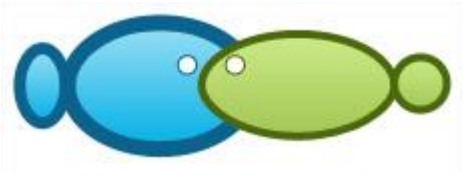

Molecular identification of lactic acid bacteria from "budu" of West Sumatera, Indonesia, as a potential probiotic

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Abstract. "Budu" is a traditional food made from fermented fish. It originates from coastal area of West Sumatra, Indonesia. The basic material of budu is mackerel (*Scomberomorus commerson*), which is fermented, salted, and dried. Budu is mostly found in Padang Pariaman, Agam, and Pasaman and has the potential to become a local feedstock based probiotic. The aim of this study is to identify lactic acid bacteria contained in budu, which act as a probiotic. Identification of lactic acid bacteria (LAB) was carried out with the help of using specific media, namely MRS Agar, antimicrobial activity using test bacteria *Escherichia coli* O157, *Sthaphylococcus aureus*, and *Listeria monocytogeneses* bacteria. Lactic acid bacteria of budu were isolated molecularly using 16S rRNA gene as marker and 27F and 1429R as primers. This study used four isolates from different locations in Padang Pariaman Regency. The method used in the research is sure with descriptive data analysis. The results obtained from this study were rod-shaped lactic acid bacteria, catalase negative, and included in the homofermentative type. The antimicrobial activity showed that the lactic acid bacteria obtained were able to inhibit the pathogenic bacteria *E. coli, S. aureus,* and *L. monocytogenes*, based on the phyloenetic analysis the bacteria obtained were closely related to Lactobacillus brevis.
Key Words: budu, fermented fish, lactic acid bacteria, *Lactobacillus brevis*, probiotic.

Introduction. Lactic acid bacteria can be isolated from the fermentation of animal and plant products, such as fermented milk, fish, etc. Budu fish is a traditional food that applies spontaneous fermentation, originating from Padang Pariaman Regency, Indonesia. Budu is salted, fermented fish. The amount of salt depends on the location of manufacturing and the storage time. Budu does not refer to a certain species, but rather the product of fermentation produced from coastal area of Padang Pariaman Regency, Agam, and Pasaman, Indonesia. Budu is mostly made of large sea fish with white meat, such as mackerels (*Scomberomorus guttatus*) and *Chorinemus tala* (Yusra et al 2014). Budu is a product of fermentation produced by people from coastal areas of West Sumatra. Budu originating from West Sumatra, Indonesia, is different from budu originating from Malaysia. Sim et al (2015) state that budu from Malaysia is a fermented, thick fish sauce, with brown color, processed traditionally by adding salt on raw anchovies (*Stolephorus* spp.).

Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Lactobacillus, Enterococcus, Aerococcus, Vagococcus, Tetragenococcus, Carnobacterium, Weisella, and *Oenococcus* are included in the lactic acid bacteria (LAB) group according to their ability to metabolize lactic acid (Ray 2005). Previous studies identified lactic acid bacteria in fermented fish, namely *Prediococcus acidilactici* bacteria from gourami (*Osphronemus goramy*) (Melia et al 2019), and *Lactobacillus plantarum* and *Pediococcus pentaceus* from *Sardinella gibbosa* traditionally prepared in Indonesia (Nurhikmayani et al 2019). *Lactobacillus plantarum* was also found in Nile tilapia (*Oreochromis niloticus*) (Nuraafi et al 2015).

The aim of this study was to identify and isolate LAB from budu, which has the potential to be a source of probiotics and, therefore, to be able to inhibit pathogenic bacteria activity.

Material and Method

Description of the study sites. Samples were collected from budu manufacturing houses from Padang Pariaman Regency, Indonesia. There are 4 isolates from Sungai Limau and Batang Gasan Sub-District used in this study (Figure 1). This research was conducted in the Laboratory of Technology of Animal Product, Faculty of Animal Science, Andalas University, Padang, Indonesia, in October 2019.

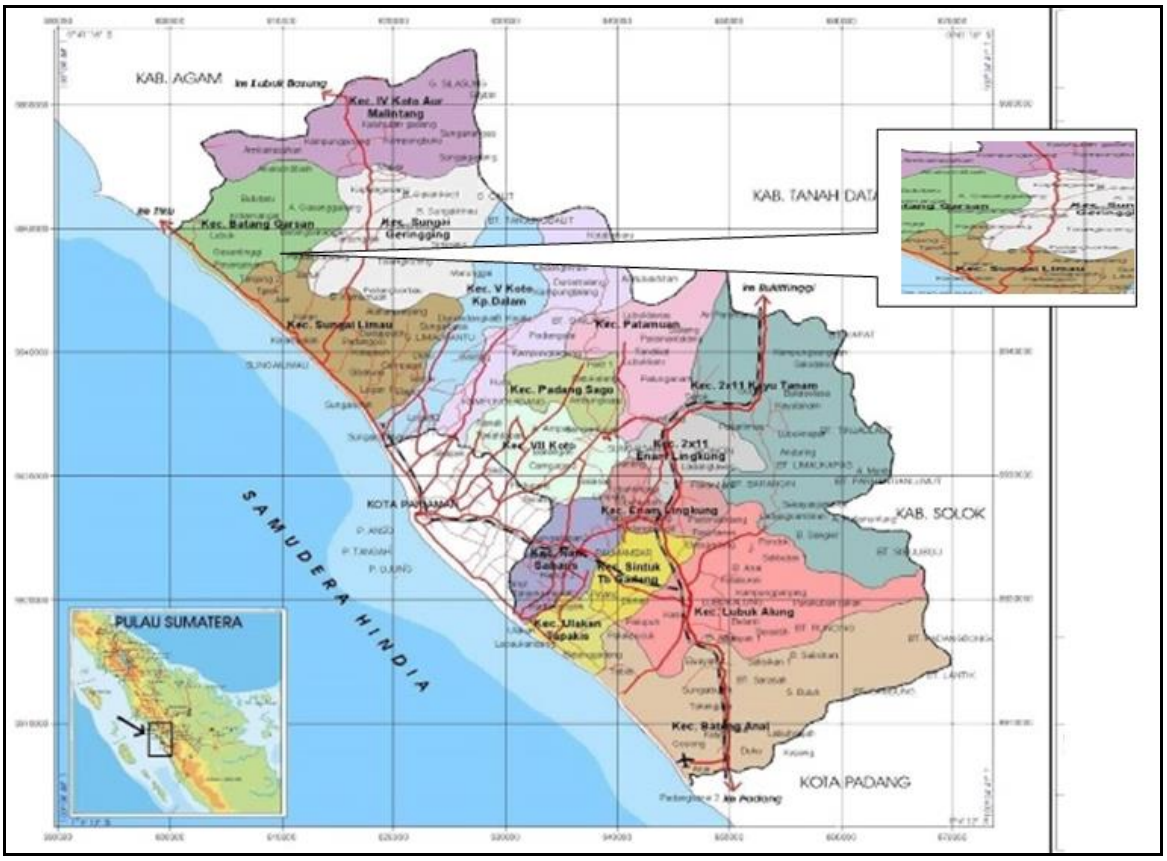

Figure 1. The location of sampling in Padang Pariaman Regency, Indonesia.

Isolation of bacteria. The isolation was conducted using the Purwati et al (2005) method. MRS broth and MRS agar (Merck, Germany) were utilized. 1 g of the sample (budu) was dissolved in 9 mL of MRS broth and incubated for 24 h at 37°C. 100 µL of the sample were planted on MRS agar using the spread plate method, and it was incubated at 37°C for 48 h. The number of bacterial colonies was counted and several single colonies were selected for further testing. The shape, color, Gram coloring, catalyst testing, and fermentation properties were observed.

Acid resistance test. 5N HCL (until it reaches pH 3) was added to 9 mL of MRS broth. A control of MRS broth was also used as a control. 1 mL of lactic acid bacteria culture was added to the prepared MRS broth and incubated at 37°C for 90 min. It was then grown on MRS agar media using the spread plate method and incubated for 48 h at 37°C. The viability was calculated with the following formula (Rashid & Hassanshahian 2014):

Viability = CFU treatment/CFU control x 100

Bile salt resistance test. 1 mL of lactic acid bacterial culture was placed into a test tube with 9 mL of MRS broth with oxgall 0.3% and 0.5%, and incubated for 5 h. The lactic acid bacterial isolates were placed on agar media with a dilution of up to 10^{-6}, and incubated for 48 h at 37°C. The viability values of the growing colonies were calculated with the next formula (Walker & Gililand 1993):

Viability = CFU treatment/CFU control x 100

Antimicrobial activity. 1 mL of culture was incubated for 24 h within 9 mL of MRS broth at 37°C. It was then centrifuged at 14000 rpm for 5 min. The supernatant was filtered with a filtration membrane of 0.22 µL. The pH of the cell-free supernatant was corrected to 6.5 with 1N NaOH to avoid the inhibition effect due to the presence of organic acid (Yang & Rannala 2012). Pathogenic bacteria grew in anaerobic conditions at 37°C for 24 h. 20 mL of cultured pathogenic bacteria (0.2%) was inserted in Muller Hinton Agar (MHA) at the temperature of 50°C. After the agar solidified, a 6 mm hole was made with a cork borer. The supernatant was collected (50 µL), inserted in each petri dish, and set aside for 15–20 min. Antimicrobial activity tests were compared using penicillin, ampicillin and kanamycin antibiotics, before being incubated for 24 h at 37°C in aerobic conditions. The reading of the results is seen from the diameter of the clear zone on the MHA media, diameter measured with a caliper.

Genomic DNA isolation of lactic acid bacteria and 16S rRNA. 1000 µL of sample of the single-colony LAB isolates from the MRS broth was pipetted and inserted in a new Eppendorf, and centrifuged in 14000 rpm for 2 min. Genomic DNA isolation was done with the Kit Promega (USA). The supernatant was discarded, and the pellet was retrieved and 480 µL 50 mM EDTA and 120 µL lysozyme were added. The mix was incubated in a water bath at 37°C for 60 min, and centrifuged at 14000 rpm for 2 min. The supernatant was discarded and the pellet was collected. 600 µL nuclei lysis solution was added. The mix was homogenized with a micropipette and incubated at 80°C for 5 min. The mix was set at room temperature. 3 µL RNase solution was added, and the mix homogenized and incubated in a water bath at 37°C for 60 min. 200 µL protein precipitation solution was added, and the new mix was vortexed, incubated in ice for 5 min and centrifuged at 14000 rpm for 3 min. Afterwards, the supernatant pipette was moved to a new Eppendorf. The pellet was discarded. 600 µL isopropanol was added and homogenized, and centrifuged at 14000 rpm for 2 min. The pellet was retrieved, supernatant discarded and 600 µL 70% ethanol was added before being homogenized and centrifuged at 14000 rpm for 2 min. The pellet was collected and supernatant discarded. The Eppendorf containing pellet was aerated for 15 min. The DNA pellet was rehydrated by adding 50 µL rehydration solution for 30 min, at 65°C. The primers R(16S-1492R, Tm 47°C, 5'GTT TAC CTT GTT ACG ACTT-3) and F (16S- 27F, Tm 54.3°C, 5'AGA GTT TGA TCC TGG CTC AG-3) were used is prepared (10 pM concentration), retrieved 90 µL dH_2O + 10µl (R and F Primer). The DNA ladder for 5 µL was inserted and voltage was set at 100 V for 40 min. Agarose gel was placed in a container and tris acetate EDTA (TAE) buffer was added until submersion. The gel was observed under a UV lamp. After the reading under UV, the sample resulted from PCR was purified to be sequenced.

Phylogenetic analysis. The sequence alignment analysis was performed by comparing the obtained sequences (query) with those in the Gene Bank with database searches in NCBI (http//:www.ncbi.nlm.nih.gov) by using BLAST (Basic Local Alignment Search Tool). Phylogenetic analysis was performed with MEGA v7.0 with phylogenetic tree displaying maximum likelihood, using Kimura-2.

Results and Discussion

Isolation and identification of lactic acid bacteria. The four isolates (BD1, BD2, BD3, and BD4) in this research (Table 1) were gram-positive bacteria, did not produce catalyst enzymes, and were rod (bacilli) shaped, homofermentative bacteria.

Table 1

Characteristics of lactic acid bacteria from budu

Isolate	*Cell Shape*	*Gram*	*Catalase*	*Fermentative type*
BD1	Bacilli	+	-	Homofermentative
BD2	Bacilli	+	-	Homofermentative
BD3	Bacilli	+	-	Homofermentative
BD4	Bacilli	+	-	Homofermentative

Acid tolerance test. LAB as a probiotic should have resistance to acid. LAB acid resistance of budu fish can be seen in Table 2.

Table 2

LAB viability of budu at pH 3

Sample	*pH 3 ($x10^6$ CFU mL^{-1})*	*Control ($x10^6$ CFU mL^{-1})*	*Viability (%)*
BD1	63	92	68.5
BD2	29	100	29
BD3	47	152	30.9
BD4	58	158	36

Bile salt tolerance. The LABs with resistance to bile salts can be seen in Table 3.

Table 3

Resistance of budu isolates against bile salts

Sample	*Oxgall 3% ($x10^6$ CFU mL^{-1})*	*Oxgall 5% ($x10^6$ CFU mL^{-1})*	*Control ($x10^6$ CFU mL^{-1})*	*Viability (%)*	
				3%	*5%*
BD1	34	26	280	12.5	9.3
BD2	26	13	212	12.3	6.1
BD3	118	68	195	60.5	34.9
BD4	21	8	142	14.8	5.6

Antimicrobial activity. The area of clear zone/zone of inhibition of each isolate varies due to the differences in their ability (Table 4).

Table 4

Diameter of the area of clear zone of antimicrobials

Sample	*Testing bacteria (mm)*		
	Escherichia coli O157	*Staphylococcus aureus* ATCC 25923	*Listeria monocytogenes* CFSN004330
BD1	10	18	0
BD2	6.11	23.32	2.7
BD3	7.12	31.36	15.2
BD4	13.18	27.32	0
Penicillin (40 µ)	2.7	2.5	2.7
Ampicillin (30 µ)	15.2	12.17	0
Kanamycin (2 µ)	16.21	8.15	0

Genome isolation and 16S rRNA reaction of lactic acid bacteria. The results of electrophoresis in Figure 2 show that LAB was successfully amplified with 16S rRNA. The successful amplification is indicated by the display of the PCR 1.5 kB product, with the

expected fragment size when using the primers 27 F AGAGTTTGATCCTGGCTGAG and 1492 R GTTTACCTTACGACTT. The result of sequencing using the reverse primer resulted in up to 885 nucleotides.

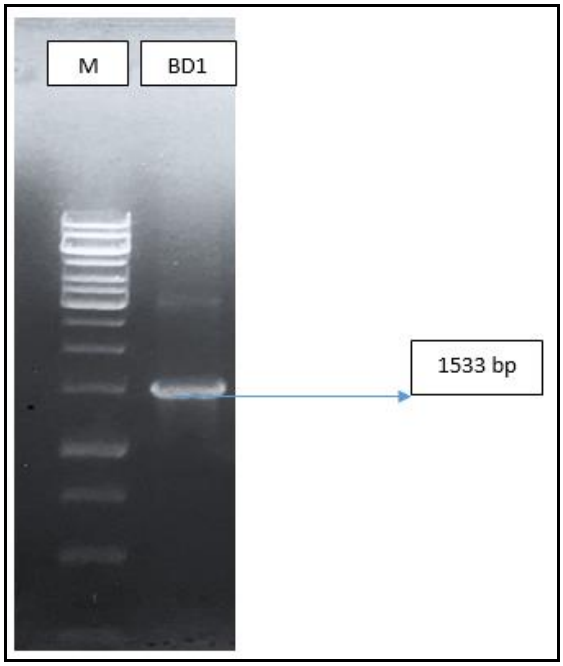

Figure 2. The result of amplification of ribosome RNA gene by using 1429R and 27F. Isolation of lactic acid bacteria from budu (M=1 kB DNA ladder).

Phylogenetic tree analysis. Based on the result of the BLAST analysis, the BD1 isolate bacteria found in budu has a 99% similarity with *Lactobacillus brevis* NRIC 0138, showing a close kinship with it. Figure 3 presents the phylogenetic tree.

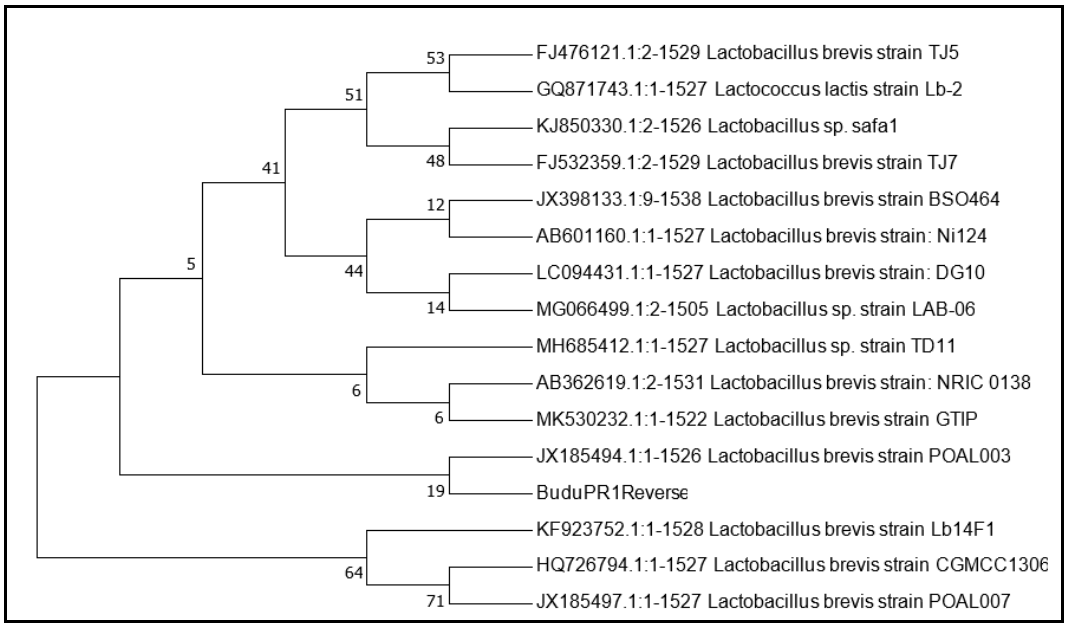

Figure 3. Phylogenetic tree of budu sample (BD1).

Identified LAB from budu fish are rod shaped, purple gram-positive bacteria, the results being similar with those of other studies on budu (Yusra et al 2014). According to Juliyarsi et al (2018), the coloring test conducted on LAB isolated from tempoyak (a

regional condiment) showed rod shaped and homofermentative LAB. This study supports the hypothesis that homofermentative LAB uses the Embden Meyernof-Parnas pathway, which produces lactic acid, 2 mol ATP from 1 glucose/hexose molecule in normal conditions, produces no CO_2, and twice as much biomass as heterofermentative LAB (Syukur & Purwati 2013).

Decrease in value viability of each isolate was in the range of 29-68%. The viability values varied in each isolate because the ability of each type of bacteria to survive at low pH also differed depending on the bacterial strain. Cotter & Hill (2003) stated that differences in the resistance to acid of several LAB species were related to the permeability of cell walls to protons (H^+). The permeability of the cell to H^+ is determined by the outflow of protons activated by membrane ATPase for Translocation H. The difference in the activity of the ATPase enzyme from each bacteria will determine the permeability of H^+. In addition, the same authors explained that several LAB species can induce a response to acid (acid tolerance response). This response will induce a homeostatic pH system, along with protection and repair mechanisms. High acidic conditions will cause damage to cell membranes and release of intracellular components, resulting in cell death (Cotter & Hill 2003).

The ability of lactic acid bacteria to survive in bile salts is related to the ability of the isolates to produce bile salt hydrolase (BHS). Isolates presented the enzyme BSH, so they were able to change the physico-chemical properties of bile salts to non-toxic. This is demonstrated by the growth of lactic acid bacteria in bile salt media, as demonstrated by Surono (2004), who claims that, while lactic acid bacteria cells are resistant to bile salts, growth still happens and does not occur when incubated in media containing 0.3% bile salts; however, a small amount of intracellular medium will seep out.

Research that has been carried out using the test bacteria *L. plantarum* by measuring the resistance of bile salts with a percentage ranging from 0.05-0.015% for 4 hours resulted in damage to the morphology of these bacteria (Bron et al 2004). A higher percentage of bile salts brings greater damage to the bacterial cell wall and, finally, cell lysis. The difference in resistance to bile salts indicates that each isolate was strain-dependent. There is a relationship between the fatty acid composition of the bacterial cell wall and its ability to withstand bile salts (Kimoto-Nira et al 2007). Cell viability can be seen in the fact that some LAB are capable of producing exopolosaccharides (EPS) which function as protection against bile salt stress (0.15-0.3%) and acidic pH (2.0-3.0) (Boke et al 2010).

The largest clear zone when testing *S. aureus* was around 18–31.36 mm. The clear zone of the BD3 isolate was 31.36 mm, and the capacity of BD2 isolate to suppress *S. aereus* bacteria was superior to penicillin (2.5 mm), ampicillin (12.17 mm), and kanamycin (8.13 mm). If compared to antibiotic control, BD1 isolate has the upper hand in inhibition ability when compared to penicillin (no clear zone) and kanamycin (8.13 mm) antibiotics. The results from this study place the BD1 samples in the moderate activity category for the *E. coli* test bacteria and strong activity in the test bacteria *S. aereus*. The activity zones of inhibition are grouped into four categories: weak activity (<5 mm), moderate activity (5–10 mm), strong activity (11–20 mm), and very strong activity (21–30 mm) (Morales et al 2003). There are several compounds produced by LAB with antibacterial properties, such as organic acids, hydrogen peroxide, and protein compounds or a specific protein complex known as bacteriocin (Yang & Rannala 2012).

According to Yusra et al (2013), the antimicrobial activity of LAB from budu fish against *E. coli* had a clear zone of 10–20 mm and against *S. aureus* of 13–24 mm. Melia et al (2019) obtained a clear zone of 21.36 mm for *E. coli*, 18.23 mm for *S. aureus*, and 5.1 mm for *L. monocytogenes* using LAB from "bekasam". The comparison with antibiotics can be seen in the Table 3. LAB from budu is able to inhibit *S. aureus* better than penicillin (2.5–2.7 mm), kanamycin (8–16 mm), and ampicillin (12–16 mm).

The phylogenetic tree constructed shows the close kinship with *L. brevis*. The pattern of the sample of bacteria forming a monophyletic group with *L. brevis* strain POAL003, BRIC 0138, TD11 and GTIP is clearly seen. Several strains, despite their close ancestry, have the ability to grow and develop under specific conditions specific to their

native habitat. The results of Wikandari et al (2012) differ from ours, identifying in bekasam *L. plantarum*, *L. pentosus,* and *Pediococcus pentosaceus*.

The results of Nurhikmayani et al (2019) also differ, identifying *L. plantarum* in isolates from fermented Nile tilapia. Several strains of LAB such as *Enterococcus faecalis, Tetragenococcus muriaticus, Lactobacillus delbrueckii* subp. *delbrueckii,* and *Carnobacterium divergens* were found in the study conducted on the isolation of LAB of Philippines' traditional food manufactured from fermented milkfish (*Chanos chanos*), chanos-rice mixture (named burong bangus) (Arcales & Alolod 2018). In the study conducted by Bagunu et al (2018), isolation of LAB from Nile tilapia resulted in the LAB species *Pediococcus pentosaceus* and *Enterococcus avium*. The different species of bacteria found occur due to the different type of fermented fish used, the material used to ferment the fish, or the method used for fermentation.

Conclusions. The ability of LAB to inhibit pathogenic bacteria characterizes it as a potential probiotic. The results of the sequencing showed that the base length of the BD1 sample bacteria was 1533 bp. Phylogenetic analysis was carried out, showing its proximity to *Lactobacillus brevis*. It is necessary to carry out further research on the application of these bacteria in food products, to determine their status as a source of probiotics.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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