**Lactococcus lactis** and **Lactococcus garvieae** identified from Aceh buffalo (**Bubalus bubalis**) associated lactic acid bacterial isolates

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**Abstract.** Lactic acid bacteria (LAB) are one of the beneficial bacterial groups due to their metabolic activities, including production of bacteriocin that are potentially applied as biopreservation. The objectives of the study were to identify LAB isolate coded as BK-03 using biochemical and molecular approach as well as to determine the potency of the isolate in producing bacteriocin. The isolate of BK-03 recovered from the meat of Aceh indigenous buffalo was subjected to biochemical and molecular identification. Based on biochemical identification using the API 50 CHL, the LAB isolate of BK-03 was identified as **Lactococcus lactis** ssp lactis 1 with 98.6% proximity percentage. However, different results were obtained from molecular approach using 16S rRNA, as the isolate was identified as **Lactococcus garvieae** with a similarity index of 99%. The results of the antimicrobial assay showed that the crude extracts of BK-03 bacteriocin did not show an inhibition zone to **Escherichia coli**, but had an inhibitory zone against **Staphylococcus aureus** with an average diameter of 7.45 mm.

**1. Introduction**

Lactic acid bacteria (LAB) are increasingly explored due to their potentials in terms of the production of lactic acids and bacteriocin. These group of bacteria residing as animal intestinal microbiota and produce lactic acids as part of their carbohydrate metabolism through fermentation. Furthermore, these bacteria might secrete antimicrobial substances especially bacteriocin, i.e. small ribosomally-synthesized antimicrobial peptides or proteins, that might kill or inhibit closely related (narrow spectrum) or non-related bacteria (broad spectrum) [1].

This bacteriocin is potentially applied as a food preservative agent since it can reduce or eliminate the growth of major pathogenic bacteria causing food spoilage [2] without deteriorating the eukaryotic cells or intestinal microbiomes [3]. Other investigators demonstrated that the bacteriocin of LAB, i.e. **Lactobacillus** sp. strain SCG 1223, were effectively applied to preserve fresh meat of chicken and beef from the contamination of **Salmonella typhimurium**, **Escherichia coli**, and **Listeria monocytogenes** [4]. Not surprisingly, LAB is explored to be alternatives for preservative agents and applied in various fields, including food industries as well as pharmaceuticals [5,6].
LAB can be recovered from various sources, including from buffalo’s milk \[7,8\] or meat \[9,10\]. Previously, the LAB of BK-03 was recovered from Aceh buffalo meat \[11\]. The objectives of the present study were to identify a LAB isolate of BK-03 using biochemical and molecular approaches and evaluate its potential for bacteriocin producer.

2. Materials and Methods

2.1. Bacterial Isolate Cultivation, Morphology, and Characterization

The isolate of LAB BK-03 recovered from Aceh buffalo meat was re-cultivated on the De Man, Rogosa and Sharpe (MRS) agar (Merck, Germany) followed by morphological characterization. The isolate was then subjected to bacteriocin activity assay using paper-disk diffusion method against Staphylococcus aureus and Escherichia coli as bacterial target.

2.2. Bacteriocin assay

In the assay, the LAB isolates of BK-03 grown overnight in MRS broth at 37 °C were centrifuged at 10,000 rpm for 5 min. The supernatant was then collected and inoculated into a sterile paper disk that was placed on Mueller Hinton agar plates that had been previously inoculated with the target bacteria. A tetracycline paper disk was used as positive control before the plates were incubated for 24h at 37 °C followed by inhibition zone measurement.

2.3. Biochemical-Molecular Identification and Phylogenetic Analysis

The isolate of LAB BK-03 was tested for its activity of catalase and sugar fermentation using API® 50 CHL (bioMérieux, Inc, USA) test kit according to the manufacturer instruction. DNA extraction was conducted using a QiAmp DNA minikit (Qiagen, USA) following the instruction from the manufacture with modifications. The modifications were made in the initial sample volume and final elution volume. In the study, a total 1 mL of LAB BK-03 overnight-grown on MRSB media was used as initial sample volume and a total of 200 uL was used as final elution volume.

Amplification for 16srRNA gene was performed using universal primer sets of Bact 27F-Uni 1492R (F: 5’-AGAGTTTGATCCTGGCTCAG-3’; R: 5’-GGTTACCTTGTTACGACTT-3’). The PCR amplification was performed in 50 μL reactions containing 1 μL primer Bact 27F (20 pmol/mL), 1 μL primer Uni 1492R (20 pmol/mL), 3 μL sampel DNA, 1,25 μL Taq DNA polymerase, 5 μL buffer 10x, 0,4 μL dNTP mix (dATP, dGTP, dCTP, dTTP), 4 μL MgCl2, and 34,35 μL ddH2O. The mixture was then run in a thermocycler with condition as follow: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, annealing 57.1°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were analysed on 1 % (w/v) agarose gels with SYBR safe DNA stain at 100V for 45 min in 1X TBE buffer and visualized using gel documentation to assess bands of the expected size (1.500 bp).

The PCR products showing the expected size were then purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Germany) prior to sequencing. DNA sequencing was performed by an external provider (PT. Genetika Science). The sequence result was then compared to other sequences in gene bank database using BLAST-N program. After the alignment, the gene sequence of 16s rRNA was subjected to its homology percentage determination with other sequences followed by a generation of phylogenetic tree with neighbour joining approach using MEGA 7.0.

3. Results and Discussion

Generally, the morphological features of LAB isolates used in this study were macroscopically round-shaped colony with cream pigmentation, smooth (entire) margin, and convex elevation grown on MRS agar (Figure 1a). Microscopic observation, the isolates were Gram-positive cocci (Figure 1b). For the catalase assay, the isolate showed a negative determinant. In the current research, the LAB isolate of BK-03 is characterized to have Gram-positive coccus with negative catalase and round-shaped colony with cream pigmentation, smooth (entire) margin, and convex elevation grown on MRS agar. These characteristics are commonly associated with LAB isolate that is mainly indicated from the Gram positive, non-spor forming, negative catalase with rod or coccus-shaped cells, and producing lactic acid as main products from sugar fermentation. For the catalase assay, the isolate showed a negative
determinant once the isolated mixed with H$_2$O$_2$ 3%. This aligns with previous study signifying that sixty-two isolates identified as LAB having negative-catalase test [12].

![Figure 1](image1.png)

**Figure 1.** The isolate of LAB BK-03 (a) colony morphology on the MRS agar (b) microscopic observation with 1000X magnification showing Gram positive cocci

Based on the biochemical assay using API® 50 CHL test kit, the LAB isolate of BK-03 was identified as *Lactococcus lactis* ssp *lactis* 1 with the percentage of significance of 98.6%. The isolate showed the ability to ferment 17 sugars in the test, i.e. ribose, galactose, glucose, fructose, mannose, mannitol, acetyl glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, trihaloes, amidon, β-gentiobiose, and potassium gluconate.

The crude extract of the LAB isolates of BK-03 showed different inhibitory activity against indicator bacteria used in the study. The isolates had positive inhibition against *S. aureus* (7.45 mm), but not *E. coli* (Table 1). LAB are widely known as one of the bacteriocin-producing bacteria by means of secretion of 20-60 amino acid containing anti-microbial peptides (AMP) that inhibit pathogenic bacteria [13]. In the present study, the LAB Isolates of BK-03 inhibited the growth of *S. aureus* indicated by the clear zone surrounding the isolates with diameter of 7.45 mm. Yet, the isolates showed no inhibition of *E. coli*. The amplification of the 16srRNA gene using the extracted DNA of the LAB isolate of BK-03 as a template showed products at the molecular size of 1.500 bp (Figure 2).

![Figure 2](image2.png)

**Figure 2.** Visualization of the amplified 16srRNA gene of LAB isolate of BK-03 (Lane 1) on 0.8% agarose gel electroporated for 45 min at 100 v, M is marker.

| Target Bacteria           | Zone of inhibition (mm) | Positive control (tetracycline) |
|---------------------------|-------------------------|---------------------------------|
| *Staphylococcus aureus*   | 7.45 ± 0.49             | 26.8 ± 1.13                     |
| *Escherichia coli*        | 0                      | 21.55 ± 0.64                    |

**Table 1.** Inhibition activity of the LAB isolates of BK-03 against indicator bacteria
The construction of phylogenetic tree using a full sequence of 16s rRNA gene of LAB isolate of BK-03 is shown in Figure 3. Based on the NCBI BLAST proximity, the LAB isolate of BK-03 had the highest values to *Lactococcus garvieae* with a similarity index of 99%. Moreover, in the phylogenetic tree analysis, the LAB isolate of BK-03 indicated the high relationship with *Lactococcus garvieae* strain CAU:204, strain CAU:172, and strain CAU:178 with bootstrap value of 61 (Figure 3).

![Figure 3. Phylogenetic tree of the LAB isolate of BK-03 (red box) based on the full sequence of 16s rRNA gene with neighbour joining approach using MEGA 7.0.](image)

In terms of identification, the LAB isolate of BK-03 was identified as *Lactococcus lactis* ssp *lactis* 1 with the percentage of significance of 98.6%. Phenotypic test using API® 50 CHL kit is useful to discriminate LAB isolates to the genus or species level based on the database owned and managed by *bioMérieux, Inc* [14].

Despite its discriminatory power, the phenotypic identification based on biochemical tests might have variability. The variability might be related to physiological features among isolates as well as limited species database, so the test requires further confirming tests, i.e. molecular identification [15]. Based on the 16s rRNA, the LAB isolate in the present study might be *Lactococcus garvieae*. *Lactococcus garvieae* is commonly found as fish pathogens [16], whereas others also isolated the bacteria from Bovidae animal, i.e., Indian bison [17] or other meat [18].

4. Conclusion
It is concluded that the LAB isolate of BK-03 was identified as *Lactococcus lactis* ssp lactis 1 with 98.6% proximity percentage using biochemical approach or as *Lactococcus garvieae* with a a similarity index of 99% using molecular approach of 16S rRNA analysis. The LAB isolates had positive inhibition against *S. aureus*, but not *E. coli*.

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