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Full Length Research Paper

Probiotic Potency and Molecular Identification of Lactic Acid Bacteria Isolated from Bali Cattle's Colon, Indonesia

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Lactic acid bacteria (LAB) is a group of bacteria which able to produce lactic acid as a main product. LAB normally live in the digestive tract of humans and animals, and generally use as probiotics. On the other hand, Bali cattle are known has highly adaption to poor quality of feed so that it is suggested that specific types of LAB can be found. The aims of this study was to determine the probiotics producing by LAB isolates 18A isolated from colon of Bali cattle as well as to identify of isolate by analysis of the 16S rRNA gene sequence. Probiotic potency analysis was performed by cultivation of LAB isolates 18A on the Man Rogosa Sharpe Broth (MRSB) medium, continued by Gram staining and catalase test. Antimicrobial activities of isolate were done against pathogenic bacteria (*E. coli* KL 48 (2) and *S. aureus*). Furthermore, genetic analysis was initiated by DNA isolation, amplification of the 16S rRNA gene, and sequencing. The result showed that LAB isolate 18A has inhibitory efficacy against gastrointestinal pathogens such as *Escherichia coli* KL 48 (2) and *Staphylococcus aureus* by 18,8% and 28,06% respectively. Moreover, genetic analysis showed the LAB isolate 18A as *Enterococcus durans* with the similarity level of 99%, and a value of 100 bootstrapping.

Keywords: Lactic acid bacteria, 16S rRNA gene, antimicrobial activity, Bali cattle'colon.

INTRODUCTION

Lactic acid bacteria (LAB) is a group of bacteria which able to produce lactic acid as the main products through homofermentation and heterofermentation process. The

acidification caused by enzymatic processes of the growing LAB affect the taste, texture and durability of a fermented foods (Klaenhammer *et al.*, 2002). Lactic acid bacteria can be found in pickled fruits and vegetables (Bae *et al.*, 2006; Chambel *et al.*, 2006; Nyanga *et al.*, 2007; Duangjitcharon *et al.*, 2007), fruit drinks (Plessis *et al.*, 2004), sausage (Ammor *et al.*, 2005), rumen fluid of Bali cattle (Suardana *et al.*, 2007), milk (Imen *et al.*, 2016) and wheat, rice, cassava (Reddy *et al.*, 2008).

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Several strains of LAB normally found as flora in the digestive tract, both in humans and animals (Tannock, 1998). Furthermore, Bali cattle is known has highly adaptable to poor quality of feed (Sutarno and Setyawan, 2015), so that it was possibly to find a specific LAB in its digestive tract (Suardana, 2007).

Lactic acid bacteria is mainly use in food fermentation technology, however this microorganism has also a clinical important regarding human health. Research showed that a live LAB which was consumed by human, have a positive contribution to health through the activity of metabolism which is known as probiotics. As probiotics, the LAB must be fulfilling the requirements as a good probiotic i.e. an antagonistic effects against pathogenic bacteria. In addition, the probiotic candidates have to be well known taxonomically, ranging from genus to species and even to the level of sub-species (Holzapfel *et al.*, 2001).

Identification of lactic acid bacteria can be carried out phenotypically and genotypically. Phenotypic identification consist of cell morphology observation, Gram staining test, motility test, catalase test, gas production test, acid production and growth tests under several temperature, pH and salinity (Pyar and Peh, 2014). However, the phenotypic identification methods is less specific, which mean there is a possibility that different bacteria identified as the same species. A more accurate test is based on genotype of bacteria using molecular analysis, especially to differentiate a bacteria at the species level (Gonzales *et al.*, 2001).

Genotypic identification of LAB is based on molecular analysis using 16S rRNA sequence. This method is the most popular technique for analyzing bacterial genome (Gonzales and Saiz, 2005). This method also has been successfully used by researcher previously to identification molecularly of local isolates of *E. coli* (Suardana, 2014).

MATERIAL AND METHODS

Probiotic Analysis of Lactic Acid Bacteria

The research was done initiating by cultivation of LAB isolates 18A which known had widely antimicrobial activity compare with others according to the previously study. Pure culture of LAB isolates 18A from previous study were thawed at 4°C for 15 minutes before recultured on 5 mL sterile MRS broth. Positive growth marked by turbidity on MRS broth medium. Gram staining was done using standarized Gram staining procedure, and catalase test was done using H₂O₂ 3% (Harrigan and McCance, 1976).

Antimicrobial test was done using modified Schillinger and Luke (1989) againts gastrointestinal pathogenic bacteria (*Escherichia coli* KL48(2) and *Streptococcus*

aureus). Antimicrobial activity of LAB isolate 18A was showed by the formation of inhibition zone around the well. Inhibition zone was measured using calipers for three times and the inhibition percentage compared to chloramphenicol as a control.

$$\text{Inhibition Percentage of LAB} = \frac{A - B}{A} \times 100\%$$

$$\text{Inhibition Efficacy} = 100\% - \text{Inhibition Percentage}$$

A : Inhibition Zone of chloramphenicol
B : Inhibition Zone of LAB

Molecular Identification of Lactic Acid Bacteria

Genomic DNA isolation were performed using QIAamp DNA Mini Kit[®]. The procedure of DNA isolation was following QIAamp DNA Mini Kit[®] protocols. DNA amplification was performed using PCR method. One set of universal primers namely oligonucleotides B27F 5'-AGAGTTTGATCCTGGCTACG-3' and U1492R 5'-GGTTACCTTGTTACGACTT -3' was used in this study (Lim *et al.* 2009). Each DNA sample was diluted with aquabidest by 1: 4 ratio. One microliter (1 mL) of genomic DNA was mixed into the PCR tube, then 25 mL dream taq green, 1 mL of forward primer B27F (10 pmol), 1 mL of reverse primer U1492R (10 pmol) and 10 mL aquabidest were added. The PCR amplification had initial DNA denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, finished by final extension at 72°C for 5 min at the end of amplification. Amount 5 µl of PCR product were examined by electrophoresis in 1.5% (w/v) TAE agarose gel at 100 v for 45 min. The gel then stained by 1% solution of ethidium bromide (50 µl/L) and distained with aquedest for 10 min. Gel was visualized by UV transillumination and it was recorded by digital camera (Suardana, 2014).

The sequencing of 16S rRNA gene was performed using genetic analyzer (ABI Prism 3130 Genetic Analyzer) at Eijkman Institute for Molecular Biology, Jakarta. The sequences were edited using MEGA 4.0 version software. The nucleotide sequences of 16S rRNA gene were aligned against some nucleotide sequence database of LAB which appeared in the NCBI. The sequences were aligned using Clustal W and the phylogenetic analysis was constructed using neighbor joining algorithm (Saitou and Nei, 1987; Tamura *et al.*, 2007). Criteria for species identification is 99% sequence similarity or higher for species assignment, and 95% sequence similarity or higher for genus assignment (Bosshard *et al.*, 2003) or minimum 99% sequence similarity and ideally 99.5% sequence similarity or < 1% divergence (Janda and Abbott, 2007).

RESULTS

Probiotic Analysis of Lactic Acid Bacteria

Isolates 18A obtained from previous study was confirmed as lactic acid bacteria in MRS Broth showed the growth characterizing by the turbidity of medium. The LAB isolates 18A belong to positive Gram bacteria indicated by violet appearance under the microscope observation. Catalase test using H₂O₂ 3% showed a negative reaction marked by the absence of bubbles formation of oxygen (O₂).

Antimicrobial activity test of LAB isolates 18A was performed by measuring the diameter of inhibition formed around the well. Based on antimicrobial activity test the LAB isolates 18A can inhibit pathogens bacteria growth, such as *E. coli* KL 48(2) and *S. aureus*. The LAB isolates 18A showed an inhibitory activity against pathogenic *Escherichia coli* KL48(2) with inhibitory zone diameter range from 0.21 - 0.75 cm (0.41 ± 0.24 cm). Inhibitory zone diameter against *Streptococcus aureus* was 0.32 -

1.08 cm (0.59 ± 0.34 cm). The inhibition efficacy of LAB isolates 18A against pathogenic *E. coli* KL 48(2) and *S. aureus* was 18.8% and 28.06% respectively (Table 1).

Molecular Identification of Lactic Acid Bacteria

Molecular identification of LAB isolates 18A showed a positive results with amplicon size of 1502 base pairs (bp) which is the expected size of 16S rRNA gene amplification using an U1492R and B27F primers (Figure 3). According to Case *et al* (2007), 16S rRNA can be used as molecular markers because these molecules are ubiquitous with identical functions among organism. However, the variance of this molecule can also be undergo by time, so that can be used as an evolutionary chronometer. Genotypic characterization of LAB is based on the similarity (homology) of 16S rRNA sequences compared to other isolates in GenBank database.

The comparison of the nucleotide sequence among LAB isolate 18A and other isolates obtained from GenBank are as follows (Table 2).

Table 1. Antimicrobial test of LAB isolates 18A against gastrointestinal pathogenic bacteria

Isolate	Inhibitory Zone Diameter Against	Inhibition Efficacy (%)	Inhibitory Zone Diameter Against	Inhibition Efficacy (%)
	<i>E. coli</i> KL 48(2) (cm)			
LAB isolates 18A	0.75	33.78	0.55	25.94
	0.44	19.81	1.08	50.94
	0.21	9.45	0.43	20.28
	0.27	12.16	0.32	15.09
Mean±SD	0.41 ± 0.24	18.8	0.59 ± 0.34	28.06

Table 2. Alignment of 16S rRNA gene of LAB isolate 18 A and other isolates obtained from GenBank

#Isolat_18	CTTACACCCC	GCGAAGGCGA	AATGATCTAA	GTCTG	[176]
#L._rhamnosus_D16552.1	ACG...ATA.	.TAT.....G	[176]
#L._sunkii_AB366385	ACG...TT.	.A.....	[176]
#B._animalis_sp.lactis_AB050136	ACG...G.T.	.G.G.....	.G.C...GGC	...C.	[176]
#B._saguini_AB559504	ACG...G.Y.	.G.G.....	.G.C...AGC	...C.	[176]
#B._reuteri_AB613259	ACG...G.TT	.G.....	.G.C...AGC	...C.	[176]
#L._lactis_sp.lactis_AB008215	ACG.TG.TA.	...G.A....	.G.....G	.C...	[176]
#L._lactis_subsp._cremoris_AB008214	ACG.TGATA.	...G.A....	.G.....G	.C...	[176]
#L._lactis_bv.diacetylactis_AB100805	ACG.TG.TA.	...G.A....	.G.....G	.C...	[176]
#L._mesenteroides_sp.mesenteroides_AB596935	ACG....A.	..CG.....	...A...AGT	[176]
#L._mesenteroides_sp.mesenteroides_AB596937	ACG....A.	..CG.....	...A...AGT	[176]
#E._durans_AB596943	ACG.....	[176]
#E._cecorum_AB681217	ACG....T..	.A.G.....G	[176]
#Isolat_18	AGGAAGTCTA	CCGTTAGGTT	CTAGCCTAAT	TGATC	[290]
#L._rhamnosus_D16552.1ACGC...G.C...	[290]
#L._sunkii_AB366385C.A...C...	[290]
#B._animalis_sp.lactis_AB050136TGC	T..GGGA..C	.G..T.C...	..G.T	[290]
#B._saguini_AB559504G..TAT	T..CCGA..C	.G..T.C...	A.G..	[290]
#B._reuteri_AB613259G..TGGGA...	.G..T.C...	A.G..	[290]
#L._lactis_sp.lactis_AB008215	.A.....ACT	A.....AC	.A.A.....	...A	[290]
#L._lactis_subsp._cremoris_AB008214	.A.....CT	AT.....AC	.A.A.....	...A	[290]
#L._lactis_bv.diacetylactis_AB100805	.A.....ACT	A.....AC	.A.A.....	...A	[290]
#L._mesenteroides_sp.mesenteroides_AB596935A.G	T..A.....C	.G.....	..G.A	[290]
#L._mesenteroides_sp.mesenteroides_AB596937A.G	T..A.....C	.G.....	..G.A	[290]
#E._durans_AB596943	[290]
#E._cecorum_AB681217TC?	...AA...CG..	[290]

Table 2. continue

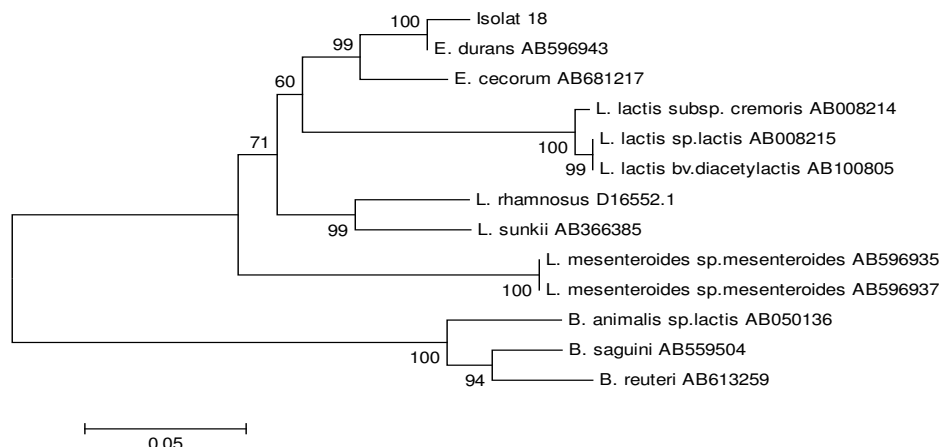
#Isolat_18	CAAGAACACC	GAGAGCAGAG	ACCACCGGAA	AGTTG	[398]
#L._rhamnosus_D16552.1A	..T.....A	[398]
#L._sunkii_AB366385A	[398]
#B._animalis_sp.lactis_AB050136T.G...	G...A	[398]
#B._saguini_AB559504G.T.G...	G...A	[398]
#B._reuteri_AB613259G.T.G...	G...A	[398]
#L._lactis_sp.lactis_AB008215	[398]
#L._lactis_subsp._cremoris_AB008214	[398]
#L._lactis_bv.diacetylactis_AB100805	[398]
#L._mesenteroides_sp.mesenteroides_AB596935A.....G	..C.....T.A	[398]
#L._mesenteroides_sp.mesenteroides_AB596937A.....G	..C.....T.A	[398]
#E._durans_AB596943	[398]
#E._cecorum_AB681217G.....	[398]
#Isolat_18	AGGACAGCCC	GAGGGTCAGA	ACTTGAACT	CTCAA	[524]
#L._rhamnosus_D16552.1?....G..TG.	[524]
#L._sunkii_AB366385C..T.	[524]
#B._animalis_sp.lactis_AB050136C...	.C...C.G..	C...AG.CT.	GGTT.	[524]
#B._saguini_AB559504C...C.G..	C.....CG.	G..T.	[524]
#B._reuteri_AB613259C...C.G..	C.....TT.	G..G.	[524]
#L._lactis_sp.lactis_AB008215G...A.C.	[524]
#L._lactis_subsp._cremoris_AB008214G...A.C.	[524]
#L._lactis_bv.diacetylactis_AB100805G...A.C.	[524]
#L._mesenteroides_sp.mesenteroides_AB596935T.....G..	G.....TG.	[524]
#L._mesenteroides_sp.mesenteroides_AB596937T.....G..	G.....TG.	[524]
#E._durans_AB596943	[524]
#E._cecorum_AB681217	[524]
#Isolat_18	ACCCAATCCC	CTTGGGACTC	GTTGGACGCG	GTTGT	[629]
#L._rhamnosus_D16552.1	[629]
#L._sunkii_AB366385	[629]
#B._animalis_sp.lactis_AB050136TG...GTT.	.CC.C	[629]
#B._saguini_AB559504C.....GTT.	.CC.C	[629]
#B._reuteri_AB613259C.....GTT.	.CC.C	[629]
#L._lactis_sp.lactis_AB008215	.G.....CG...	[629]
#L._lactis_subsp._cremoris_AB008214	.G.....CG...	[629]
#L._lactis_bv.diacetylactis_AB100805	.G.....CG...	[629]
#L._mesenteroides_sp.mesenteroides_AB596935	.G.....TCG...A	[629]
#L._mesenteroides_sp.mesenteroides_AB596937	.G.....TCG...A	[629]
#E._durans_AB596943	[629]
#E._cecorum_AB681217	[629]
#Isolat_18	TGACCCACGG	TGAGACGTAA	GGTATATAGG	ACATA	[737]
#L._rhamnosus_D16552.1AC.....	[737]
#L._sunkii_AB366385AA	..A.....	.C.....	[737]
#B._animalis_sp.lactis_AB050136T.G.T	C...G...G.C...	.T.C	[737]
#B._saguini_AB559504T.G.T	C...G...G.C...	.T.C	[737]
#B._reuteri_AB613259T.G.T	C...GY.G.C...	.T.C	[737]
#L._lactis_sp.lactis_AB008215	.A.T...TA	[737]
#L._lactis_subsp._cremoris_AB008214	.A.T...TA	[737]
#L._lactis_bv.diacetylactis_AB100805	.A.T...TA	[737]
#L._mesenteroides_sp.mesenteroides_AB596935G..T.AT.....	.A.....	[737]
#L._mesenteroides_sp.mesenteroides_AB596937G..T.AT.....	.A.....	[737]
#E._durans_AB596943	[737]
#E._cecorum_AB681217AC.....	[737]
#Isolat_18	GGCCTCACCT	GCTCACACGA	GGAACGTACT	G	[830]
#L._rhamnosus_D16552.1A..G.....	T	[830]
#L._sunkii_AB366385A..G.....	T	[830]
#B._animalis_sp.lactis_AB050136G.....A.....G.....	T	[830]
#B._saguini_AB559504G.....A.....G.....	T	[830]
#B._reuteri_AB613259G.....A.....G.....	T	[830]
#L._lactis_sp.lactis_AB008215	T	[830]
#L._lactis_subsp._cremoris_AB008214	T	[830]
#L._lactis_bv.diacetylactis_AB100805	T	[830]
#L._mesenteroides_sp.mesenteroides_AB596935A	..C..T.....	T	[830]
#L._mesenteroides_sp.mesenteroides_AB596937A	..C..T.....	T	[830]
#E._durans_AB596943	T	[830]
#E._cecorum_AB681217	T	[830]

The data in Table 2 showed nucleotide sequence of isolate 18A has 27, 19, and 65 nucleotides different against *L. rhamnosus*, *L. sunkii*, and *B. animalis sp. lactis*, respectively. The difference also showed with

others including species of *Bacillus*, *Lactobacillus*, and *Enterococcus*. The pairwise distance of isolate 18A and other isolates obtained from Genbank was showed in Table 3.

Tabel 3. Pairwise distance analysis of LAB isolates 18A and other isolates obtained from GenBank

Isolat_18	L._rhamnosus_D16552.1	L._sunkii_AB366385	B._animalis_sp.lactis_AB050136	B._saguini_AB559504	B._reuteri_AB613259	L._lactis_sp.lactis_AB008215	L._lactis_subsp._cremoris_AB008214	L._lactis_bv.diacetylactis_AB100805	L._mesenteroides_sp.mesenteroides_AB596935	L._mesenteroides_sp.mesenteroides_AB596937	E._durans_AB596943	E._cecorum_AB681217
L._rhamnosus_D16552.1	0.12											
L._sunkii_AB366385	0.10	0.07										
B._animalis_sp.lactis_AB050136	0.32	0.30	0.31									
B._saguini_AB559504	0.31	0.30	0.32	0.08								
B._reuteri_AB613259	0.33	0.32	0.32	0.08	0.05							
L._lactis_sp.lactis_AB008215	0.13	0.16	0.16	0.36	0.36	0.36						
L._lactis_subsp._cremoris_AB008214	0.13	0.16	0.16	0.36	0.35	0.37	0.01					
L._lactis_bv.diacetylactis_AB100805	0.13	0.16	0.16	0.36	0.36	0.36	0.00	0.01				
L._mesenteroides_sp.mesenteroides_AB596935	0.18	0.16	0.17	0.34	0.33	0.34	0.19	0.19	0.19			
L._mesenteroides_sp.mesenteroides_AB596937	0.18	0.16	0.17	0.34	0.33	0.34	0.19	0.19	0.19	0.00		
E._durans_AB596943	0.01	0.11	0.09	0.30	0.30	0.31	0.12	0.12	0.12	0.17	0.17	
E._cecorum_AB681217	0.06	0.11	0.12	0.29	0.30	0.31	0.14	0.14	0.14	0.16	0.16	0.05

**Figure 1.** The phylogenetic tree of LAB isolates 18A based on 16s rRNA sequence.

The data in Table 3 showed that LAB isolates 18A has one per 100 nucleotides different or having 99% similarity value with *Enterococcus durans* AB596943. However, LAB isolates 18A showed a reasonable distance with *Lactococcus lactis* sp. *Lactis* AB008215, *L. lactis* subsp. *Cremoris* AB008214, *L. lactis* bv. *diacetylactis* AB100805, *Lactobacillus rhamnosus* D16552.1 and *L. sunkii* AB366385, with nucleotide different were 13, 13, 13, 12, 10 per 100 nucleotides or it has similarity value were 87, 87, 87, 88, and 90%, respectively. On the other hand, isolate 18 A showed similarity more far compared to *Bifidobacterium animalis* sp. *lactis* AB050136, *B. saguini*

AB559504 and *B. reuteri* AB613259 i.e. 68, 69, and 67%, respectively.

Further analysis of data Table 2 in the form of phylogenetic tree of LAB isolates 18A was showed in Figure 1.

The phylogenetic tree in Figure 1 showed isolate 18A was shared clade with *Enterococcus durans* AB596943, and it was grouped differently with *Lactococcus lactis* spp., *Lactobacillus rhamnosus* D16552.1 and *L. sunkii* AB366385, and *Bifidobacterium animalis* sp. *lactis* AB050136, *B. saguini* AB559504 and *B. reuteri* AB613259, respectively.

DISCUSSION

The isolate 18A which has been confirmed as a species of LAB, known have antimicrobial activity against pathogenic bacteria as due to various metabolites produced by this species. Most of LAB metabolites is undisposed organic acids compound which can decrease the extracellular pH. A low extracellular pH will cause cell cytoplasm acidification. In addition, the undisposed organic acids compound will become lipophilic which then diffuse into the cell via cell membrane. the undisposed organic acids would also cripple the electrochemical proton gradient and altering the permeability of the membrane resulted on substrate transport system detriment. The other antimicrobial property of LAB is through H₂O₂ activity bind oxygen (O₂) that lead to an anaerobic atmosphere, so that aerobic bacteria can not survive. Besides, it is also produced antimicrobial compounds known as bacteriocins that are bacteriostatic agent and effectively against many positive Gram bacteria. Bacteriocins contain nisin which does not inhibit the growth of negative gram bacteria, fungi or yeasts, but inhibit the growth of positive Gram bacteria (Christensen and Hutkins, 1992).

Genetic analysis of 16S rRNA gene showed isolate 18A has closely similarity with *Enterococcus durans* AB596943. *Enterococcus spp.* is a positive Gram bacteria, cocci, facultative anaerobic, optimum growth temperature of 35°C, can be grown in NaCl 6.5%, pH of 9.6%, and able to survive at 60°C for 30 minutes (Schleifer and Kilpper, 1984). The strain was known having an important role in creating the aroma and the taste of the traditional cheese-making in many countries. In addition, some strains of *Enterococcus* today is used as a probiotic (Foulquie' Moreno *et al.*, 2006).

Enterococcus is a homofermentatif lactic acid bacteria (Madigan *et al.*, 2006). According to Papagianni (2012), homofermentatif lactic acid bacteria involve in Embden Meyerhof pathway known as glycolysis, produces lactic acid, 2 moles of ATP from one molecule of glucose/hexose under normal conditions, does not produce CO₂ and produce biomass cells twice as heterofermentatif lactic acid bacteria. Homofermentatif LAB is widely used in the fermented dairy products.

Jafari *et al.* (2011) and Acurcio *et al.* (2014) isolated and identified the *Enterococcus durans* from cow's milk and from sheep's milk respectively. Both revealed that *E. durans* is a potential probiotics due to the ability to withstand in the low pH (pH 2.0), bile salts, as well as antimicrobial activity against several pathogenic bacteria such as *E. coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *thypimurium Salmonella* and *Pseudomonas aeruginosa*. In addition, Pieniz *et al.* (2014) showed that *E. durans* have a high antioxidant activity that can be used to reduce oxidative damage of food and feed. In

conclusion, the present study found that lactic acid bacteria isolates 18A isolated from Bali cattle' colon confirmed as *Enterococcus durans* with a similarity of 99% and has the inhibitory efficacy against gastrointestinal pathogens such as *Escherichia coli* KL48 (2) and *Staphylococcus aureus* by 18.8% and 28.06%, respectively. The isolate was potential to use as a probiotic candidate.

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