



Selection of potential lactic acid bacteria from fermented Sumbawa mare's milk as starter cultures

Ilmiyatus Safitri Devi and Yoga Dwi Jatmiko*

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya,
Veteran Street, Malang, East Java, 65145 Indonesia.
Email: jatmiko_yd@ub.ac.id

Received 30 December 2019; Received in revised form 22 May 2020; Accepted 4 September 2020

ABSTRACT

Aims: The aim of this study was to screen lactic acid bacteria (LAB) isolates from fermented Sumbawa mare's milk that meet the requirements as starter cultures, and to evaluate the effect of the selected starter culture in improving the organoleptic quality of mare's milk fermentation.

Methodology and results: The LAB isolates (13 isolates) derived from naturally fermented Sumbawa mare's milk were firstly screened for acidification activity. Afterwards, the selected isolates were evaluated for the starter culture criteria such as technological properties (proteolytic test, lipolytic test, and exopolysaccharide production), food safety test (hemolytic test and antibiotic sensitivity test), antimicrobial activity test. The selected culture (SC) together with yogurt starter cultures (YC) and combination between the selected isolate and a mixture of both (MC) were used to ferment fresh mare's milk. Six LAB isolates (DB7, BC10, DC4, BC9, DC10, and BC7) were obtained from the acidification screening. Isolate BC10 was the most potential isolate as starter culture due to its ability in terms of acidification and proteolytic activity, lack of lipolytic activity, no indication of pathogenic potency, as well as able to inhibit the growth of *Escherichia coli* ATCC 25922. However, this isolate was resistant to antibiotics kanamycin, trimethoprim, and cinoxacin. The isolate BC10 presented 99.99% sequence similarity with respect to *Lactobacillus plantarum*.

Conclusion, significance and impact of study: The selected starter culture (isolate BC10) was able to improve the organoleptic quality of fermented mare's milk especially aroma compared to the other starter cultures. Therefore, *Lactobacillus plantarum* BC10 is a potential isolate to be used as starter culture for mare's milk fermentation.

Keywords: Lactic acid bacteria, starter cultures, technological characterization, organoleptic, Sumbawa mare's milk

INTRODUCTION

Mare's milk has more advantages over cow's milk and has similarities with human milk in terms of nutritional content. Mare's milk contains lactose and immunoglobulin (IgG) which is equivalent to human milk, so it is recommended to substitute human milk for toddlers who were allergic to cow's milk. Its fat content is also lower than human milk, thus it is low in calories (Malacarne *et al.*, 2002). In addition to high nutrition, mare's milk also contains active compounds such as lactoferrin, oleic acid, and lysozyme. Lactoferrin has anti-inflammatory, immunomodulatory, antibacterial, antifungal, and antiviral activities (Dankow *et al.*, 2012). The calcium and phosphorus ratio of mare's milk (1.6–1.8: 1) is higher than cow's milk (1.4: 1), and is almost similar to that in human milk (1.9: 1) (Sheng and Fang, 2009). Mare's milk also contains vitamins A, D₃, and E which are similar to human milk, and has a high vitamin C content (Csapo *et al.*, 1995; Jastrzębska *et al.*, 2017).

Although the nutrition and health benefits of mare's milk is categorized as good, however, in Indonesia, the types of mare's milk food products are still limited to traditional fermented milk products. This traditional fermented milk products made from unpasteurized fresh mare's milk and fermented without the addition of starter cultures, so called naturally fermented milk. The fermented Sumbawa mare's milk is part of naturally fermented milk (NFM) products that categorized as functional foods due to its health promoting potential, in terms of nutritional values and probiotic microorganism sources (Jatmiko *et al.*, 2018). Traditional fermented mare's milk products through spontaneous fermentation require a longer time in the production process, with high possibility of microbial contamination, and lack of consistency of product quality due to the lack of control over the microbes involved in fermentation. Besides, traditional natural fermentation allows the fermented products to have taste and flavor variations due to diverse microbes playing a role in the fermentation process. Starter culture is a selected microbial strain with a

*Corresponding author

constant characteristic for the production of the expected characteristic under controlled conditions (Ravindra, 2015). The expected characteristic of fermented products used is product quality consistency, either in taste, flavor, or other organoleptic parameters. Application of starter cultures for Sumbawa mare's milk will not only able to increase product diversification, but also increase product safety because lactic acid bacteria (LAB) in starter culture will inhibit the growth of undesired microbes.

Lactic acid bacteria in the starter cultures also could increase the value added of the product through increasing technological characteristics (acidification activity, and production of bioactive compounds such as diacetyl, exopolysaccharide and enzymes) (de Souza and Dias, 2017). Studies of LAB from Sumbawa mare's milk was still limited to the LAB diversity, and study on the potency of LAB isolated from Sumbawa mare's milk has not been elucidated yet (Jatmiko *et al.*, 2019; Mulyawati *et al.*, 2019). Therefore, this paper is aimed to determine some technological properties of the LAB isolates from fermented Sumbawa mare's milk to be used as a starter culture to develop fermented mare's milk products with high quality.

MATERIALS AND METHODS

Lactic acid bacteria cultures

This study used 13 LAB strains derived from previous research (Mulyawati *et al.*, 2019). These strains were isolated from fermented Sumbawa mare's milk, collected from three regency (Dompu, Sumbawa, and Bima) in Sumbawa Island, Province of West Nusa Tenggara using two different isolation media, namely De Man, Rogosa, and Sharpe agar (MRS) agar and M17 agar containing 1% calcium carbonate (CaCO₃). Seven isolates (DB4, DB9, DB2, DB6, DB3, DB7, DB11) isolated using MRS agar, were re-cultured in MRS agar containing 2% lactose, while the six isolates (DC13, BC10, BC7, DC10, BC9, and DC4) isolated using M17 agar, were re-cultured in M17 agar media containing 2% lactose. Before the examination was performed, all isolates were grown in the respective media at 37 °C for 24 h to obtain the similar density.

Screening LAB as starter culture

The LAB isolates with rapid acidification rate were selected as starter culture candidates. The cultures (3 mL) were transferred to 27 mL of 1% skim milk broth (containing 0.3% yeast extract and 0.2% glucose). The cultures were then incubated at 37 °C for 24 h. The pH value was measured at 0, 2, 4, 6, and 24 h to determine the acidification rate/ pH change (Equation 1). The pH reduced by 0.4 after 3, 3.5, and >5 h of incubation was categorized fast, medium and slow acidification rate, respectively (Akabanda *et al.*, 2014). This test was performed in triplicates. The isolates with the fastest acidification were used for further tests, namely technological characterization (proteolytic test, lipolytic

test, and exopolysaccharide production), food safety properties (hemolytic test and antibiotic sensitivity test), protective property (antimicrobial activity test).

Acidification rate/ pH change (Δ pH) =

$\text{pH}_{\text{at time}} - \text{pH}_{\text{zero time}}$ (Equation 1)

Proteolytic activity test

Proteolytic activity was tested using agar well-diffusion method in triplicates. A 8-mm-diameter well was made in 1% skim milk agar containing 0.05% sodium chloride (NaCl), 0.1% yeast extract, 0.2% tryptone, 0.01% calcium chloride (CaCl₂), and 1.5% bacteriological agar. The LAB cultures (50 μ L) were inoculated into the wells, and then incubated at 37 °C for 24 h. This test was performed in triplicates, and the wells filled with the sterile distilled water were served as the negative control. The proteolytic activity was shown by the presence of clear zones around the wells, and it was measured using Equation 2 as shown in below (Vijayaraghavan and Vincent, 2013).

Diameter of clear zones (mm) =

Diameter of total clear zone – Diameter of the well
.....(Equation 2)

Lipolytic activity test

Lipolytic activity was tested using agar well-diffusion method in four replicates. The selected LAB cultures with 50 μ L were inoculated in the 8-mm-diameter wells on Sierra agar containing 1% peptone, 0.5% NaCl, 0.01% CaCl₂, 1.5% Bacto agar, and 1% Tween 80 (Sierra, 1957). The plates were incubated at 37 °C for 96 h. This test was performed in triplicates, and wells with the sterile distilled water served as the negative control. The lipolytic activity was indicated by the presence of clear zones around the wells, and it was determined using Equation 2 (Akabanda *et al.*, 2014).

Exopolysaccharide (EPS) production test

Production of EPS of LAB was detected qualitatively by the presence of ropy colony on the agar surface. One loopful of selected LAB isolates were streaked onto MRS or M17 agar medium supplemented with 5% sucrose, then incubated at 37 °C for 24–48 h. This test was conducted in triplicates, and the medium without inoculum served as the negative control.

Antimicrobial activity test

Screening of antimicrobial activity was evaluated using agar disk-diffusion method in triplicates. The selected LAB cultures with density 10⁷ CFU/mL were centrifuged at 10,000 \times g at 4 °C for 10 minutes. The cell free supernatant (CFS) was separated from the pellet, and the

pH of CFS was adjusted to 6.5–6.8. The neutralized CFS was filtered using 0.22 µm-millipore membrane. The 50 µL of sterile neutralized CFS was inoculated onto the blank-disk, and the disks were placed onto NA agar containing 100 µL (10⁶ CFU/mL) of indicator bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* Typhi). Inhibition zones were measured based on Equation 3 after incubation at 37 °C for 24 h. Sterile distilled water and streptomycin were served as the negative and positive control, respectively.

Diameter of inhibition zones (mm) =

Diameter of total clear zone – Diameter of the disk
.....(Equation 3)

Antibiotic susceptibility test

The selected LAB cultures (100 µL) were spread on MRS or M17 agar plates. Then, antibiotic disks (kanamycin 30 µg, erythromycin 15 µg, cinoxacin 100 µg, trimethoprim 5 µg) were placed on the agar plate. The diameter of inhibition zones was measured after incubation at 37 °C for 48 h. The test was conducted in triplicates.

Hemolytic test

Hemolytic test was performed by streaking LAB isolates on sheep blood agar media. Hemolytic activity was observed after incubation at 37 °C for 24 h. Partial hydrolysis of red blood cells forms a greening zone around the colony and the edge of the colony which is labeled as α-hemolysis. A clear zone around the colony was detected as β-hemolysis. The absence of color changes on blood agar was detected as γ-hemolysis, and the isolates with this character was selected as starter culture candidate (Monika *et al.*, 2017).

Mare's milk fermentation

One of the LAB isolates was selected as the most potential LAB starter culture was selected based on the tested criteria, such as highest proteolytic activity, lowest lipolytic activity, produced EPS, sensitive to antibiotics, exhibited antimicrobial activity, and no hemolytic activity. Three types of starter cultures were used in mare's milk fermentation namely SC (selected culture), YC (yogurt cultures), and MC (mixture of both starter cultures or mixed cultures). The commercial yogurt starter contains *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus acidophilus*. Each starter culture (3%) was added into 900 mL of pasteurized mare's milk. Fermentation was carried out at room temperature for 24 h. The change of pH value was measured to determine the acidification rate (Equation 4), and the total LAB was calculated using total plate count (TPC) method on MRS agar.

Acidification rate (%) =

(initial pH – final pH) / initial pH × 100%.....(Equation 4)

Organoleptic test

The organoleptic characteristics was evaluated by 31 unskilled panelists (12 male and 19 female panelists) aged between 18–22 years old using hedonic scale method. This panelist size has met the requirement of sensory panels for hedonic method (Watts *et al.*, 1989). Panelists tasted three mare's milk fermentation products and determined the score (scale 1–7) of a range of organoleptic parameters namely color, taste, aroma, mouthfeel, and overall. Score 1 is dislike very much, score 2 is dislike moderately, score 3 is dislike slightly, score 4 is neither like nor dislike, score 5 is like slightly, score 6 is like moderately, score 7 is like very much (Granato *et al.*, 2010).

Molecular identification

Total DNA of the selected LAB isolate was extracted using Zymo-Spin™ Lysis Kit by following the manufacturer's instruction. The DNA was amplified using 16S rDNA universal primers namely 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT CTG ACT ACT T-3') (Chen *et al.*, 2015). The PCR reaction was run with initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 52 °C for 45 sec, 72 °C for 90 sec, with a final extension at 72 °C for 5 min. The 16S rDNA amplicon was confirmed using agarose gel electrophoresis (1.5%). The phylogenetic tree was constructed using MEGA 6 software for windows based on Neighbor-Joining algorithm and Tamura-Nei model with bootstrap of 1000.

Data analysis

All assays (acidification, proteolytic activity, antimicrobial activity, and antibiotic susceptibility) were conducted in triplicates. The quantitative data were analyzed using one-way analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$). Meanwhile, the organoleptic test was analyzed using Kruskal Wallis test and Tukey's HSD test ($p < 0.05$). All statistical analyses were completed using SPSS for windows version 20.0.

RESULTS AND DISCUSSION

Screening of potential LAB as starter culture

Rapid acidification is the main characteristic of starter culture for the development of fermented milk products (Akabanda *et al.*, 2014). Six of the thirteen isolates had the fastest acidification because they achieved ΔpH of 0.4 after 6 h. The six isolates were DC4, BC10, BC9, DC10, DB7 and BC7 (Figure 1, Table 1). Despite having the fastest rate of acidification, these isolates were

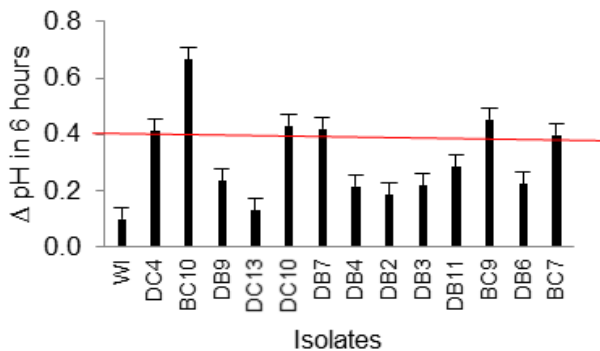


Figure 1: Acidification rate of selected LAB (WI: without inoculum). Data were expressed as the mean from three replicates of each isolate while error bar indicates its standard deviation.

Table 1: Technological characteristics of selected LAB isolates.

Isolate	Acidification rate in 6 hours/pH change	Proteolytic activity (mm)	Lipolytic activity (mm)	EPS production*
Control	0.10 ± 0.00 ^a	0 ± 0.00 ^a	0.00	-
DB7	0.42 ± 0.03 ^b	2.13 ± 0.10 ^{bc}	0.00	-
BC10	0.67 ± 0.02 ^c	2.29 ± 0.27 ^{bc}	0.00	-
DC4	0.41 ± 0.03 ^b	1.97 ± 0.15 ^b	0.00	-
BC9	0.45 ± 0.01 ^b	2.38 ± 0.27 ^c	0.00	-
DC10	0.43 ± 0.03 ^b	2.16 ± 0.16 ^{bc}	0.00	-
BC7	0.40 ± 0.04 ^b	2.07 ± 0.08 ^{bc}	0.00	+

Data were expressed as the mean from three replicates of each isolate while error bar indicates its standard deviation. Different letters were significantly different ($p < 0.05$).

*(-) soft colony texture (did not produce EPS); (+) ropy colony texture (produce EPS).

categorized as slow acidification (after 5 h of incubation). Strains with slow acidification can be used as adjunct cultures depending on other characteristics (Ayad *et al.*, 2004). The rate of acidification of isolates depends on their metabolic pathway. Isolates with homofermentative metabolic pathways produced higher lactic acid as a consequence they had faster acidification rates (Mahmoudi *et al.*, 2013).

The six selected isolates were subsequently evaluated for the technological properties [proteolytic tests, lipolytic tests, and exopolysaccharide production (Table 1)], protective properties (antimicrobial activity tests), and food safety tests (antibiotic sensitivity tests and hemolytic tests).

Proteolytic activity

Proteolytic activity of selected LAB was observed from the formation of clear zones around the wells. The isolates which had the highest proteolytic activity was isolate BC9, then followed by BC10, and DC10 (Table 1). Therefore, these three isolates with the highest proteolytic activity were selected and suitable candidates for starter culture. Lactic acid bacteria as starter culture were expected to have a high proteolytic activity so it can improve the organoleptic characteristics of the fermented milk products (Christensen *et al.*, 1999; Moslehisad *et al.*, 2013) as well as increase the production of bioactive peptides (Chaves-López *et al.*, 2014).

Lipolytic activity

Lipolytic activity of selected LAB was observed from the formation of clear zones around the wells. However, none of the isolates formed clear zones on the media (Table 1). According to Tsakalidou *et al.* (1994), lipolytic activity in LAB was low even though some strains of *Enterococcus* showed higher lipolytic activity. The lipase enzyme activity of the starter culture used is needed to hydrolyze milk fat in the cheese ripening process (Aravindan *et al.*, 2007). However, high lipolytic activity can cause rancidity in milk. Therefore, LAB isolates with no lipolytic activity was suitable character for starter culture of milk fermentation.

Exopolysaccharide (EPS) production

Production of EPS qualitatively can be observed from the forming of ropy colonies. Only isolate BC7 had ropy colony texture (Table 1). According to Dinoto *et al.* (2011), isolates that did not exhibit ropy colony character did not mean they did not produce EPS. Isolates with soft colony texture do not form sticky strand but produce EPS with quite high concentration. EPS production is a desirable character in starter cultures for dairy products because EPS role as a natural thickener which will increase product consistency and viscosity (Ruas-Madiedo, 2005). Based on the results of EPS production test, isolate BC7 was the most potential isolate as a starter culture candidate.

Susceptibility of selected LAB to antibiotics

Each selected LAB isolate had different susceptibility to the four antibiotics tested (Table 2). Some isolates that have sensitivity to antibiotics were isolate DC4 to kanamycin (30 µg), and isolates DB7, BC9, DC10, BC7 to trimethoprim (5 µg). While isolate BC10 showed character that was resistant to the three antibiotics tested, but this isolate was intermediates to erythromycin. Therefore, among the six isolates, isolates DB7, BC9, DC10, BC7, and DC4 were the most qualified isolates as starter cultures based on their level of sensitivity to antibiotics.

The risk of antibiotic resistance was the ability of antibiotic-resistant strains to transfer resistant genes to pathogenic bacteria (Mathur and Singh, 2005). These

Table 2: Susceptibility of selected LAB isolates to antibiotics and its hemolytic test.

Isolates	Antibiotics				Hemolytic test
	Kanamycin (30 µg)	Erythromycin (15 µg)	Trimethoprim (5 µg)	Cinoxacin (100 µg)	
DB7	R	I	S	R	γ-hemolysis
BC10	R	I	R	R	γ-hemolysis
DC4	S	I	R	R	γ-hemolysis
BC9	R	I	S	R	γ-hemolysis
DC10	R	I	S	R	β-hemolysis
BC7	I	I	S	I	β-hemolysis

R = resistance, I = intermediate, S = sensitive.

genes could be transferred to other bacteria through conjugation mechanisms. This resistance was due to the presence of D-Ala-D-Lactate in peptidoglycan in greater amounts than D-Ala-D-Ala dipeptide which is the target of antibiotics (Danielsen and Wind, 2003). *Lactobacillus* sp. has been reported resistant to the class of aminoglycoside (kanamycin) (Gueimonde *et al.*, 2013), trimethoprim (Danielsen *et al.*, 2004), and quinolones (Li *et al.*, 2015). Generally, the location of the resistant gene was in the plasmid, but the detection of the location of the antibiotic resistant gene in isolate BC10 still requires to be conducted to ascertain whether the antibiotic resistant gene could be transferred to bacteria in the digestive tract, especially pathogenic bacteria. The strains with mobile genetic element carrying gene encoding resistant to antibiotics should not be used as starter cultures (Duškova and Karpiskova, 2013).

Antimicrobial activity

Antimicrobial activity was observed from six LAB isolates by the agar disk-diffusion agar method. There were two out of six isolates that showed antimicrobial activity, namely BC10 and BC9 (Figure 2). Each isolate showed inhibition against different indicator bacteria. Isolate BC10 could inhibit the growth of *E. coli* ATCC 25922 (0.88 mm), while isolate BC9 could inhibit the growth of *B. cereus* (4.74 mm) ($p < 0.05$). The selected LAB as a starter culture of fermented milk products need to have antimicrobial activity, so it could reduce the contaminant microbial population and hence could improve food safety. Therefore, isolates BC9 and BC10 were the most potential candidates as starter culture.

Antimicrobial compounds such as lactic acid, bacteriocin, hydrogen peroxide, and diacetyl produced by LAB could inhibit the growth of pathogenic bacteria in fermentation products. The bacteriocin which is a protein was stable when the pH value is near neutral (Apolonio *et al.*, 2007).

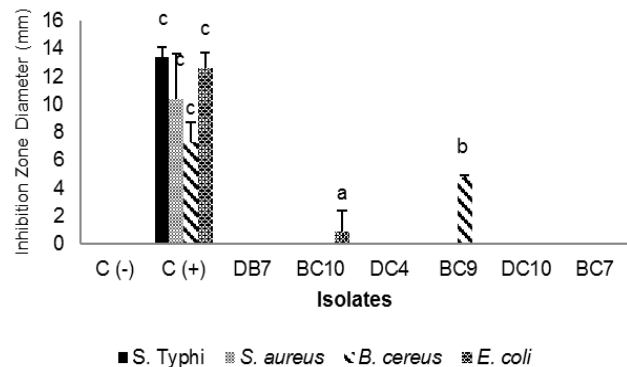


Figure 2: Antimicrobial activity of selected LAB isolates. Data were expressed as the mean from three replicates of each isolate while error bar indicates its standard deviation. Different letters were significantly different ($p < 0.05$). C(-): negative control (sterile distilled water), C(+): positive control (streptomycin).

Hemolytic activity

Isolates DC10 and BC7 could lyse red blood cells which were characterized by the formation of clear zones around bacterial colonies, so that they were categorized as having β-hemolysis. Isolates BC10, BC9, DB7 and DC4 were reported as γ-hemolysis which were characterized by the absence of clear zones formed around bacterial colonies (Table 2). Most LAB isolated from food have been reported as gamma-type hemolysis (Kumar and Murugalatha, 2012). Hemolysis is known as an indication of virulence factors in pathogenic microbes (Tallapragada *et al.*, 2018). Therefore, bacteria with gamma type hemolysis are the main requirement for the selection of strains that are safe for human consumption (Bassyouni *et al.*, 2012).

Organoleptic evaluation of fermented mare's milk

Isolate BC10 was selected as isolates that was used as starter cultures with the consideration that this isolate had the fast acidification properties (initial screening), had high proteolytic activity, antimicrobial activity, and did not demonstrate hemolytic activity. There were three types of fermented mare's milk tested organoleptically, namely YC (yogurt cultures), SC (selected culture: isolate BC10), and MC (mixture of yogurt starter culture and isolate BC10 or mixed culture).

The results of the organoleptic evaluation showed that mare's milk fermented using SC (BC10) had the highest score on almost all parameters tested except color (Figure 3). The panelists like very much the aroma of mare's milk fermented by SC (isolate BC10). Mare's milk fermented using SC produced distinctive aroma, which was similar to young coconut. The aroma of fresh mare's milk is like straw and has an after taste like coconut (Potočnik *et al.*, 2011). Overall, SC fermented milk was the most preferred fermented mare's milk. Therefore, isolate BC10 was a potential candidate to be used as a

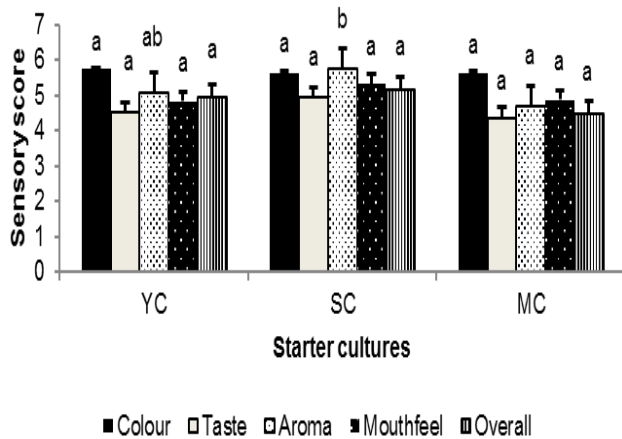


Figure 3: Scoring of organoleptic evaluation of fermented mare's milk. Data were expressed as the mean from 31 unskilled panelists while error bar indicates its standard deviation. SC: selected culture, YC: yogurt cultures, and MC: mixed cultures.

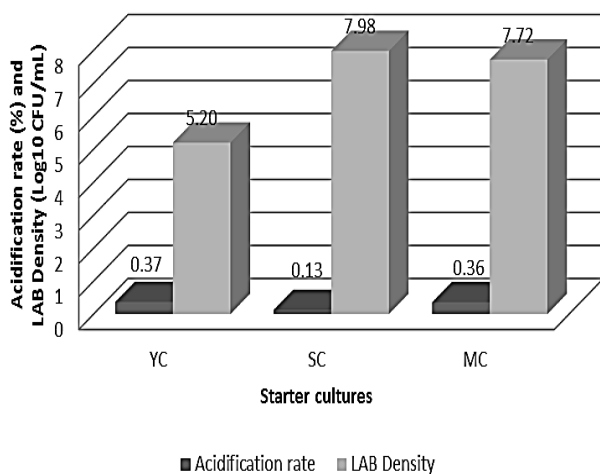


Figure 4: Acidification rate and LAB density of starter cultures in mare's milk fermentation product. SC: selected culture, YC: yogurt cultures, and MC: mixed cultures.

starter culture for mare's milk fermentation products. In addition, the advantage of BC10 as a starter culture was its antibacterial activity against *E. coli* ATCC 25922 to enhance the microbiological safety of the fermented products. The survival of food-borne pathogens, especially *E. coli* during fermentation was demonstrated in yogurt and kefir due to a high level of contamination (Gulmez and Guven, 2003). Even if the starter cultures with antibacterial activities are ingested, they will not interfere a dramatic effect on the overall intestinal microbial populations (Guinane *et al.*, 2016).

Although the lowest acidification rate was determined from the fermented mare's milk using SC starter culture (isolate BC10), however, it produced the highest LAB cell

density by 7.98 log₁₀ CFU/mL, while the lowest LAB density found in mare's milk fermented using YC starter culture by 5.2 log₁₀ CFU/mL (Figure 4). The acidification rate is not merely interfered by the cell density, but it is depended on the metabolic pathway. Starter culture consisting of homofermentative LAB produces higher acidity than heterofermentative LAB. Moreover, MC reduced pH value more rapidly as a result of lactose fermentation compared to SC. Isolate BC10 as starter culture demonstrated slow acidification properties compared to other starter culture type as can be seen in the rate of acidification of SC (Figure 4). As a result, when isolate BC10 was combined with YC, the rate of acidification of MC starter culture was relatively similar to YC starter culture. Strain with slow acidification rate is suitable to be used as adjunct cultures in fermented products (Sarantinopoulos *et al.*, 2001; Akabanda *et al.*, 2014).

Molecular identification 16S rDNA of the selected LAB strain

Based on the molecular identification, isolate BC10 was identified as *Lactobacillus plantarum* with similarity value of 99.99% towards *Lactobacillus plantarum* NBRC 15891^T (Figure 5). The evolutionary distance between isolate BC10 and the reference strain was 0.001, so that the similarity was 99.99%. The smaller the evolutionary distance among isolates, the greater the similarity value. A similarity value of 89–98% indicates that isolates belong to the same Genus, while 99–100% similarity values indicate isolates belong to the same species with the reference species (Suharjono *et al.*, 2010). A high similarity value between isolate BC10 and reference species does not mean that they are same strain. This is due to the analysis of the 16S rDNA sequence, which cannot distinguish species until strain level.

Lactobacillus is perhaps the most predominant genus of LAB (Elagoz *et al.*, 1996). *Lactobacillus plantarum* is the most versatile species/strain with useful properties and usually found in numerous fermented food products (Guidone *et al.*, 2014). Some of these fermented products include vegetables, cereals, meat, fish, milk, several ethnic fermented products, novel foods, mainly plant based foods, and beverages (Behera *et al.*, 2018). Moreover, *L. plantarum* is widely employed in industrial fermentation and processing of raw foods and generally recognized as safe (GRAS) and has qualified presumption of safety status (Ricci *et al.*, 2017). *Lactobacillus plantarum* strains must have a high ability to survive in the gastrointestinal tract (GI) and adhere to epithelial cells and most importantly be a safe strain towards animals and human (Jia *et al.*, 2017). Two concerns were noticed in *L. plantarum* BC10 as the potential starter culture, namely a low acidification rate and multidrug resistance (kanamycin, trimethoprim and cinoxacin). It would be of interest to determine the optimum fermentation conditions to increase the acidification rate by using the selected strain, as well as to

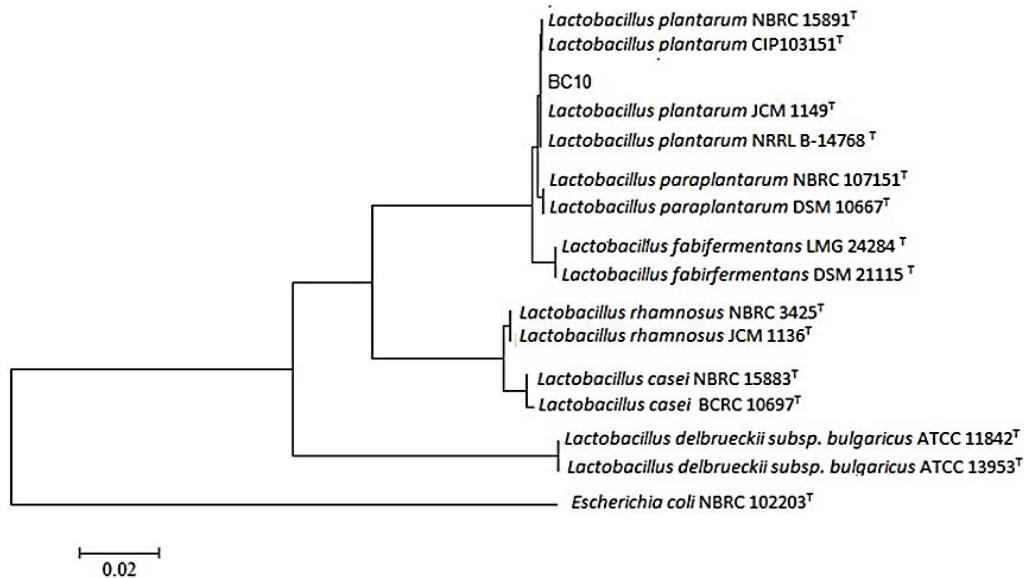


Figure 5: Phylogenetic tree of BC10 isolate and the reference strain based on similarity 16S rDNA sequence with Neighbor-Joining algorithm and Tamura-Nei model with bootstrap of 1000.

identify the encoding genes responsible for resistance and its transferability.

CONCLUSION

Six LAB isolates isolated from fermented Sumbawa mare's milk showed the fastest acidification activity. Isolate BC10 was selected as starter culture candidate since it exhibited the best ability in acidification and proteolytic activity, no lipolytic activity, and no hemolytic activity, and were able to inhibit *Escherichia coli* ATCC 25922. Isolate BC10 also demonstrated its ability to improve the organoleptic quality of mare's milk fermentation particularly the aroma. This isolate was identified as *Lactobacillus plantarum* with sequence similarity value of 99.99%. Therefore, *L. plantarum* BC10 is a potential isolate to be used as starter cultures for fermenting mare's milk products with still considering the possible existence of transferrable antibiotic resistance genes.

ACKNOWLEDGEMENTS

We would like to thank Indofood Sukses Makmur Tbk. for supporting this research through Indofood Riset Nugraha Program (IRN) 2018/2019 No. SKE.517/CC/VIII/2018.

REFERENCES

- Akabanda, F., Owusu-Kwarteng, J., Tano-Debrah, K., Parkouda, C. and Jespersen, L. (2014). The use of lactic acid bacteria starter culture in the production of Nunu, a spontaneously fermented milk product in Ghana. *International Journal of Food Science* **2014**, 721067.
- Apolonio, A. C. M., Carvalho, M. A. R., Bemquerer, M. P., Santoro, M. M., Pinto, S. Q., Oliveira, J. S., Santos, K. V. and Farias, L. M. (2007). Purification and partial characterization of a bacteriocin produced by *Eikenella corrodens*. *Journal of Applied Microbiology* **104**(2), 508-514.
- Aravindan, R., Anbumathi, P. and Viruthagiri, T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology* **6**(2), 141-158.
- Ayad, E. H. E., Nashat, S., El-Sadek N., Metwaly, H. and El-Soda, M. (2004). Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. *Food Microbiology* **21**(6), 715-725.
- Bassyouni, R. H., Abdel-all, W. S., Abdel-all, M. G. F. S. and Kamel, Z. (2012). Characterization of lactic acid bacteria isolated from dairy products in Egypt as a probiotic. *Life Science Journal* **9**(4), 2924-2933.
- Behera, S. S., Ray, R. C. and Zdolec, N. (2018). *Lactobacillus plantarum* with functional properties: An approach to increase safety and shelf-life of fermented foods. *BioMed Research International* **2018**, 9361614.
- Chaves-López, C., Serio, A., Paparella, A., Martuscelli, M., Corsetti, A., Tofalo, R., & Suzzi, G. (2014). Impact of microbial cultures on proteolysis and release

- of bioactive peptides in fermented milk. *Food microbiology* **42**, 117-121.
- Chen, Y. L., Lee, C. C., Lin, Y. L., Yin, K. M., Ho, C. L. and Liu, T. (2015).** Obtaining long 16S rDNA sequences using multiple primers and its application on dioxin-containing samples. *BMC Bioinformatics* **16**, S13.
- Christensen, J. E., Dudley, E. G., Pederson, J. A. and Steele, J. L. (1999).** Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **76(1-4)**, 217-246.
- Csapo, J., Stefler, J., Martin, T. G., Makray, S. and Csapo-Kiss, Z. (1995).** Composition of mares' colostrum and milk. Fat content, fatty acid composition and vitamin content. *International Dairy Journal* **5(4)**, 393-402.
- Danielsen, M., Andersen, H. S. and Wind, A. (2004).** Use of folic acid casei medium reveals trimethoprim susceptibility of *Lactobacillus* species. *Letters in Applied Microbiology* **38(3)**, 206-210.
- Danielsen, M. and Wind, A. (2003).** Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology* **82(1)**, 1-11.
- Danków, R., Pikul, J., Osten-Sacken, N. and Teichert, J. (2012).** Characteristics and salubrious properties of mare milk. *Nauka Przyroda Technologie* **3(6)**, 1-12.
- de Souza, J. V. and Dias, F. S. (2017).** Protective, technological, and functional properties of select autochthonous lactic acid bacteria from goat dairy products. *Current Opinion in Food Science* **13**, 1-9.
- Dinoto, A., Saputra, S., Nugroho, A. J. and Rahayu, R. D. (2011).** Keanekaragaman bakteri penghasil eksopolisakarida asal saluran cerna manusia. *Berkala Penelitian Hayati* **16**, 195-201.
- Duškova, M. and Karpiskova, R. (2013).** Antimicrobial resistance of lactobacilli isolated from food. *Czech Journal of Food Science* **31(1)**, 27-32.
- Elagoz, A., Abdi, A., Hubert, J. C. and Kammerer, B. (1996).** Structure and organisation of the pyrimidine biosynthesis pathway genes in *Lactobacillus plantarum*: A PCR strategy for sequencing without cloning. *Gene* **182(1-2)**, 37-43.
- Granato, D., Ribeiro, J. C. B., Castro, I. A. and Masson, M. L. (2010).** Sensory evaluation and physicochemical optimisation of soy-based desserts using response surface methodology. *Food Chemistry* **121(3)**, 899-906.
- Gueïmonde, M., Sanchez, B., de los Reyes-Gavilan, C. G., Margolles, A. (2013).** Antibiotic resistance in probiotic bacteria. *Frontiers in Microbiology* **4**, 202.
- Guidone, A., Zotta, T., Ross, R. P., Stanton, C., Rea, M.C., Parente, E., Ricciardi, A. (2014).** Functional properties of *Lactobacillus plantarum* strains: A multivariate screening study. *LWT - Food Science and Technology* **56(1)**, 69-76.
- Guinane, C. M., Lawton, E. M., O'Connor, P. M., O'Sullivan, O., Hill, C., Ross, R. P. and Cotter, P. D. (2016).** The bacteriocin bactofencin A subtly modulates gut microbial populations. *Anaerobe* **40**, 41-49.
- Gulmez, M. and Guven, A. (2003).** Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b and *Yersinia enterocolitica* O3 in different yogurt and kefir combinations as prefermentation contaminant. *Journal of Applied Microbiology* **95(3)**, 631-636.
- Jastrzębska, E., Wadas, E., Daszkiewicz, T., Pietrzak-Fiećko, R. (2017).** Nutritional value and health-promoting properties of mare's milk – a review. *Czech Journal of Animal Science* **62(12)**, 511-518.
- Jatmiko, Y. D., Howarth, G. S. and Barton, M. D. (2018).** Naturally fermented milk and its therapeutic potential in the treatment of inflammatory intestinal disorders. *AIP Conference Proceedings* **2019**, 060009-1-060009-16.
- Jatmiko, Y. D., Mustafa, I. and Ardyati, T. (2019).** Profile of microbial community of naturally fermented Sumbawa mare's milk using next-generation sequencing. *Journal of Biological Researches* **24(2)**, 58-62.
- Jia, F. F., Zhang, L. J. and Pang, X. H., Gu, X. X., Abdelazez, A., Liang, Y., Sun, S. R. and Meng, X. C. (2017).** Complete genome sequence of bacteriocin-producing *Lactobacillus plantarum* KLDS1.0391, a probiotic strain with gastrointestinal tract resistance and adhesion to the intestinal epithelial cells. *Genomics* **109(5-6)**, 432-437.
- Kumar, A. M. and Murugalatha, N. (2012).** Isolation of *Lactobacillus plantarum* from cow milk and screening for the presence of sugar alcohol producing gene. *Journal of Microbiology and Antimicrobials* **4(6)**, 16-22.
- Li, S., Li, Z., Wei, W., Ma, C., Song, X., Li, S., He, W., Tian, J. and Huo, X. (2015).** Association of mutation patterns in GyrA and ParC genes with quinolone resistance levels in lactic acid bacteria. *The Journal of Antibiotics* **68**, 81-87.
- Mahmoudi, F., Miloud, H., Bettache, G. and Mebrouk, K. (2013).** Identification and physiological properties of *Bifidobacterium* strains isolated from different origin. *Journal of Food Science and Engineering* **3(4)**, 196-206.
- Malacarne, M., Martuzzi, F., Summer, A. and Mariani, P. (2002).** Protein and fat composition of mare's milk: Some nutritional remarks with reference to human and cow's milk. *International Dairy Journal* **12(11)**, 869-877.
- Mathur, S. and Singh, R. (2005).** Antibiotic resistance in food lactic acid bacteria – a review. *International Journal Food Microbiology* **105(3)**, 281-295.
- Monika, Savitri, Kumar, V., Kumari, A., Angmo, K. and Bhalla, T. C. (2017).** Isolation and characterization of lactic acid bacteria from traditional pickles of Himachal Pradesh, India. *Journal of Food Science and Technology* **54(7)**, 1945-1952.
- Moslehshad, M., Mirdamadi, S., Ehsani, M. R., Ezzatpanah, H. and Moosavi-Movahedi, A. A. (2013).** The proteolytic activity of selected lactic acid bacteria in fermenting cow's and camel's milk and the resultant sensory characteristics of the products.

- International Journal of Dairy Technology* **66(2)**, 279-285.
- Mulyawati, A. I., Jatmiko, Y. D., Mustafa, I., Ardyati, T. and Suharjono (2019).** Diversity of lactic acid bacteria isolated from fermented mare's milk products based on PCR-RFLP analysis. *IOP Conference Series: Earth and Environmental Science* **230**, 012104.
- Potočnik, K., Gantner, V., Kuterovac, K., Cividini, A. (2011).** Mare's milk: Composition and protein fraction in comparison with different milk species. *Mljekarstvo* **61(2)**, 107-113.
- Ravindra, P. (2015).** Advances in Bioprocess Technology. Springer. pp. 435-454.
- Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R. et al. (2017).** Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 5: Suitability of taxonomic units notified to EFSA until September 2016. *EFSA Journal* **15(3)**, 4663-4684.
- Ruas-Madiedo, P. (2005).** Methods for the screening, isolation and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science* **88**, 853-866.
- Sarantinopoulos, P., Andrighetto, C., Georgalaki, M. D., Rea, M. C., Lombardi, A., Cogan, T. M., Kalantzopoulos, G. and Tsakalidou, E. (2001).** Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal* **11**, 621-647.
- Sheng, Q. and Fang, X. (2009).** Bioactive components in mare milk. In: *Bioactive Components in Milk and Dairy Products*. Park Y. W. (ed.). Wiley-Blackwell, Oxford, UK. pp. 195-213.
- Sierra, G. (1957).** A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek* **23(1)**, 15-22.
- Suharjono, S., Agung, P. W. M., Triwiratno, A., Wuryantini, S. and Oktavia, L. (2010).** Sistematis filogenetik isolat-isolat kapang indigenous Indonesia sebagai entomopatogen Kutu Sisik (*Lepidosaphes beckii* Newman) hama tanaman jeruk. *Biota* **15(2)**, 231-236.
- Tallapragada, P., Rayavarapu, B., Rao, P. P., Ranganath, N. N. and Veerabhadrapa, P. P. (2018).** Screening of potential probiotic lactic acid bacteria and production of amylase and its partial purification. *Journal of Genetic Engineering and Biotechnology* **16(2)**, 357-362.
- Tsakalidou, E., Manolopoulou, E., Kabaraki, E., Zoidou, E., Pot, B., Kersters, K. and Kalantzopoulos, G. (1994).** The combined use of whole-cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic acid bacteria isolated from traditional Greek dairy products. *Systematic and Applied Microbiology* **17(3)**, 444-445.
- Vijayaraghavan, P. and Vincent, S. G. P. (2013).** A simple method for the detection of protease activity on agar plates using bromocresol green dye. *Journal of Biochemical Technology* **4(3)**, 628-630.
- Watts, B. M., Ylimaki, G. L., Jeffery, L. E. and Elias, L. G. (1989).** Basic sensory methods for food evaluation. The International Development Research Centre. Ottawa, Canada. pp. 66-78.