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Identification and Characterization Indigenous of *Lactobacillus sp* from Bovine Rumen Fluid of Slaughterhouse

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Abstract

The discovery and characterisation of indigenous lactic acid bacteria (LAB) are important for diversity microbes as candidate probiotic. This research was aimed to identify lactic acid bacteria isolate from isolation process of local bovine rumen fluid from slaughterhouse in Surabaya Indonesia. Genotypic testing was conducted by analyzing 16S rDNA and biochemical identification. DNA of sample isolate was isolated and then amplified in vitro through the PCR method. Determination of nucleotide sequence of 16S rDNA was performed with sequencing method. The result of nucleotide sequence was than compared with *GenBank* database. The BLAST was then applied to identify the phylogenetic tree. Based on the biochemical characterization and nucleotide sequences, that isolate was identified as *Lactobacillus rhamnosus subsp* TG15. The result of this research showed that *L.rhamnosus subsp* TG15 showed viability bacteria in MRSA as control as much as 1.1×10^8 CFU/ml, mean while in MRSA pH 2, *L.rhamnosus subsp* TG15 showed its viability as much as 9.3×10^6 CFU/ml. Viability of isolate on bile tolerance 0.3% was 2.4×10^7 CFU/ml). Index antagonist bacteria test on *S.aureus* showed inhibition diameter as much as 2.0 mm and in antagonist test on *E.coli* as much as 2.5 mm. Based on the result, it could be concluded that this research found a new strain of lactic acid bacteria, *L.rhamnosus subsp* TG15 and that isolate has ability as the probiotic candidate.

Keywords: *L.rhamnosus subsp*TG15; survival on acidity; bile salts;*S.aureus*and*E.coli*.

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1. Introduction

One of the biggest problems in the poultry farming is feed. Feeding costs is the biggest component in the production cost composition of poultry industry which is around 65-70% from the total costs. The poor continuity of the feed supply availability becomes one obstacle in producing competitive poultry product. In other words, feeding cost

is a crucial factor that determines the competitiveness of the poultry industry [1]. Probiotic is defined as a mono culture or mixed culture from living microorganism that has advantages for the living of indigenous microbiota in the human and animal [2]. Another definition probiotics are viable, nonpathogenic

microorganisms (bacteria or yeast) that are able to reach the intestines in sufficient numbers to confer benefit to the host [3, 4]. The most common microorganism species used as probiotic are *Lactobacillus*, *L.acidophilus*, *L. plantarum*, *L. rhamnosus*, *L.reuteri*, *L fermentum*, *L.lactus*, *L.brevis*, *L.casei*, Bifidobacterium, *Lactococcus*, *Leuconostoc* [5–7]. Commonly used bacterial probiotics include *Lactobacillus* species, *Bifidobacterium* species, *Escherichia coli*, and *Streptococcus* species. *Lactococcus lactis* and some *Enterococcus* species have also been used [8].

Probiotics may be able to decrease colonization by pathogens that exploit inflammation to enhance colonization by decreasing innate inflammatory responses, including macrophage activation phenotypes. Probiotics are also well documented to increase modulation of adaptive immunity [9]. Microorganism in digestive tract will be balanced when probiotic has already administered in the digestive system, so digestion process and feed digestibility of the livestock will be perfect. Lactic acid bacteria (LAB) not only influences the large intestine by affecting the intestinal flora but also by affecting other organs via the modulation of immunological parameters and intestinal permeability or via the production of bioactive or regulatory metabolites. LAB are capable of producing inhibitory substances, other than organic acids, that have inhibitory activities against different microorganisms [10].

The discovery and characterisation of indigenous lactic acid bacteria (LAB) are important for diversity microbes as candidate probiotic. This research aimed to identify lactic acid bacteria isolate from local bovine rumen fluid from slaughterhouse in Surabaya Indonesia by assay on acidity survival, bile salts survival, pathogenic bacteria survival.

2. Materials and Methods

LAB were isolated from liquid rumen, ten grams of each sample were put into 50 ml of MRS (Oxoid) broth and incubated at 37°C for 3 days. For the isolation of LAB, the supernatants were diluted and plated in MRS (Oxoid) agar medium, [11]. Colonies characterized by different morphologies were randomly picked from the agar plates at the highest dilution. Gram-positive, catalase-negative, non-motile, rod shape and

cocci isolates were cultivated in the broth medium at 37°C for 24 h, and re-streaked into the same agar medium.

2.1. DNA isolation and Amplification by PCR

DNA isolation was performed using the Ausubel method withsome modification. DNA Amplification by PCR was conducted using: Kit Platinum Taq DNA Polymerase High Fidelity (Invitrogen) The 16S rDNA of bacteria was amplified by PCR using universal primers for general bacteria. DNA Amplification by PCR was performed using the Ausubel method with some modification [12].

2.2. Sequence analysis of 16S rDNA

The DNA sequencing of 16S rDNA coding was performed by the 1st BaseMalaysia. Analysis of sequencing results was carried out by BLAST, using the database available at www.ncbi.nlm.nih.gov.

2.3. Acid tolerance test

Acid tolerance test was performed through total plate count method with modification on centrifugation condition and pH media [13]. Stock culture was transferred to 10 ml MRSB for 24 hours. The culture centrifuged at 3500 rpm for 15 minutes. After that, the pellet was washed with 0.85% of sterile NaCl solution and the re-suspensed cell was added 1% (v/v) in 10 ml MRSB as control, as well as MRSB that was adjust in pH 2 by using HCl, and then incubated for 90 minutes at 37°C. After incubation ended, total plate count on MRSA was performed by using pouring method and re-incubated for 48 hours at 37°C. **Antagonistic Test on Enteric Pathogen Bacteria.** Antagonistic test on enteric pathogen was performed with agar diffusion method with modification in the pouring of pathogenic bacteria culture [14]. LAB culture was grown on MRSB medium at 37°C for 18-20 hours. After that, pathogenic bacteria were inoculated as much as 1 ose in the nutrient broth media, to be incubated for 24 hours at 37°C. After incubation ended, 0.2 ml of the incubated bacteria was taken and placed in to 100 ml nutrient agar media (0.2%) to be mixed well (homogen), and then placed in to petri dish with 1-20 ml for each dish until solid. After agar plate became solid, hole was created in the agar plate media with 6 mm diameter. Five holes were created for each petri dish. LAB culture from MRSB was spotted into the hole as much as 50 µl to be then incubated for 24 hours at 37°C. MRSB medium without LAB was used as the control.

3. Result and Discussion

3.1. Genotypic identification: DNA Amplification with PCR and identifying coding genes based on nucleotide sequence of 16S rDNA genomes

Genotypic characterization of lactic acid bacteria isolate TG15 was carried out based on 16S rDNA gene coding DNA to determine its genus and strain. 16S rDNA coding DNA can be applied as a molecular marker for species definition, since this molecule exists in every organism with identical function in all organisms [15]. The biochemical characteristics and 16S rDNA gene were analyzed to identify the taxonomic position of the strain [16]. Nucleic acid molecule type on identification of isolate using 16S rDNA has a Query Length of 1315 bp. The isolate's nucleotide structure obtained was then identified using BLAST (Basic Local Alignment Search Tool) program in www.ncbi.com. An advanced test was conducted on code TG15 lactic acid bacteria isolate with 16S rDNA and phylogenetic tree structure with 90-98% degree of similarity. The majority of bacteria resembling TG15 isolate originated from *Lactobacillus* genus. Based on the degree of similarity of nucleotide structure, the closeness in position with *Lactobacillus rhamnosus* GG (ATCC 53103) (accession NC_013198.1; 98% identity), and inherited traits in congruence with microbe identification system, the isolated strain was identified as *Lactobacillus rhamnosus* TG15.

The LAB was successfully isolated from the samples using a selective medium of MRS agar. Lactic acid produced by *Lactobacillus* creates acid environment that can inhibit pathogenic bacteria growth. *Lactobacillus* is a heterogeneous bacteria group that consists of 135 species and 27 subspecies [17]. The other research showed that some LAB strains have function as competitive inhibitors on pathogenic organism [18].

3.2. Biochemistry assay of *Lactobacillus rhamnosus* TG15

In this research, colony of TG15 lactic acid bacteria were capable of growing on MRSA selective medium. Based on Gram staining, this lactic acid bacteria isolate was Gram positive, rod shaped. Biochemistry assay was applied in examining lactic acid bacteria isolates from liquor rumen of beef cattle. Based on biochemistry assay show that the isolate was capable of performing fermentation for several carbohydrates: lactose, arabinose, cellobiose, raffinose, and manitol and positive esculin, but negative fermentation to sucrose, gluconate, ribose, xylose, arginin, galaktose and rhamnose. This was comparable with the study of the other researcher show that strain *L. rhamnosus* GG

Table 1: Viability tolerance of *L. rhamnosus* TG15 in pH 2 and bile salt.

Isolate	Viable bacteria (CFU/ml)		
	MRS Agar (control)	MRS Agar pH 2	Bile tolerance 0.3%
Lactobacillus rhamnosus	1.1×10^8	9.3×10^6	2.4×10^7

(ATCC 53103) and HN001 for several carbohydrates that were distinct for the different metabolic clusters: L-Rhamnose, Cellobiose, L-Sorbose and α-Methyl-D-Glucoside [19]. Based on morphology and biochemistry assay, lactic acid bacteria isolated from liquor rumen beef cattle could be identified as *L. rhamnosus*

3.3. Lactid Acid Bacteria Survival Test on acidity and bile Salts

The result of this research show that *L. rhamnosus* TG15 have viability tolerance in low pH. The results showed that the concentration of *L. rhamnosus* TG15 in pH 2 was 9.3×10^6 CFU/ml, compared viability of *L. rhamnosus* TG15 in pH 7 (control) was 1.1×10^8 CFU/ml in MRS Agar and the viability tolerance of *L. rhamnosus* TG15 in bile salt 0.3% was 2.4×10^7 CFU/ml (table 1). The viability tolerance in the bile salt condition is one of the main criteria for in vitro selection of potentially probiotic bacteria and microbes [20]. Because the bacterial cell wall is comprised mainly of phospholipids, bile salt which is an emulsifier and solubilizes the lipid that can damage the bacterial cells [21]. The result of this research show that *L. rhamnosus* TG15 have bile salt tolerance. The results showed that the concentration of *L. rhamnosus* TG15 in bile tolerance 0.3% was 2.4×10^7 CFU/ml. The other researcher was done to isolate, select and identify lactic acid bacteria (LAB) for the probiotic properties in cattle. The results showed that the concentration of LAB from small intestine, large intestine and feces were 5.15×10^7, 5.85×10^7 and 1.25×10^2 CFU/g, respectively, tolerated to pH 3 and tolerated to bile salt [22].

3.4. Antagonistic test of *L. rhamnosus* TG15 against *E.coli* dan *S.aureus*

The result of this research show that *L. rhamnosus* TG15 have antagonistic effect against *E. coli* and *S. aureus*, index antibacterial show diameter inhibition are 2.5 mm and 2 mm, respectively. This was comparable with the study of the other researcher show that *Lactobacillus* and *Bifidobacterium* strains can directly kill *Salmonella typhimurium* in vitro [23, 24]. Probiotic bacteria can lower the luminal pH through secretion of acetic

and lactic acids, which inhibits the growth of some pathogens, including enterohemorrhagic *E. coli* (EHEC) [25]. Probiotics can directly inhibit growth or killing of pathogens by production of antimicrobial molecules including short-chain fatty acids (SCFA) and bacteriocins or microcins [26]. Short chain fatty acids, particularly butyric acid is the main energy source for intestinal epithelium cells and can stimulate the release of gastrointestinal peptide or growth factors which may affect cell proliferation [27]. In general, *Lactobacillus rhamnosus* has non-pathogen character. *Lactobacillus rhamnosus* is one of lactic acid bacteria that balance the bowel ecosystem, which later can increase *Lactobacillus* and *Bifidobacterium* number, decrease procarcinogenic enzyme activity, and inhibit pathogenic bacteria growth in the bowel and urogenital through bacteriocin mechanism such as lactate acid production [28].

4. Conclusion

Based on 16S rDNA genome nucleotide sequence result and biochemical characterization, the isolates which isolated from indigenous bovine rumen fluid was identified as *Lactobacillus rhamnosus* TG15. That isolate has tolerant characteristic on acidity, bile salt, and have antagonistic effect against *E. coli* and *S. aureus* so it has potency as probiotic candidate.

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