Identification of lactic acid bacteria as antimicrobial from milk Toraja Belang buffalo

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Abstract. Lactic acid bacteria can produce antimicrobial compound. The bacteria can be found in milk. The objective of this research is to study antibacterial activity and identification of lactic acid bacteria from milk of Toraja Belang Buffalo South Sulawesi. The lactic acid bacteria were isolated by pour plate method using MRSA containing 1% CaCO₃. Antimicrobial activity of the isolates against Salmonella typhi, Staphylococcus aureus and EPEC were assayed using agar well diffusion method. The result showed that among 14 isolates of LAB, the most potent antibacterial activity of Cell Free Supernatant against to EPEC is K1B, Staphylococcus aureus is K4A2 and Salmonella typhi is K5B2. Based on the 16S rDNA sequence, K1B, K4A2 and K5B2 isolates were identified as Enterococcus faecalis ATCC19433 with similarity 99.758%, 100% and 99.759% respectively.

1. Introduction
The use of antibiotics for the long term can lead to resistance and damages commensal microflora in the human intestine. This can affect increasing the occurrence of allergies and autoimmune diseases than influence effectiveness and cost of treatment [1]. Use of antibiotics for the long term can lead to resistance and damages commensal microflora in the human intestine. This can affect increasing the occurrence of allergies and autoimmune diseases than influence effectiveness and cost of treatment [2].

Lactic acid bacteria are non-pathogenic and can be found in a variety of habitats, such as fermented foods and milk [3]. The LAB of milk as an antimicrobial has been widely practiced, among them [4] who found Lactobacillus fermentum, Pediococcus spp, Leuconostoc mesenteroides subsp. mesenteroides, and Lactococcus from fermented milk. Enterococcus faecium and Lactobacillus lactis from Camel milk in Egypt [5]. Pediococcus shahsavar, Enterococcus alborz, Pediococcus sigal and Enterococcus faecalis from cow milk in Iran [6]. Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus pentosus, Lactococcus lactis from buffalo milk in North Sumatra [7].

One of animal capable producing milk and never been reported diversity and potential those antimicrobial its from Belang Buffalo Toraja (Bubalus bubalis Linn). Belang Buffalo is an endemic
animals Tana Toraja, Makassar South Sulawesi. The population of Toraja Belang Buffalo has decreased, so it needs to be conserved. One of the conservation ways is to study the diversity and potential of lactic acid bacteria from the milk of Belang Buffalo. Therefore, this research needs to be done. The objective of the research is to isolate and identify lactic acid bacteria from the milk of Toraja Belang Buffalo which has potential as antimicrobial.

2. Materials and Methods
Pathogen bacteria are EPEC ATCC 25922, Staphylococcus aureus 134-P, and Salmonella typhi ATCC 58105535 obtained from Microbiology Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.

2.1 Isolation of lactic acid bacteria from fresh milk of Toraja Belang buffalo
Fresh milk samples were obtained from Belang Buffalo, South Sulawesi Province, Indonesia. Lactic acid bacteria were isolated by pour plate technique using MRSA containing 1% CaCO₃ [8]. Fresh milk 25 mL was diluted with 225 mL NaCl, 0.86 % and it made dilution series up to 10⁻⁶ dilutions. The suspension of each dilution 0.1 mL inoculated into MRSA medium in Petri dish according pour plate method. Bacterial cultures from fresh milk were incubated at 37° C for 48 hours. Each isolate was purified and it carried out Gram stained and catalase test. The selected isolates were stored at -20° C containing 20% glycerol.

2.2 Assay of pathogenicity
Pathogenicity assay was carried out based on hemolytic activity of LAB. Hemolysis assay was carried out using Columbia agar plate containing 5% sheep blood. The Culture of each bacterial isolate on the MRSA medium at 24 hour incubation was taken 1 loop and inoculated on the blood agar surface. Cultures were incubated at 37° C for 24 hours, then observed β-hemolysis (clear zone), α-hemolytic (green zone) and γ-hemolytic (without clear zone).

2.3 Antimicrobial activity assay of lab cell free supernatant against pathogenic bacteria
Antimicrobial activity of LAB were tested against pathogenic bacteria well diffusion method [9, 10]. Each isolate of LAB from culture stock was taken one loop and inoculated into 10 mL MRS broth and incubated at 37 °C for 24 h. Bacterial culture was centrifuged at 10,000 rpm, 4 °C for 10 min. The supernatant was filtered using 0.22 μm milipore membrane. Pathogenic bacteria consist of Salmonella typhi ATCC58105535, Staphylococcus aureus 134-P, and EPECATCC 25922 were grown in Nutrient Broth at 37 °C till logarithmic phase with 10⁶ cell/mL density. The bacteria cultures 100 μL of each pathogenic bacteria was spread on the surface of NA medium and then incubated at 4°C for one hour. The well was made on the NA medium with 5 mm diameter and filled with 50 μL of LAB. The culture was incubated 37° C for 24 hours. The antimicrobial activity was determined by measuring of the clear zone around the wells with three replications. The data of antimicrobial activity was analysed one-way analysis of variance (ANOVA) with α = 5% using SPSS 16.0 software program.

2.4 Identification of lactic acid bacteria as antimicrobial
The LAB isolate was identified based on phenotype character and similarity of 16S rDNA sequences. Phenotypes of bacterial isolates were characterized based on Bergey's Manual of Systematic Bacteriology. The DNA of LAB was extracted with modified of i-genomic Soil DNA Extraction Mini Kit. The 16S rDNA sequence of LAB was amplified [9, 11]. Sequence of 16S rDNA was amplified using 27f primer (5'-AGAGTTTGATCCTGGCTC AG-3') and 1492r (5'CTACGGGCTACCTTGTTACGAG-3'). The PCR mix reaction consist of 25 μL PCR master mix, 2.0 μL each primer, 19.0 μL ddH2O, and 2.0 μL DNA template. The sequence of 16S rDNA was amplified using a 35-cycle PCR program consist of the initial denaturation at 94 °C for 5 min; denaturation at 94 °C for1 min; annealing 54 °C for 30 seconds, extension at 72 °C for 1 min, and final
extension 72 °C for 7 minutes. Amplicon of 16S rDNA was purified and sequenced at First base Malaysia. The 16S rDNA sequence was alignment with the 16S rDNA sequence reference to construct phylogeny tree based on Neighbor Joining algorithm with bootstrap 1000 replications using MEGA program [12].

3. Results and Discussion

3.1. Isolation of lactic acid bacteria from fresh milk of Toraja Belang buffalo

Lactic acid bacteria can be found in nutrient-rich habitats such as milk. Milk contains many nutrients, which are good for LAB growth, such as proteins, fats, carbohydrates (lactose), vitamins and minerals. Lactose is utilised by the bacteria for its metabolism, producing lactic acid so milk becomes acidic.

Lactic acid bacteria from fresh milk of Toraja Belang Buffalo was obtained 14 isolates that grown in MRSA medium at 37 °C. The isolated are K1A, K1B, K2B1, K2B2, K2C1, K2C2, K3A, K4A1, K4A2, K5A1, K5A2, K5B1 and K5B2. The whole of isolates were positive gram, negative catalase. The whole isolates were gamma haemolytic or non haemolytic, which means the isolates were nonpathogenic. Lactic acid bacteria as antimicrobial should be non-pathogenic. Hemolytic means that the isolates are virulence and causing high infection [10, 12].

3.2. Antimicrobial activity of lab cell free supernatant against pathogenic bacteria

The fourteen isolates of LAB were tested their activity against to pathogens ie EPEC, Salmonella typhi and Staphylococcus aureus. The results of antimicrobial activity that LAB, have different ability inhibiting pathogenbacteria (Figure 2). Isolate K5B2 has inhibition zone 9.23 mm greater than K5A2 was 4.53 mm against Salmonella typhi ATCC58105535 (p <0.05). The LAB supernatant of K1A, K1B, K2B1, and K2B2 were able to inhibit the growth of EPEC ATCC25922 with inhibition zone 11.4, 13.2, 10.13, and 10.87 mm respectively (p> 0.05). The supernatant inhibition activity of LAB against Staphylococcus aureus 134-P were varies (p <0.05) among isolate of K1B, K2B1, K2C1, K2C2, K4A1, K4A2, K5A1, and K5B2. The highest antimicrobial activity capable of inhibiting EPEC is K1B, Staphylococcus aureus is K4A2 and Salmonella typhi is K5B2.
The ability of lactic acid bacteria producing antimicrobial compounds such as organic acids (lactic acid and acetic acid) and or bacteriocin that can suppress the growth of pathogens [14]. Antibacterial activity from lactic acid bacteria is caused by the production of organic acids (acetic acid and lactic acid) so as to decrease the pH of the medium or due to competition of nutrients. In addition, antibacterial activity is also due to bacteriocin. The differences ability of isolates to inhibit pathogen may be due to differences in the component or the amount of antimicrobial produced by the LAB isolates [13].

The mechanism of inhibition of bacterial growth by organic acids is due to its hydrophobic nature, thus facilitating the diffusion of protons into cells through cell membranes. This causes the intracellular pH to be higher than the extracellular pH. In the cell, the organic acid will dissociate and decrease the intracellular pH by means of proton release. This proton release results in disruption of metabolic functions (such as substrate translocation and oxidative phosphorylation), so that bacterial cell growth becomes inhibited [15].

3.3. Identification of lactic acid bacteria as antimicrobial  
The K1B, K4A2 and K5B2 isolates from fresh milk of Toraja Belang buffalo as antimicrobil were identified based on 16S rDNA sequence. The phylogenetic tree (Figure 3) showed that these isolates are closely related to the Enterococcus faecalis ATCC 19433. Based on the 16S rDNA sequence, K1B, K4A2 and K5B2 isolates were identified as Enterococcus faecalis ATCC19433 with similarity 99.758 %, 100% and 99.759%, respectively (Figure 3).

Enterococcus as antimicrobial was found from camel milk in Egypt [5] and cow milk from Iran [6]. Although some studies suggest that Enterococcus faecalis can cause urinary tract infections, it is common in immune compromised cases or as opportunistic cases [15].
Figure 3. Phylogenetic tree of K2B1 isolate and reference strain based on 16S rDNA sequence with Neighbor-Joining, Tamura-Nei algorithm with bootstrap 1000.

4. Conclusion
The isolate K1B, K4A2 and K5B2 can inhibit the pathogen growth and were identified as Enterococcus faecalis ATCC19433T.

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