure 2. T-RFLP gene profile 16S rRNA BAL broiler chicken digested with HaeIII restriction enzyme.](image_url)

Description: P0 = commercial feed control; P1 = commercial + prebiotic bran; P2 = commercial feed + prebiotic bran fermentation of *R. Oryzae*.

The relative abundance of LAB filotypes in ileum broiler chickens, with three treatments having different values (Figure 3). Relative abundance analysis uses T-RFs of *Hae*III, because the number of T-RFs is higher than of *Msp*I. Relative abundance analysis of T-RF profiles used which more T-RFs [15]. The types of BAL found in almost all samples were unidentified bacteria (50bp; 74bp; 82bp; 131bp; and 157bp), uncultured bacteria (87bp; 280bp; and 331bp), *Lactobacillus sp.*, *L. delbrueckii* subsp. *Bulgarius*, *L.intermedius*, and *L. gallinarum*. The type of *Lactobacillus sp.*, *L. delbrueckii* subsp. *Bulgarius* and uncultured bacteria 87bp have a higher relative abundance than other types. This is different from the reported that population of *L. salivarius* [16] was more dominant in cecum and ileum at 36 days, and in ileal mucosa at 35 days [17].
Figure 3. Relative abundance of phylotypes LAB in the ileum

Description: \( P_0 \) = commercial feed control; \( P_1 \) = commercial + prebiotic bran; \( P_2 \) = commercial feed + prebiotic bran fermentation of \( R. \text{Oryzae} \).

The analysis results from the sample (Table 1) illustrated that the value of diversity index (H) of LAB ileum, it is included in low to high category. The high and low of diversity index influenced by the number of species, evenness, and dominance index in each environment. The more number of species, the more diverse of community and the more individuals of each species, that are greater the role of that type in the community [18].

Samples of ileum with prebiotic bran treatment had the highest diversity index (\( H' = 1.343 \)) compared to other samples. The evenness index (E) has a value of 0.853 which means the community is evenly distributed and stable, and the dominance index (0.312) indicates that no species dominate in the community. This can be due to feed and environmental factors in the ileum. Giving prebiotic bran and bran fermentation can increase the value of diversity index on ileum, compared to control. Prebiotics are able to selectively stimulate the growth and activity of beneficial microorganisms in the intestine, such as \( \text{Bifidobacteria} \) and \( \text{Lactobacillus} \) so beneficial to health [4].

Table 1. The Result of Number of Phylotypes, Diversity, Evenness index and Dominance index

|                  | \( P_0 \) | \( P_1 \) | \( P_2 \) |
|------------------|-----------|-----------|-----------|
| Number of Phylotypes (S) | 3.5       | 5         | 3.5       |
| Shannon diversity index (H’) | 0.838     | 1.343     | 1.044     |
| Pielous evenness index (E) | 0.786     | 0.853     | 0.709     |
| Dominance Index (D)      | 0.512     | 0.312     | 0.406     |

Description: \( P_0 \) = commercial feed control; \( P_1 \) = commercial + prebiotic bran; \( P_2 \) = commercial feed + prebiotic bran fermentation of \( R. \text{Oryzae} \).

4. Conclusion

Based on T-RF analysis, any difference diversity of community of lactic acid bacteria (LAB) on ileum broiler chickens after given prebiotic bran and prebiotic bran fermentation by \( R. \text{oryzae} \). The addition of prebiotics bran further increases the diversity of lactic acid bacteria in ileum, compared with prebiotic giving of \( R. \text{oryzae} \) fermented bran.
References
[1] Konig, H. and J. Frohlich. 2009. Biology of microorganisms on grapes, in must and in wine. Springer-Verlag Berlin Heidelberg. 1-4.
[2] Azat, R., Y. Liu, W. Li, A. Kayir, D. Lin, W. Zhou, and X.Zheng. 2016. Probiotic properties of lactic acid bacteria isolated from traditionally fermented Xinjiang cheese. Journal of Zhejiang University Science B. 17(8):597-609.
[3] Pandey, K.R., Naik, S.R., and Vakil, B.V. 2015. Probiotics, prebiotics and synbiotics- a review. Journal Food Science Technolog. 52(12): 7577–7587.
[4] Cummings, J.H. and G.T. MacFarlane. 2002. Gastrointestinal Effects of Prebiotics. British Journal Nutrition., 87(2): 145-151.
[5] Rao, B.S. N. 2000. Nutritive Value of Rice Bran. Nutrition Foundation of India Bulletin, 21. 5–7.
[6] Pelizer, H.L., H.M. Pontieri, and O.I. Moraes. 2007. Utilizac¸ a˜ o de Resı´duos Agroindustriais em Processos Biotecnol´ ogicos como Perspectiva de Reduc¸ a˜ o do Impacto Ambiental. Journal of Technology Management and Innovation (2):118–127.
[7] Caffaro-Filho, R.A., Fantinatti-Garboggini, F., Durrant, L. R. 2007. Quantitative analysis of Terminal Restriction Fragment Length Polymorphism (T-RFLP) microbial community profiles: peak height data showed to be more reproducible than peak area. Braz. J. Microbiol. 38 (4).
[8] Tiquia, S. M. 2009. Using Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis to Assess Microbial Community Structure in Compost Systems. In: (Ed). Stephen P. Cummings. Bioremediation: Methods and Protocols. Springer protocol. Springer link.
[9] Sambrook J, E. Fritsch, T. Maniatis. 1998. Molecular cloning: A Laboratory Manual. 2nd ed. N.Y., Cold Spring Harbour Laboratorary Press.
[10] Kurniati, T., L. Nurlaila, and Im. 2017. Effect of Inoculum Dosage Aspergillus niger and Rhizopusoryzae mixture with Fermentation Time of Oil Seed Cake (Jatropha curcas L) to the content of Protein and Crude Fiber. Journal of Physics 824(1):1-8.
[11] Yaşar, S., M. S. Gök, Y. Gürbüz. 2016. Performance of Broilers Fed Raw or Fermented and Redried Wheat, Barley, and Oat Grains. Turkish Journal of Veterinary and Animal Science 40: 313-322.
[12] Ştef, L., D. Drinceanu, N. Corcionivoschi, C. Julean, D. Ştef, D. Moţ, E. Simiz. 2009. The Effect of Dietary Non-starch Polysaccharides on the Intestinal Viscosity and on the Cecal Microflora of Broiler Fed with Various Protein Sources. Archiva Zootecnica 12(3):22-29.
[13] Annett, C. B., J. R. Viste, M. Chirino-Trejo, H. L. Classen, D. M. Middleton and E. Simko. 2002. Necrotic enteritis: Effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Journal Avian Pathology. 31(6): 598-601.
[14] Lu, J., U. Idris1, B. Harmon, C. Hofacre1, J. J. Maurer and M. D. Lee. 2003. Diversity and Succession of the Intestinal Bacterial Community of the Maturing Broiler Chicken. Applied and Environmental Microbiology 69(11): 6816-6824.
[15] Pangastuti, A. 2008. Analisis Komunitas Bakteri selama Tahapan Perkembangan Larva Udang Putih (Litopenaeus vannamei). Disertasi. IPB Press, Bogor.
[16] Ranjitkar, S., B. Lawley, G. Tannock, R. M. Engberg. 2016. Bacterial Succession in the Broiler Gastrointestinal Tract. Applied and Environmental Microbiology. 82(8):2399-2410.
[17] Wang, L., Lilburn, M., and Yu, Z. 2016. Intestinal microbiota of broiler chickens as affected by litter management regimens. Front. Microbiol. 7:593.
[18] Estradivari, E. Setywawan, dan S. Yusri. 2009. Terumbu Karang Jakarta: Pengamatan Jangka Panjang Terumbu Karang Kepulauan Seribu (2003-2007). Yayasan Terumbu Karang Indonesia, Jakarta.