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Isolation of Lactic Acid Bacteria with Cholesterol-Lowering Activity from Digestive Tracts of Indonesian Native Chickens

H. Julendra[a], A. E. Suryani[a]*, L. Istiqomah[a], E. Damayanti[a], M. Anwar[a], & N. Fitriani[b]
[a]Research Center for Natural Product Technology (BPTBA), Indonesian Institute of Sciences (LIPI)
Gading, Playen, Gunungkidul DI. Yogyakarta, Indonesia
[b]Student of Department of Biology, Faculty of Mathematics and Natural Sciences, Sebelas Maret University (UNS)
Jalan Ir. Sutami No. 36A, Jebres, Kota Surakarta, Jawa Tengah 57126, Indonesia
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ABSTRACT

The aim of the study was to identify the cholesterol-lowering activity of indigenous lactic acid bacteria isolated from the small intestine, cecum, and colon of Indonesian native chickens and evaluated for bile salt hydrolase (BSH) activity *in vitro* by using MRS media added taurodeoxycholic acid (TDCA) and $CaCl_2$. The quantitative measurement of cholesterol-lowering activity of LAB was investigated by using soluble cholesterol containing MRS broth (100 µg/mL of cholesterin) and incubated at 37 °C for 48 h. Cholesterol content in supernatant was analyzed using microplate reader. The highest percentage of cholesterol reduction found in isolates from colon of native chicken with the value of 17.43% and identified as *Lactobacillus plantarum*. Based on phylogenetic tree analysis, this isolate was closely related to *L. plantarum* strain LGFCP4 (accession number KM199683.1) isolated from GIT of Guinea fowl from India. It could be concluded that *L. plantarum* AKK-30 had cholesterol-lowering activity.

Keywords: lactic acid bacteria (LAB), cholesterol, bile salt hydrolase (BSH), native chicken

ABSTRAK

Penelitian ini bertujuan untuk mengidentifikasi aktivitas penurunan kolesterol oleh bakteri asam laktat *indigenous*. Bakteri asam laktat (BAL) diisolasi dari usus halus, sekum dan kolon ayam kampung Indonesia dan dievaluasi terhadap aktivitas hidrolase garam empedu secara in vitro dengan menggunakan media MRS yang ditambahkan asam taurodeoxycholic (TDCA) dan $CaCl_2$. Pengukuran secara kuantitatif aktivitas penurunan kolesterol oleh BAL dilakukan dengan menggunakan MRS Broth yang mengandung kolesterol terlarut (100 µg/mL cholesterin) dan diinkubasikan pada suhu 37 °C selama 48 jam. Kolesterol yang terkandung di dalam supernatan dianalisis menggunakan *microplate reader*. Persentase penurunan kolesterol tertinggi dengan nilai 17.43% ditemukan pada isolat yang berasal dari kolon ayam kampung dan diidentifikasi sebagai *Lactobacillus plantarum*. Berdasarkan analisis pohon filogeni, isolat tersebut memiliki kekerabatan yang dekat dengan *L. plantarum* strain LGFCP4 (accession number KM199683.1) yang diisolasi dari saluran pencernaan ayam mutiara asal India. Dapat disimpulkan bahwa *L. plantarum* AKK-30 memiliki aktivitas menurunkan kolesterol.

Kata kunci: bakteri asam laktat (BAL), kolesterol, hidrolase garam empedu, ayam kampung

INTRODUCTION

Cholesterol contained in broiler meat without skin is around 133-202 mg/100 g of dry matter (DM) basis and in the whole meat is around 261-407 mg/100 g of DM (Ismoyowati & Widyastuti, 2003). Native chicken has the ability to suppress the levels of cholesterol in meat; it is probably due to activity of lactic acid bacteria that live in the intestines. The cholesterol-lowering mechanism for some types of beneficial bacteria including lactic acid bacteria that thought to have the ability to metabolize cholesterol from food in the intestines, so it was not absorbed by the gut (de Ross & Katan, 2000). According to Ooi & Liong (2010), an alternative method to reduce levels of cholesterol and triglycerides in poultry meat was the use of lactic acid bacteria as probiotics.

*Corresponding author:
E-mail: deyan02@gmail.com

Bile-salt hydrolase (BSH) are enzyme deconjugates bile acid into glycine or taurine from the steroid moiety, producing free bile salts. Deconjugated bile salt is not easy to be absorbed by small intestine then it is excreted in feces that eventually reduces the number of bile acids returned to the liver. BSH activity is detected in some strains related to the gastrointestinal tract (GIT), representing several species of LAB (*Bifidobacterium* and *Lactobacillus*) indigenous of the digestive tract (Kumar *et al*., 2012). However, probiotics derived from endogenous bacteria of chicken is still inefficient. Probiotics that do not originate from the indigenous bacteria when entering through the digestive tract will be damaged before it reaches the small intestine, therefore the use of probiotic from indigenous bacteria are expected to be more tolerant to change in pH, temperature, and enzyme activity of digestive tracts.

Based on research conducted by Agaliya & Jeevaratnam (2012) studying cholesterol lowering effect of 8 lactobacilli, the strain *L. plantarum* JJ18 showed a higher cholesterol removal capacity from media and tolerance towards acid and bile. Cenesiz *et al.* (2008) also reported that supplementation of LAB as probiotic in broiler birds significantly (P≤0.05) reduced total cholesterol serum level.

The aim of the study was to identify the cholesterol-lowering activity of indigenous lactic acid bacteria isolated from the small intestine, cecum, and colon of Indonesian native chickens and evaluated for bile salt hydrolase (BSH) activity *in vitro* by using MRS media added taurodeoxycholic acid (TDCA) and $CaCl_2$. This LAB will be used for chicken as a feed additive to reduce the cholesterol level in poultry meat.

MATERIALS AND METHODS

Isolation and Identification of Lactic Acid Bacteria

Strains of LAB were collected from digestive tract of Indonesian native chicken (Ayam Kampung) according to Torshizi *et al*. (2008) method. Small intestine, cecum, and colon were cut, and the contents of lumen were taken and then diluted in NaCl solution (Merck) 0.85% up to 105 dilutions. de Mann Rogosa Sharpe (MRS) Agar media (Oxoid) with pH 6.2 and 0.2% CaCO3 (Merck) was plated and incubated at 37 °C for 24 h with the addition of each serial dilution. The LAB colonies were identified by clearing zone appearance. LAB characterization procedures consisted of morphology, Gram staining, catalase, gas production, and motility tests according to Krieg *et al*. (2010). API 50 CHL kit (bioMērieux) were used to observe biochemical identification of the selected LAB. The observation data were determined by API web software (bioMērieux).

The identification of LAB was also confirmed using alignment of 16S rRNA gene sequence available in GenBank. DNA genome from LAB was isolated according to PeqGOLD Bacterial DNA kit. DNA was amplified using primer sequences of 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Gong *et al*., 2007). Sequencing of 16S rRNA gene was conducted by First BASE laboratories (Singapore). Data of DNA sequence was edited by FinchTV program and contiq with BioEdit, and kept in FASTA format. The sequence result was analyzed by basic local alignment search tool (BLAST) program by accessing data in NCBI. Phylogenetic analysis was performed by comparing several sequences with high similarity in BLAST result. Statistical method was using Maximum-likelihood. Stability of grouping was measured using 1000 bootstrap replicates. Phylogenetic tree was performed by MEGA5 program (Cobos *et al*., 2011).

LAB isolates were kept at −80 °C in MRS broth consisting of 15% (v/v) glycerol. An MRS-agar plate was streaked from the frozen stock to ensure purity for any assay.

Bile Salt Hydrolase Activity

Prior to determining the ability of LAB in lowering of cholesterol, the selected isolates were tested to make sure whether they have bile salt hydrolase (BSH) activity. The test was conducted by impregnation around sterilized paper disks (Oxoid) on the MRS agar plates added with sodium salt of taurodeoxycholic acid 0.5% (w/v) (TDCA, Sigma, USA), and 0.37 g/L $CaCl_2$ (Merck). Plates were incubated in anaerobic condition for 72 h at 37 °C, and the precipitation zones diameter around the disks was calculated (Lim *et al*., 2004; Sirilun *et al*., 2010).

Cholesterol-lowering Activity

Cholesterol (C75209, Sigma) was dissolved in Tween 80, then added to MRS at the final concentration of 100 μg/mL. One percent of LAB in the broths were anaerobically by giving CO_2 gas into the tube and incubated for 24 h at 37 °C. After incubation, the broths containing LAB were centrifuged at 4000 rpm for 10 min at 4 °C (*Centofriger*® BL II, Selecta) and the supernatants were collected. Cholesterol concentrations in supernatants were determined using Tomaro-Duchesnau *et al.* (2014) method. Briefly, 500 μL of 33% (w/v) KOH and one mL absolute ethanol were added to 500 μL of the supernatant. The solutions were then vortexed and incubated at 37 °C for 15 min followed by cooling to room temperature. For phase separation, one mL of aquadest and 1.5 mL of hexanes were added to the solutions and vortexed. The phases were then allowed to separate at room temperature. Subsequently, 500 μL of the hexane layer was transferred into a glass tube, and the solvent was evaporated by using nitrogen gas. Once dried, one mL of 50 mg/dL o-phthalaldehyde reagent prepared in acetic acid was added, and the samples were mixed. Following mixing, 250 μL of concentrated H_2SO_4 was added to each tube, and the solutions were vortexed then incubated for 20 min at room temperature. The absorbance was read at 570 nm using a microplate reader (Thermo Scientific). A standard curve of absorbance versus cholesterol concentrations was generated using the cholesterol concentrations: 0, 4, 8, 12, 16, 24, 32, 40, 52, 64, 128, 256 μg/mL cholesterol in MRS (R2= 0.998). Cholesterol lowering effect was calculated following the equation:

Cholesterol lowering effect= (C–C')/C,

where the concentrations of cholesterol existing in the supernatants of uninoculated and inoculated were C and C' (Guo *et al.*, 2011). Cholesterol-lowering percentage by each *Lactobacillus* strain was also calculated as follow:

% Cholesterol-lowering= [(cholesterol-lowering (µg/mL)/cholesterol (µg/mL)] × 100%

RESULTS

Isolation of Lactic Acid Bacteria Candidates from Chicken's Gastrointestinal Tract

Lactic acid bacteria isolation from chicken's digestive tract was successful in selecting 26 (8 isolates derived from the small intestine, eight isolates from the cecum, and ten isolates from the colon). The selected LAB had a single colony, and clear zone appearance on MRS-agar supplemented with 0.2% $CaCO_3$. These isolates did not have catalase, did not produce gas on Glucose Yeast Peptone (GYP) medium, non-motile on Sucrose Yeast Peptone (SYP) medium, Gram-positive and had coccus and rod shape.

Characterization of Selected Lactic Acid Bacteria

Table 1 showed that 26 isolates characterized as LAB produced negative results on catalase test. Observations of 26 isolates of LAB by staining showed that all isolates were Gram-positive (Damayanti *et al.*, 2012). Motility test indicated that all of isolates had non-motile properties showing no movement that resembles a vine-propagation besides the needle puncture area and there was no cloud formation at SYP medium.

Bile Salt Hydrolase Activity

For 26 isolates tested, all have the precipitation zones (Figure 1) with different diameters (Table 2). Isolates with the largest diameter values were isolated from chicken intestine (AKU-4), while isolates that have the smallest diameter values were isolated from chicken colon (AKK-21).

In vitro Test for Cholesterol-lowering Activity

The results showed that the ten isolates of LAB have the action for lowering cholesterol levels (Table 3). The activity of LAB to decrease cholesterol varied in a range of 16.6-34.4 µg/mL. The highest activity was shown by AKK-30 with percentage of 17.43% followed by AKU-4 at 15.24%, and AKS-16 at 15.02%, while the lowest cholesterol reduction rate present in isolates AKS-19 with 8.41% compared to control.

Identification of Selected LAB with the Highest Cholesterol-lowering Activity

The chosen lab isolate was identified based on biochemical procedures by using API 50 CHL kit (bioMerieux) as shown in Table 4. In biochemical identification, AKK-30 isolate was identified as *Lactobacillus plantarum* (92.3% of similarity), and 27 types of carbohydrates were able to be fermented. Based on 16s rRNA sequences analysis, AKK-30 was identified as *L. plantarum*.

Tabel 1. Characteristics of lactic acid bacteria isolated from digestive tracts of native chicken

Isolate	Morphology	Gram staining	Catalase test	Motility test	Gas production assay
Intestine of native chicken					
AK U 1	Coccus	+	-	-	-
AK U 2	Short rod	+	-	-	-
AK U 3	Coccus	+	-	-	-
AK U 4	Short rod	+	-	-	-
AK U 5	Short rod	+	-	-	-
AK U 6	Coccus	+	-	-	-
AK U 7	Coccus	+	-	-	-
AK U 8	Coccus	+	-	-	-
Caecum of native chicken					
AK S 9	Coccus	+	-	-	-
AK S 10	Short rod	+	-	-	-
AK S 11	Short rod	+	-	-	-
AK S 14	Coccus	+	-	-	-
AK S 16	Short rod	+	-	-	-
AK S 17	Short rod	+	-	-	-
AK S 18	Short rod	+	-	-	-
AK S 19	Coccus	+	-	-	-
Colon of native chicken					
AK K 21	Short rod	+	-	-	-
AK K 22	Short rod	+	-	-	-
AK K 23	Coccus	+	-	-	-
AK K 24	Short rod	+	-	-	-
AK K 25	Short rod	+	-	-	-
AK K 27	Coccus	+	-	-	-
AK K 29	Short rod	+	-	-	-
AK K 30	Short rod	+	-	-	-
AK K 31	Short rod	+	-	-	-
AK K 32	Long rod	+	-	-	-

Note: (-)= Negative reaction, (+)= Positive reaction.

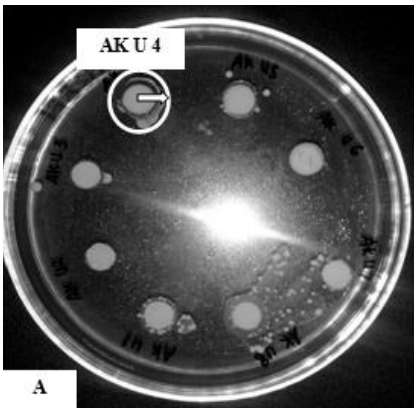

Figure 1. Precipitation zone of AKU-4 isolate

Table 2. Diameter precipitation zone of 26 isolates lactic acid bacteria from digestive tracts of native chicken

Isolate	Diameter zone of precipitation (mm)		
	control	d1	d2
Intestine			
AK U 1	6	9.9	9.9
AK U 2	6	6.1	4.6
AK U 3	6	7	8.3
AK U 4	6	13.4	10.5
AK U 5	6	9.1	8.3
AM 6	6	8	8
I 7	6	7.6	7.6
ME 8	6	6.7	8.9
Caecum			
AK Graduate 9	6	11	9.2
AK S 10	6	7.1	7.1
AK S 11	6	7.3	6.7
AK S 14	6	7.4	7.4
AK Graduate 16	6	8.1	10.4
AK S 6 10.9 10.9 15			
AK S 6 7.6 7.4 16			
AK S 19	6	9	9.8
Colon			
AK K 21	6	5	6.3
AK K 6 7.7 7.2 19			
AK K 23	6	6.5	6.3
AK K 6 7.2 6.8 21			
AK K 6 6.4 5.7 22			
AK K 6 5.4 5.4 23			
AK K 6 6.4 6.9 24			
AK K 6 10.2 8.6 25			
AK K 6 7.3 6.2 26			
AK K 32	6	6.8	6.8

DISCUSSION

Isolation of Lactic Acid Bacteria Candidates from Chicken's Gastrointestinal Tract

Supplementation of $CaCO_3$ into the media was intended to obtain early estimation colonies of LAB with the clear zone presence appeared after the incubation period. Calcium carbonate reacted with the lactic acid produced by LAB thus forming Ca-lactate acids and identified by clear zone around the colonies on media (Harimurti *et al*., 2005). According to Jannah *et al*. (2014), microbiota diversity and abundance varied at a particular location along the gastrointestinal tract, the number of the microbiota declined in less favorable condition for bacterial growth. The presence of microorganisms in the poultry's digestive tract due to the interaction of bacteria with the environment that entry by feeding. Differences age of the chicken also gave differences in the amount and type of bacteria present in the gastrointestinal tract. Heravi *et al*. (2011) also stated that the bacteria isolated from chicken digestive was *Lactobacillus* which had specification such as positive Gram stain, catalase negative reactions, and form of bacillus and the amplification of 16s rRNA fragments (0.24 kbp) using specific primers assured that all isolated bacteria belongs to the genus of Lactobacillus.

Characterization of Selected Lactic Acid Bacteria

Negative results on catalase test that were found in 26 isolates indicated by the formation of oxygen after H_2O_2 addition. This lead to accordance with Adams & Nout (2001) who stated that LAB had negative catalase activity. The gas production assay from all isolates showed negative result hence the isolates were homofermentative (Shazali *et al*., 2014). Based on biochemical point of view, LAB consist of both homofermenters, yielding mainly lactic acid, and heterofermenters, which, apart from lactic acid, produce a large variety of fermentation metabolites such as acetic acid, carbon dioxide, ethanol, and formic acid (Kleerebezem & Hugenholtz, 2003).

In this research 26 isolates of LAB included in the class of gram-positive bacteria when it formed purple color under a microscope. Negative results from motility test showed that the lactic acid bacteria had no flagella for movement.

Bile Salt Hydrolase activity

Precipitation zone that was formed in BSH enzyme activity was caused by the deposition of cholesterol as a bile acids constituent that has been conjugated (Sirilun *et al*., 2010). In the qualitative test of BSH enzyme activity, *sodium salt taurodeoxycholic* acts as a substrate that will be conjugated by BSH from lactic acid bacteria, while the addition of $CaCl_2$ ion intended to optimize BSH enzyme activity. Bile salt hydrolase is produced by lactic acid bacteria, these enzymes can produce conjugated bile acids in the form of free cholic acids that are less absorbed by the intestine. A qualitative test intended to determine the activities of bile salt hydrolase that produced by lactic acid bacteria in conjugate sodium salt taurodeoxycholic acid were added in the media. If detected positive for BSH enzyme activity, precipitation zone will appear around the paper disk that contains lactic acid bacteria (Lim *et al*., 2004).

Table 3. Cholesterol value from *in vitro* test using ELISA Microplate Reader (ppb)

AK U 1	AK U 4	AK U 5	AK U 6	AK U 8	AK S 9	AK S 16	AKS 17	AK S 19	AK K 30	CONTROL
183.8	171.2	166.4	185.0	179.0	175.7	169.6	162.5	198.1	166.2	207.8
175.1	168.9	185.3	170.4	178.8	180.7	164.2	170.2	173.3	164.0	194.0
177.6	161.7	174.2	170.6	170.4	173.6	169.3	173.2	170.8	158.6	190.2

Table 4. Fermentation of 49 types of carbohydrate based test identification using API KIT 50 CHL for AKK-30

No.	Type of carbohydrates	AKK- 30	No	Type of carbohydrates	AKK-30
0	Temoin	-	25	Esculine	+
1	Glycerol	+	26	Salicin	+
2	Erythritol	-	27	D-Cellibiose	+
3	D-arabinose	-	28	D-Maltose	+
4	L-arabinose	+	29	D-Lactose	+
5	D-ribose	+	30	D-Melibiose	+
6	D-xylose	-	31	D-Sacharose	+
7	L-xylose	-	32	D-Trehalose	+
8	D-adonitol	-	33	Inulin	-
9	Methyl-βD-xylopyranoside	-	34	D-Melezitose	+
10	D-galactose	+	35	D-Raffinose	+
11	D-glucose	+	36	Amidon	-
12	D-fructose	+	37	Glycogen	-
13	D-mannose	+	38	Xylitol	-
14	L-sorbose	-	39	Gentibiose	-
15	L-rhamnose	+	40	D-Turanose	+
16	Dulcitol	-	41	D-Lyxose	-
17	Inositol	-	42	D-Tagatose	+
18	D-mannitol	+	43	D-Fucose	-
19	D-sorbitol	+	44	L-Fucose	-
20	Methyl-αD-mannopyranoside	+	45	D-arabitol	-
21	Methyl-αD-glucopyranoside	+	46	L-arabitol	-
22	N-acetylglucosamine	+	47	Potassium gluconate	-
23	Amygdaline	+	48	Potassium 2 ketogluconate	-
24	Arbutine	+	49	Potassium 5 ketogluconate	-

Note: Specification (+) = fermentable; (-) = nonfermentable.

In Vitro Test for Cholesterol-lowering Activity

Top ten isolates with the highest precipitation zone diameters were determined for cholesterol-lowering activity. The cholesterol-lowering test of the isolates was investigated upon incubation with 100 µg/mL water-soluble cholesterol-Tween 80 in MRS. Compared to the results of BSH enzyme activity, the higher precipitation zone providing opportunities to the higher cholesterol reduction activity, but it was not always linear with the results from this study. Isolates with the greatest precipitation zone (AKU-4) did not show the highest activity in the cholesterol-lowering test, while AKK-30 which had smaller precipitation zone than AKU-4 produced the highest cholesterol-lowering activity. This discrepancy might be due to different cholesterol-lowering mechanisms in each test.

There are several mechanisms of LAB in lowering cholesterol. LAB will produce the BSH enzyme which played a role as an anti-cholesterol. BSH enzyme catalyzed a reaction in which glycine and taurine were deconjugated from bile salts. The corresponding unconjugated bile acids precipitate at low pH, which can not be reabsorbed by liver in the cycle of enterohepatic (Seeley *et al*., 2000) so it is excreted in the feces, resulting in reduced cholesterol levels (Huang *et al*., 2013).

Identification of Selected LAB with the Highest Cholesterol-Lowering Activity

The selected LAB isolate was identified based on biochemical procedures by using API 50 CHL kit (bioMerieux) as shown in Table 4. In biochemical identification, AKK-30 isolate was identified as *L. plantarum* (92.3% of similarity), and 27 types of carbohydrates were able to be fermented.

Characterization of LAB was based on 16S rRNA sequences analysis to determine the genus and strain. This gene was amplified using universal primer 27f and 1492r, and the amplification result was shown in Figure 2. PCR product of 16S rRNA gene was 1500 bp.

BLAST homology of 16S rRNA sequences in NCBI site revealed that AKK-30 isolate was identified as *L. plantarum* with a length of 1414 bp in DNA bank database (NCBI) had 100% of max identity, 2612 of the max score, 2612 of the total score, 100% of query coverage, and 0.0 of E-value. The bacteria were classified in Kingdom of Bacteria, Division of Firmicutes, Class of Bacilli, Order of Lactobacillales, Family of Lactobacillaceae, Genus of Lactobacillus, and Species of *L. plantarum*. According to Stackebrandt & Ebers (2006), when the similarity percentage of 16S rRNA gene sequence between 2 organism was less than 98.7%-99.0%, then both of them was different species.

Phylogenetic analysis of AKK-30 isolate by nucleotide reference from Gen Bank data in NCBI was shown in Figure 3. Branch lengths are proportional to genetic distances (Becher *et al*., 1997). Bootstrap values of over 70% is shown for 1,000 replicate datasets (Coenye & Vandamme, 2003). The tree describes the relationship between selected sequences retrieved from the GenBank database and nucleotide sequences in this study. The phylogenetic tree showed that *L. plantarum* AKK-30 isolated from Indonesian native chicken was closely related to *L. plantarum* strain LGFCP4 isolated from GIT of Guinea fowl from India. Gong *et al.* (2007) stated that microbial ecology of chicken intestinal, as well as the diversity, phylogeny, distribution, and success of the bacteria as well as bird age, diet, and managing of dietary antibiotics affect the microbiota composition in these two GI tract regions. The complexity and diversity of the community structure of cecal bacteria were much higher than that of reported by culture-based studies. Moreover, a significant number of 16S rRNA genes isolated from cecal microbiota were also found to be related to uncultured bacteria identified in human feces and the intestine of other animals (including ruminants, pigs, and mice).

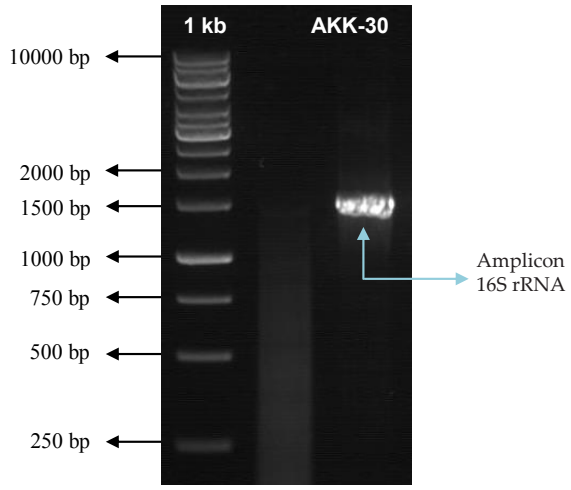

Figure 2. PCR Product 16S rRNA of AKK-30 isolate and DNA ladder (1 kb DNA ladder)

CONCLUSION

Lactic acid bacteria isolated from the gastrointestinal tract of native chicken had the cholesterol reductase activity with various percentages. Bile salt hydrolase activity qualitatively was shown by precipitation zone around LAB colonies, while quantitatively cholesterol reductase activity indicated by the ability of lactic acids bacteria to assimilate cholesterol. The highest percentage of cholesterol reduction was shown by bacteria isolated from the colon of native chicken with the value of 17.43% and identified as *L. plantarum*. Based on phylogenetic tree analysis, this isolate was closely related to *L. plantarum* strain LGFCP4 (accession number KM199683.1) isolated from GIT of Guinea fowl from India. It could be concluded that *L. plantarum* AKK-30 had cholesterol-lowering activity.

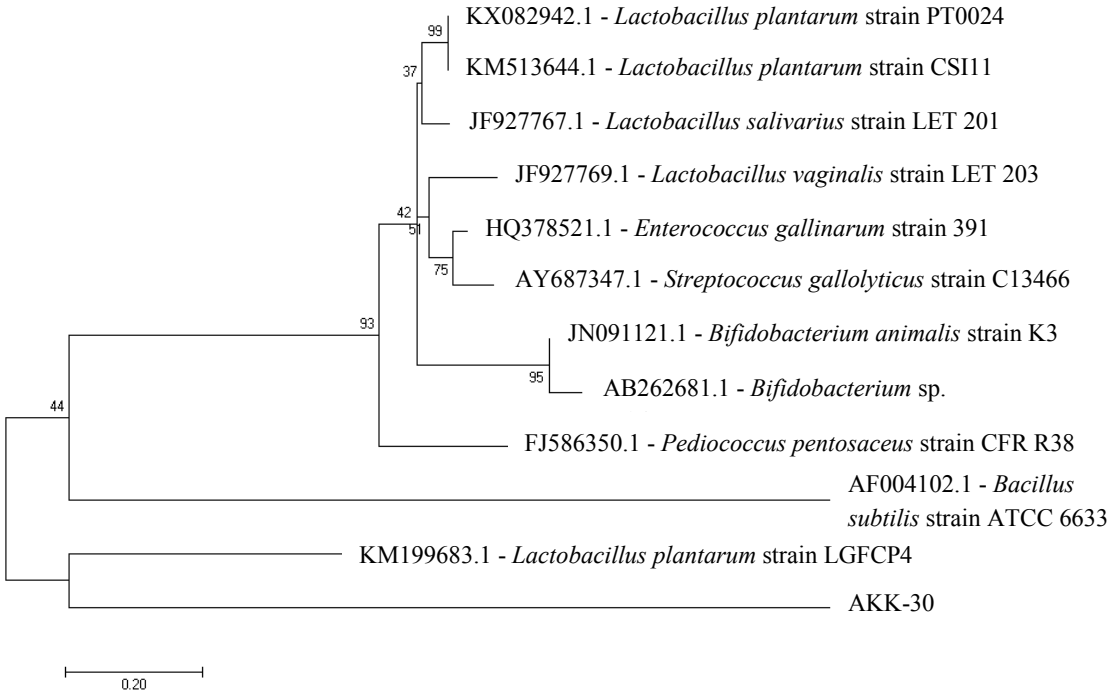

Figure 3. Phylogenetic tree of AKK-30 isolate and bacteria in the chicken's GIT constructed by a neighbor-joining method. The scale bar represents a sequence divergence of 5%.

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