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# Screening for probiotic of lactic acid bacteria isolated from the digestive tract of a native Aceh duck (*Anas platyrhynchos*)

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**Abstract.** *Risna YK, Harimurti S, Wihandoyo, Widodo. 2020. Screening for Probiotic of lactic acid bacteria isolated from the digestive tract of a native Aceh duck* (Anas platyrhynchos*). Biodiversitas 21: 3001-3007.* The lactic acid bacteria (LAB) isolated from the digestive tract of a native Aceh duck has emerged as a potential probiotic supplement for duck feed. In this study, we isolated the LAB from the digestive tract of an Aceh duck (*Anas platyrhynchos*) and identified the species and bacterial characteristics. Additionally, we isolated the LAB used in this study from the crop, proventriculus, ventriculus, duodenum, jejunum, ileum, and cecum of the native Aceh duck. Identification included the examination of morphology and physiology, followed by molecular identification by using 16S rRNA and sequence similarity tests for the bile salt hydrolase (*bsh)* gene. Bacterial isolation from the digestive tract of this duck resulted in 19 isolates with gram-positive, negative catalase, and non-motile characteristics. Of the 19 isolates, 11 isolates exhibited the ability to grow at 15°C, 37°C, and 45°C, with optimum growth at 37°C. From 11 isolates, only 6 were identified by using 16S rRNA primers and 5 were identified by using BSH primers. The identified LAB included *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Pediococcus acidilactici*. Hence, we conclude that *Lactobacillus sp* and *Pediococcus sp*. are potential probiotics that can be isolated and administered for duck feed.

**Keywords:** Aceh duck, lactic acid bacteria, probiotics, poultry

## INTRODUCTION

Since 2006, The European Union has banned the use of all antibiotic growth promoters for livestock production because the residuals from these drugs contaminate commercial products (Fernandez et al. 2014). The ban on antibiotic use occurred due to the emergence of resistant bacteria and the concern that antibiotic residues in meat and eggs might lead to potential health hazards in the consumers. The major health risk associated with antibiotics is the development of resistant microbiota in the gastrointestinal tract (Diarra et al. 2010). In Indonesia, the Indonesian Constitution no. 41 on Livestock and Animal Health had imposed a ban on antibiotics as a feed additive in 2014. In 2017, the Indonesian Ministry of Agriculture issued the Regulation of the Ministry of Agriculture Ministerial no. 14 on Animal Drugs Classification, which explicates the ban on antibiotics for use as a feed additive.

The poultry industry has used antibiotics to prevent bacterial infection on poultry farms. More than 60% of all the produced antibiotics are used in animal production for both therapeutic and non-therapeutic purposes (Van Boeckel et al. 2015). In Indonesian poultry production, antibiotics have been effectively used to inhibit the actions of poultry-associated pathogens from the various serotypes of *Escherichia coli*, *Campylobacter jejuni*, and *Salmonella enterica* (Sri-Harimurti and Hadisaputro 2015). However, the use of antibiotics in poultry production had been reported to increase antibiotic residues in meat and eggs (Mund et al. 2017, Mehdi et al. 2018). The results of a few studies have revealed that a sample of 8.3% (2/24) of positively tested chickens contained antibiotic residues of oxytetracycline. Meanwhile, 75% (18/24) of the positive egg samples contained antibiotic residues of penicillin, 12.5% (3/24) of these samples contained positive residues of aminoglycoside, and 12.5% (3/24) of these samples contained positive residues of oxytetracycline (Widiasih et al. 2019).

Because the detrimental effects of antibiotics are well documented, researchers have been prompted to think about alternatives to antibiotics. A study has proposed the use of probiotics as an alternative to antibiotics in poultry feed (Mehdi et al. 2018). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) have defined probiotics as "live microorganisms which, when administered in adequate amounts, confers a health benefit on the host" (FAO/WHO 2001; Aziz and Bonavida 2016). Lactic acid bacteria (LAB) are the commonly utilized probiotics in poultry production. LAB are mostly gram-positive, non-motile, rod- and coccus-shaped, non-spore-forming bacteria that can grow at 10°C to 45°C. They can optimally grow at a pH of 5.5–5.8 and are anaerobic (the ability to survive in the absence of oxygen) (Khalid 2011). The beneficial effects of probiotics have been attributed to their ability to suppress the growth of pathogens via the secretion of antibacterial substances such as lactic acid, peroxides, and bacteriocins (Mokoena 2017).

Vila et al. (2010) previously reported that some species of LAB produced enough hydrogen peroxide to inhibit the

growth and action of various microorganisms. *Lactobacillus acidophilus* produces acidophilin and acidolin, whereas *L. plantarum* produces bacteriocin (Pato et al. 2020, Aritonang et al. 2019). Nisin and diplococcin are among some of the antibiotics produced by Streptococci. Aritonang et al. (2019) reported that the application of bacteriocin produced by *L.plantarum* SRCM1 004 34 was able to preserve beef sausage. *Bacillus cereus* produces a bacteriocin-like substance that inhibits closely related *Bacillus* spp. and species such as *Staphylococcus aureus* and *Micrococcus luteus*, which exhibited high activity in the pH range of 3.0–9.0 (Butkhot et al. 2019).

The addition of LAB replaces the enteric pathogens by means of competitive exclusion in the poultry's intestinal tract and subsequently reduces bacterial contamination and increases productivity during poultry production. Sri-Harimurti and Ariyadi (2010) previously reported that indigenous LAB isolated from the digestive tract of healthy Indonesian native chickens (*Ayam kampung*), which consisted of *L.murinus* Ar3, *Streptococcus thermophilus* Kd2, and *Pediococcus acidilactici* Kp6, proved to be efficient as a feed supplement in improving the live performances of broiler chickens. The isolation of LAB from the digestive tract of poultry aims to obtain the endogenous probiotic bacteria that possess the ability to inhabit and grow inside the digestive tract of the poultry (Sri-Harimurti and Hadisaputro 2015). Therefore, the ideal probiotics for poultry should have a poultry origin because the probiotic bacteria can already effectively colonize the digestive tract of the host (Hibbing et al. 2010).

There has been increased development of probiotic supplementation by using LAB, but LAB isolates originated from the digestive tract of Indonesian local ducks have not yet been reported. In this study, we report the isolation and identification of strains of LAB from the digestive tract of the native Aceh duck to be used as potential probiotics.

## MATERIALS AND METHODS

### Sample collection

We obtained the samples used in the experiments from the digestive tract of a one-year-old native Aceh duck. Three ducks were obtained from a local farmer in Aceh, and was euthanized and humanely killed by severing the jugular vein. The intestines were immediately harvested, and the samples were obtained from the duck's crop, proventriculus, ventriculus, duodenum, jejunum, ileum, and cecum. The samples were collected aseptically in sterilized tubes, which were kept in ice (±4°C) during transportation to the laboratory until further analysis.

### Bacterial isolation and morphology identification

Bacterial isolation was conducted by following the method developed by Kimprasit et al. (2013) with modifications. Briefly, the freshly prepared samples were washed with phosphate-buffered saline solution and scraped. Next, 10 mL solution of the samples were inoculated onto sterilized de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) media containing 1% $CaCO_3$ and 0.05% of bile salts (Oxoid, Singapore). Thereafter, the mixture was incubated at 37°C for 24–48 h in micro-aerobic conditions. This led to the formation of a clear zone, which indicated the production of lactic acid by the putative LAB. White colonies surrounding the clear zone were subjected to morphology and physiology identification, including gram staining; shape, spore formation; motility; catalase test; growth at 15°C, 37°C, and 45°C; and production of $CO_2$.

### Bacterial growth at different temperatures

The ability of isolates to grow at different media and temperatures was tested at 15°C, 37°C, and 45°C for 24 h. The healthy culture of the selected five isolates (C1, V2, D3, J2, I1, and S4) in 1 mL was inoculated into 9-mL MRS broth and incubated overnight at 37°C. The overnight cultures were harvested, and two 2 mL ($10^8$ CFU/mL) of the cultures of each isolate were inoculated into 50-mL MRS broth and incubated at 15°C, 37°C, and 45°C for 24 h in the micro-aerobic condition. Bacterial growth was measured before and after incubation with the help of optical density (OD) spectrophotometer set at the absorbance $\lambda = 640$ nm. Biomass increase was presented in percentage (%) and was calculated as: [ (final OD − initial OD) / (initial OD)] × 100%.

### Genomic DNA extraction and amplification

Genomic DNA of the selected isolates was extracted by using a Favorgen™ kit (Favorgen, Taiwan) and complying with the manufacturer's instructions. Genomic DNA amplification was conducted by using polymerase chain reaction (PCR). The GoTag Green ready mix consisted of 25 µL of GoTag ready mix solution, 2 µL of forward primer, 2 µL of 1429R reverse primer, and 2 µL of the DNA sample. The primer pairs used in this study were 27F with 1429R (Gong et al. 2007), *bshlp*F with *bshlp*R, and *bshlf*F with *bshlf*R (Kumar et al. 2012) (Table 1). The amplification was performed by using a PCR thermal cycler with the following cycles: (i) initial denaturation at 95°C for 5 min, (ii) denaturation at 95°C for 30 s, (iii) annealing at 57°C for 30 s, (iv) elongation at 72°C for 30 s, (v) final elongation at 72°C for 10 min, and (vi) cooling at 4°C for 10 min. All of the PCR amplification procedures were repeated for 35 cycles (Xie et al. 2015). The amplified product was further confirmed via electrophoresis by using 2.0% agarose gel. It was photographed under an ultraviolet illuminator.

### DNA sequencing and phylogenetic analysis

The amplified DNA was sequenced by using 1st BASE DNA Sequencing (Genetika Science Ltd., Indonesia). Thereafter, the obtained sequence was compared with the 16S rRNA database available in GeneBank. The obtained sequences were confirmed by using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov. The DNA sequence that had close similarity up to 100% was determined and aligned with the MEGA X program to construct the phylogenetic tree.

**Table 1.** Primer pairs used for PCR amplification

| Primer | Primer | Sequences (Gong et al. 2007; Kumar et al. 2012) |
|---|---|---|
| 16S rRNA | 27 F | 5′-AGAGTTTGATCCTGGCTCAG-3′ |
| | 1429R | 5′-TAGGGTTACCTTGTTACGACTT-3′ |
| *Lactobacillus plantarum* | *bshlp* F | 5′-GCTGAATCACTCCCGGATTT-3′ |
| | *bshlp* R | 5′-CGAGCTTCGCCTGCTTAATA-3′ |
| *Lactobacillus fermentum* and | *bshlf* F | 5′-GCCGCACCTGGATCAAAT-3′ |
| *Pediococcus acidilactici* | *bshlf* R | 5′-GGGTGAAGTCTAGGTAGTCCTG-3′ |

**Data analysis**

Data obtained from the morphology, physiology, and molecular identification were analyzed by using descriptive statistics for describing the basic features of the data (Thompson 2009).

## RESULTS AND DISCUSSION

**Isolation and identification of LAB from the digestive tract of a native Aceh duck**

The results showed that all isolates obtained from the digestive tract of a native Aceh duck could grow on MRS broth media supplemented with 1% $CaCO_3$ after 24 h of incubation at 37°C. The addition of $CaCO_3$ (1%) was used to improve the selection of LAB, as indicated by the formation of clear zone. In total, 56 isolates were obtained and selected by using this method. The morphological observations showed that the isolates were either rod- or coccus-shaped; were white, brown, or pink; had convex and shiny surfaces, and were flat or fiber-edged. The obtained isolates that showed similar characteristics as LAB were selected for further identification. From a total of 56 isolates, only 19 isolates showed characteristics of LAB, such as gram-positive, catalase-negative, and non-motile (Table 2). These 19 isolates were selected for further molecular identification.

In total, 19 selected isolates from the digestive tract of a native Aceh duck were gram-positive, which was indicated by the crystal violet color retained after staining (Black and Black 2018). This phenomenon occurs because gram-positive bacteria have a thick layer of peptidoglycan (50%–90% of the cell weight) in the cell walls, whereas gram-negative bacteria have thinner layers (10% of the cell weight) (Dorr et al. 2019). LAB are categorized as gram-positive bacteria (Mattu dan Chauchan 2013; Lawalata et al. 2011, Franz et al. 2010). The bacterial cell staining also showed that 14 selected isolates were rod-shaped, whereas the other 5 isolates were coccus-shaped (Table 2). These results were similar to those reported by Xie et al. (2015) who also studied the digestive tract of ducks and reported that the isolated LAB was gram-positive and either rod- or coccus-shaped (Xie et al. 2015). Conversely, Hidayat et al. (2018) reported that the bacteria isolated from the digestive tract of broiler chickens were solely gram-positive cocci. The catalase examined the bacterial capability to produce the catalase enzyme. The catalase enzyme catalyzes the decomposition of hydrogen peroxide ($H_2O_2$) into water ($H_2O$) and oxygen ($O_2$). If the microbe is catalase-positive, then the sample shows the production of gas bubbles when 3% $H_2O_2$ was added, thereby indicating the production of $O_2$. For LAB that are categorized as catalase-negative (Khalid 2011), no gas bubble production was observed. *Lactobacillus* is a genus of LAB that is categorized as catalase-negative (Kabir et al. 2016).

The motility test was conducted to observe the bacterial motility and the dispersion of bacterial growth along the stabbed line of the media. Our data showed that all of the 19 isolates were non-motile (Table 2). This finding was in agreement with the previous findings that showed that the LAB was non-motile (Khalid 2011).

The growth of selected isolates in this study was observed at three different temperatures, namely, 15°C, 37°C, and 45°C, for 24 h. The OD test with absorbance at 640 nm ($\lambda$ = 640 nm) showed that all of the selected isolates were able to grow at all of the different temperatures (Figure 1), with an optimal growth temperature of 37°C as this temperature demonstrated the highest biomass increase following growth for 24 h. This observation suggested that LAB were mesophilic bacteria. However, some species had the ability to grow at 45°C (Mulaw et al. 2019).

As all of the 19 isolates had the potential LAB, all isolates underwent further molecular identification either by using a 16S rRNA approach or the bile salt hydrolase (*bsh*) gene to determine similarity with other LAB members. From the 19 isolates, only 11 isolates were identified; 6 isolates (C1, V2, D3, J2, I1, and S4) were identified by using 16S rRNA amplification, and 5 isolates (C1, V2, D3, I1, and S4) were identified by using *bsh* amplification. The rest of 8 isolates were not able to be identified either by using 16S rRNA or *bsh* gene. By using 16S rRNA as primers, 6 isolates (C1, V2, D3, J2, I1, and S4) had amplified bands at 1500 bp in length (Figure 2), which agreed with the target of amplification. Then, the amplified bands were sequenced.

The amplified bands obtained in Figure 2 were sequenced, and the resulting sequences were analyzed by using the BLAST program for determining the similarity of sequences across the full bacterial 16S rRNA gene bank recorded by GenBank. The BLAST analysis showed that the C1 isolate was genetically similar to *L. plantarum*; the V2 and J2 isolates were similar to *P.acidilactici*; and the D3, I1, and S4 isolates were similar to *L. fermentum* (Table 3). According to Claverie and Notredame (2007), two or more sequences are categorized as a homolog when the nucleotide show more than 70% of the similarity.

**Table 2.** Morphology and physiology of selected isolates

| Isolation spot | Code | Gram stain | Shape | Catalase | Motility | $CO_2$ detection |
|---|---|---|---|---|---|---|
| Crop | C1 | Positive | Rod | − | − | − |
| Crop | C2 | Positive | Rod | − | − | − |
| Crop | C3 | Positive | Rod | − | − | − |
| Proventriculus | P1 | Positive | Rod | − | − | + |
| Ventriculus | V1 | Positive | Coccus | − | − | + |
| Ventriculus | V2 | Positive | Coccus | − | − | − |
| Duodenum | D1 | Positive | Rod | − | − | + |
| Duodenum | D2 | Positive | Rod | − | − | − |
| Duodenum | D3 | Positive | Rod | − | − | + |
| Jejunum | J1 | Positive | Coccus | − | − | + |
| Jejunum | J2 | Positive | Coccus | − | − | + |
| Jejunum | J3 | Positive | Coccus | − | − | + |
| Ileum | I1 | Positive | Rod | − | − | − |
| Ileum | I2 | Positive | Rod | − | − | − |
| Ileum | I3 | Positive | Rod | − | − | − |
| Cecum | S1 | Positive | Rod | − | − | + |
| Cecum | S2 | Positive | Rod | − | − | − |
| Cecum | S3 | Positive | Rod | − | − | − |
| Cecum | S4 | Positive | Rod | − | − | − |

Note: Catalase test = did not show any gas bubbles (−), Motility test = isolate growth was not dispersed/non-motile (−), $CO_2$ detection test = homofermentative (−); heterofermentative (+)

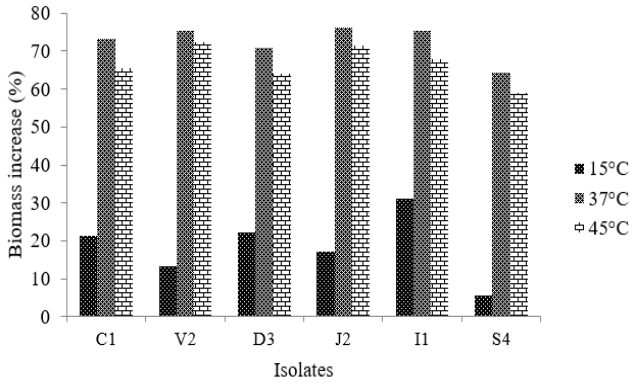


**Figure 1.** Biomass increase (%) of the isolates before and after incubation at different temperatures. (A) 15°C, (B) 37°C, and (C) 45°C for 24 h.

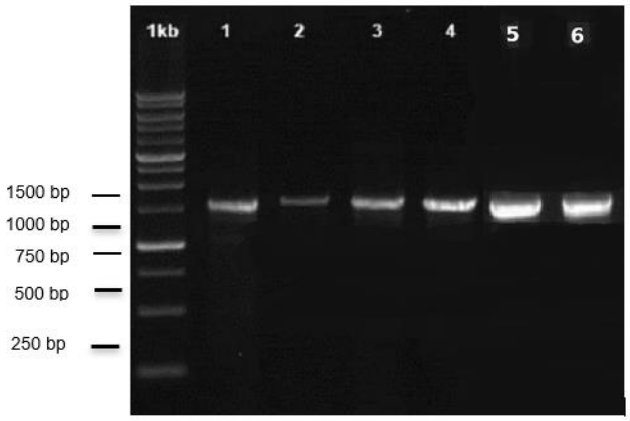


**Figure 2.** Genomic DNA amplification of six isolates by using the specific 16S rRNA primers. 1 kb = DNA marker, 1 = C1 isolate, 2 = I1 isolate, 3 = S4 isolate, 4 = V2 isolate, 5 = D3 isolate, and 6 = J2 isolate

Figure 3 depicts the comparison of the genetic relationship of the isolates C1, V2, D3, J2, I1, and S4 with several partial sequences and a complete genome for some of the strains of *Lactobacillus spp.* and *Pediococcus spp*. Herein, the sequence of *Clostridium sporosphaeroides* 16S ribosomal RNA was used as an outgroup.

**Bile salt hydrolase (bsh) gene identification**

Six selected isolates (C1, V2, D3, J2, I1, and S4) were also amplified based on the bile salt hydrolase (*bsh*) gene by using the specific primers for the *bsh* gene (Table 1), which resulted in five isolates (C1, V2, D3, I1, and S4). Furthermore, their DNA samples were amplified (Figure 4). The isolate J2, which was identified as a strain of *P. acidilactici* by using 16S rRNA primer, was unable to be identified by using the *bsh* primers. Agarose gel electrophoresis showed the amplified bands of 400 bp for C1 and 220 bp for V2, D3, I1, and S4 (Figure 4).

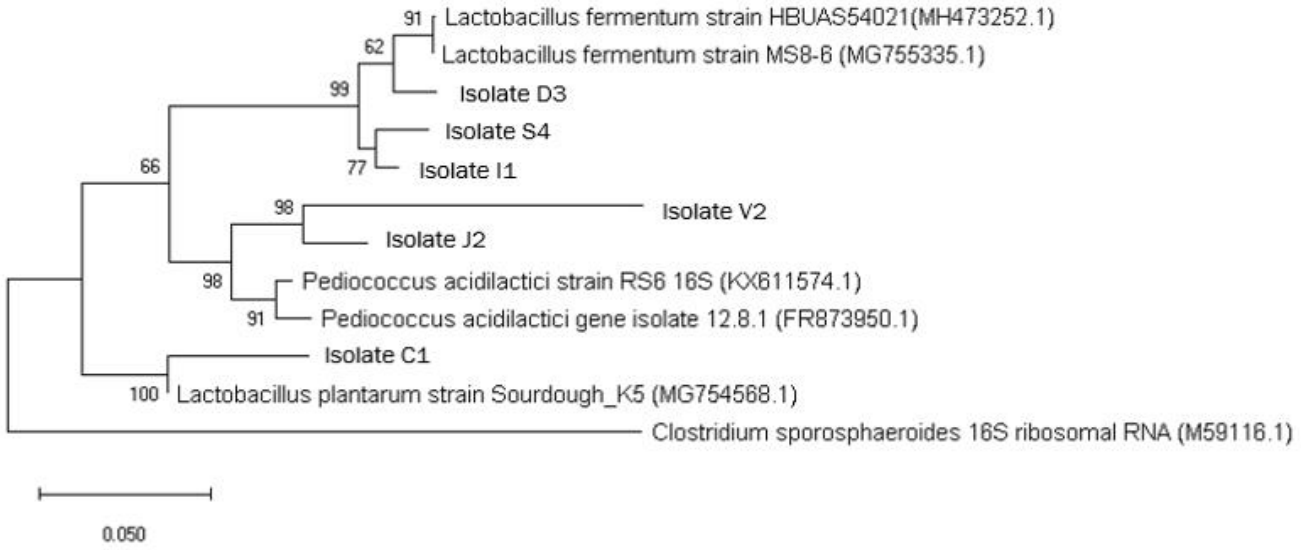


**Figure 3.** Phylogenetic tree showing the genetic relationship of the isolates D3, S4, I1, V2, J2, and C1 with other species from the genus *Lactobacillus* and *Pediococcus* based on their 16S rRNA sequences. The phylogenetic tree was produced by using the neighbor-joining method. The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site. The numbers on the branches indicate a bootstrap percentage after 1,000 replications. The access code was obtained from The National Center for Biotechnology Information (NCBI) database

**Table 3**. Comparison of the 16S rRNA sequence against the GenBank database.

| Isolate | 16S rRNA | NCBI-BLAST access no. |
|---|---|---|
| C1 | *Lactobacillus plantarum* CE7.11 96.14% | MH899287.1 |
| V2 | *Pediococcus acidilactici* RS1 90.52% | KX611572.1 |
| D3 | *Lactobacillus fermentum* HBUAS53177 95.95% | MH393037.1 |
| J2 | *Pediococcus acidilactici* isolate 12.8.1 94.53% | FR873950.1 |
| I1 | *Lactobacillus fermentum* MS8-6 96.52% | MG755335.1 |
| S4 | *Lactobacillus fermentum* HBUAS54021 96.71% | MH473252.1 |

**Table 4.** Comparison of the *bsh* gene sequences against the database in GenBank

| Isolate | *bsh* gene | NCBI-BLAST access no. |
|---|---|---|
| C1 | *Lactobacillus plantarum bsh*2O396 99.48% | KX266274.1 |
| V2 | *Lactobacillus fermentum* 845 *bsh* gene 99.46% | KM875458.1 |
| D3 | *Lactobacillus fermentum* 845 *bsh* gene 99.46% | KM875458.1 |
| I1 | *Lactobacillus fermentum* 845 *bsh* gene 100.00% | KM875458.1 |
| S4 | *Lactobacillus fermentum* 845 *bsh* gene 100.00% | KM875458.1 |

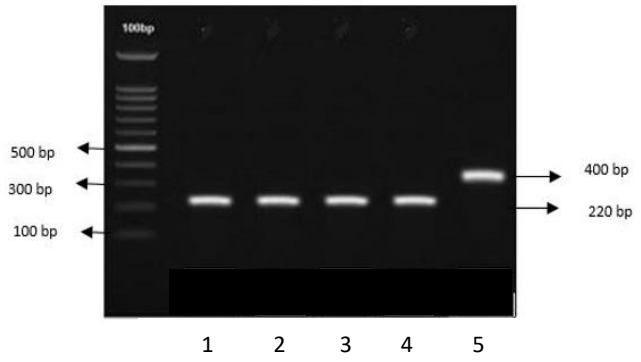


**Figure 4.** DNA amplification of the five isolates via the *bsh* gene-specific primer *bsh*lf (1 = V2, 2 = D3, 3 = I1, and 4 = S4) and the primer *bsh*lp (5 = C1)

The amplified bands were sequenced, and the resulting sequences were used for searching similarity with other bacterial *bsh* gene sequences that are available in GenBank by using the BLAST program. The results of the BLAST analysis showed a high DNA similarity (99%–100%) with several *bsh* genes for each species of *L. plantarum* and *L. fermentum*, as presented in Table 4.

The BLAST analysis of *bsh* gene sequence showed that the C1 isolate was genetically similar to *L. plantarum*, and the V2, D3, I1 and S4 isolates were similar to *L.fermentum* (Table 4). The homolog sequences and accession numbers for *L. plantarum* and *L. fermentum* from BLAST were used to construct a phylogenetic tree. Figure 5 shows the phylogenetic tree for C1, V2, D3, I1, and S4.

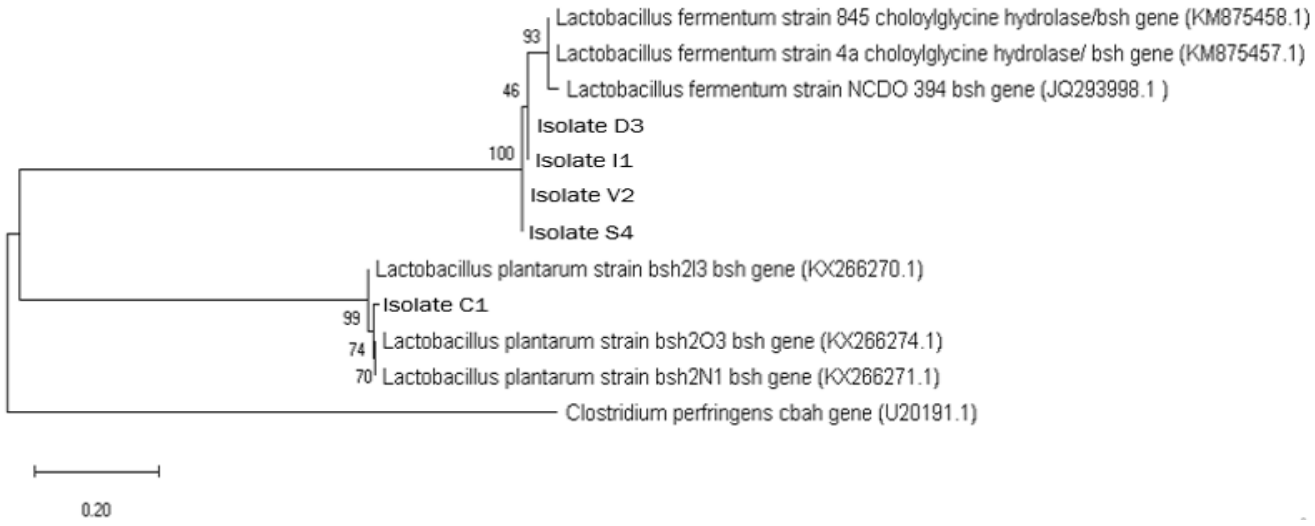


**Figure 5.** Phylogenetic tree showing a *bsh* gene relationship for the isolates D3, I1, V2, S4, C1 with other species of *L.plantarum* and *L. fermentum*. The phylogenetic tree is produced by using the neighbor-joining method. The scale bar refers to a phylogenetic distance of 0.20 nucleotide substitutions per site. The numbers on the branches indicate a bootstrap percentage after 1000 replications. The access code was obtained from the NCBI database

A comparable result of identification, except for V2, was obtained by either using 16S rRNA or *bsh* gene amplification, further suggesting that DNA sequence similarity tests are effective methods for bacterial identification. BSH is an important enzyme that is produced as a response to the bile salt. The *bsh* gene is responsible for the production of BSH enzyme in LAB. The ability to produce BSH and to tolerate high concentration of bile salt is often classified as one of important traits of probiotics. An interesting finding was observed for the V2 isolate. Based on 16S rRNA amplification sequence, this isolate was identified as a strain of *P. acidilactici* (Table 3). Conversely, based on the *bsh* gene similarity, it was identified as a strain of *L. fermentum* (Table 4). As isolate V2 was mixed within the species of *P. acidilactici* and *L. fermentum*, further identification using species-specific primers is required to confirm whether the isolate V2 was a strain of *P. acidilactici* or *L. fermentum*. Chagnaud et al. (2001) previously reported both conserved regions and variable zones of 16S rRNA gene for the identification of LAB at species level, and this finding facilitated the genetic differentiation between *P. acidilactici* or *L. fermentum*.

In conclusion, based on 16S rRNA sequences, we were able to isolate and identify one isolate similar to *L. plantarum*, two isolates similar to *P. acidilactici*, and three isolates similar to *L. fermentum* obtained from the gastrointestinal tract of Aceh duck. Identification using the *bsh* gene from the similar isolates showed one isolate similar to the *L. plantarum* strain *bsh*2O396 and three isolates similar to the *L. fermentum* strain 845. The identified LAB strains exhibited optimum growth at 37°C. In the future, commercially available probiotics of *L. plantarum*, *P. acidilactici*, and *L. fermentum* originated from ducks could be used to treat poultry against bacterial infection.

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