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# ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM PAMPANGAN BUFFALO MILK OF SOUTH SUMATERA INDONESIA

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## ABSTRACT

*Pampangan buffalo milk is one of lactic acid bacteria (LAB) source which potentially can be used as starter or probiotic starter. The objective of this research was to isolate and identify LAB derived from Pampangan buffalo, South Sumatera.* Lactic acid bacteria was isolated and grown at *De Man Rogosa Sharpe Agar (MRSA). Isolation was performed until pure culture was obtained. LAB identification was carried out through morphology, physiology and biochemical characteristic test. Morphological test was done through Gram staining and cell shape identification. Physiological characteristic was done through survivability test on pH 4.5 and temperature 45 $^{o}$C. Biochemical characteristic was carried out through catalase test, identifying the production of $CO_2$, $NH_3$, and dextran. A total of 21 LAB isolates were identified which generally known having Gram positive, rod and rounded shape. From the result of physiological and biochemical test, 10 LAB isolates from Pampangan buffalo milk were found i.e.* SKP2, SKP3, SKP5, SKP6, SKP9, SKP10, SKP14, SKP19, SKP20 and SKP21 isolates. API test CHL 50 test resulted that five LAB species were found from 10 selected isolates contained in Pampangan buffalo milk. Those LAB species were *L. brevis, L. paracasei, L. pentosus, L. plantarum* and *Lactococus lactis.*

**KEYWORDS:** *buffalo milk, LAB, isolation, identification.*

## INTRODUCTION

Milk is one of food material which naturally contains LAB and can be used for starter culture of various food products. Lactic acid bacteria from milk is also potential to be utilized as probiotic for functional foods manufacturing. According to FAO and WHO, probiotic is a life microbe which gives health benefit in a certain amount (Ljungh & Wadstrom 2006). Lactic acid bacteria is one of probiotic microbe which is commonly used for foods and drinks fermentation (de Vrese M et al. 2011).

LAB isolation from various milk types has been carried out including from breast milk (Nuraida et al. 2011), sheep milk (Iranmanesh et al. 2012), goat milk (Setyawardani et al. 2013), Bima horse milk (Antara et al. 2009), wild horse milk (Sugitha et al. 2011), Sumbawa horse milk (Sujaya et al. 2008), cow milk (Abdullah & Osman 2010), camel milk (Khedid et al. 2009; Abbas et al. 2015), and buffalo milk (Aziz et al. 2009; Tambekar et al. 2009; Singh & Sharma 2009; Patel & Patel, 2012; Sharma et al. 2013; Shafakatullah & Chandra 2014; Kumar et al. 2014). In Indonesia, LAB isolation from buffalo milk or its processed products have been carried out using river buffalo milk of North Sumatera (Rizqiati et al. 2015a and Rizqiati et al. 2015b), and using whey of West Sumatera buffalo milk (Sunaryanto & Marwoto 2013). Research related with the potency of LAB as probiotic derived from indigenous material has being continually conducted especially to be used as functional foods.

Pampangan buffalo is one of native cattle genetic resource from Indonesia which is not so popular today. However, the milking production of this buffalo reaches 8 liter per day. The milk quality is better compared with cow milk where the protein and fat content is higher but it contains lower cholesterol (Damayanthi et al. 2014). Exploration of LAB from Pampangan buffalo milk and its utilization for starter culture or probiotic has not been widely reported yet. LAB exploration is the initial stage to obtain probiotic bacteria candidate through probiotic characteristic test and then followed by its application on mozzarella cheese to produce probiotic mozzarella cheese with Pampangan buffalo milk as the raw material.

The objective of this research was to isolate LAB from Pampangan buffalo milk and to identify based on morphological, physiological and biochemical characteristic. The result of isolation and identification of LAB became the initial stage of LAB selection from Pampangan buffalo milk as probiotic candidate.

## MATERIAL AND METHOD

Material used in this research was Pampangan buffalo milk obtained from buffalo ranch in Pampangan district (Oki regency) and Rambutan district (Banyuasin regency) of South Sumatera province. Five samples were collected from each location. Buffalo milk samples were filled into sterile bottle and packed into cool box to be transported to research testing location. Testing was carried out in form of isolation and identification of LAB derived from Pampangan buffalo milk. Isolation was performed using De Man Rogosa Sharpe (MRS) medium. LAB identification consisted of identification on morphological, physiological and biochemical characteristic.

### LAB isolation

LAB isolation referred to modified Khedid et al. (2009). A 10 ml of buffalo milk sample was taken in aseptic condition and mixed into 90 ml of physiological sterile NaCl solution and then conducted suitable serial dilutions up to $10^5$. A total of 1 ml diluted sample was then placed into MRS agar (MRSA) containing *bromo cresol purple* (BCP) 0.01% at petri dish. Sample was then incubated in anaerobic condition for 24 hours at 37$^o$C. LAB colonies were determined as colonies which were surrounded by yellow color zone. Those colonies were then enumerated and streaked at MRSA. Streaking was repeatedly conducted to obtain pure colonies.

### Morphological characteristic for LAB identification

Characterizing cell morphology aimed at identifying the isolate shape and Gram staining characteristic. The cell shape was identified using microscope and after carrying out Gram staining. Gram positive with rod or round cell shape was expected in this morphology cell identification (Iranmanesh et al. 2012).

### Physiological characteristic for LAB identification

Physiological characteristic test consisted of survivability test against temperature and pH condition. Temperature survivability was conducted to select LAB which still survive at temperature 45$^o$C and 37$^o$C as control for 2-5 days. pH survivability test was carried out to select LAB isolates which can still survive at MRSB with acid condition of pH 4.5 and neutral condition of pH 7 as control and followed by incubation at 37$^o$C temperature for 7 days. Growth was justified from the muddy of the medium (Aziz et al. 2009).

### Biochemical characteristic for LAB identification

Biochemical characteristic test for LAB identification consisted of $CO_2$ production test, catalase test, dextran production test and $NH_3$ production test. $CO_2$ production test was conducted to determine the ability of isolates to produce $CO_2$ from glucose representing homofermentative or heterofermentative. Catalase test was conducted to determine the ability of isolates in producing catalase enzyme. Catalase test was conducted using peroxide hydrogen ($H_2O_2$) 3%. Dextran production test was conducted to determine the ability of isolates in producing dextran (mucous) which usually produced by *Leuconostoc* genes. $NH_3$ production test was carried out to determine the ability of isolates in producing ammonia which usually produced by *Streptococcus* genes (Setyawardani et al. 2013).

### LAB identification using kit API CHL 50

LAB identification using kit API CHL 50 (Biomerieux, France) was carried out to determine the type of LAB species. Testing was carried out by isolating one ose of LAB which placed into 10 ml of MRSB medium and then incubated at 37$^{o}$C for 24 hours. LAB culture was centrifuged 9800 x g for 10 minutes. Pellets which had been separated were placed into API medium 50 CHL with sterile pipet and homogenized using vortex. Culture was then placed into 50 wells of kit API CHL 50 strips. All wells were closed using paraffin oil to provide anaerobic condition and incubated at 37$^{o}$C for 24-48 hours. Tested parameter was blue color which turned into yellow color after incubated for 24-48 hours due to acid formation which detected by pH changes. Observed result was processed using Apiweb™ software (Gawad et al. 2010).

## RESULT AND DISCUSSION

### Lactic Acid Bacteria Isolated from Pampangan Buffalo Milk

LAB isolation was carried out using MRSA medium added with BCP as acid formation bacteria indicator where isolate producing acid will form yellow area. The presence of LAB colonies was round and white which surrounded by yellow zone. Isolation resulted 30 isolates of Pampangan buffalo milk which collected from each sample location i.e. Pampangan and Rambutan district. Each of location produced 15 isolates. LAB colonies which grown in MRSA medium added with BCP produced colonies which surrounded by yellow zone (Surono 2004).

**LAB Identification from Pampangan Buffalo Milk**

The principle of LAB isolation was to obtain single colony which will be tested to determine the LAB characteristic. A total of 30 isolates was able to be isolated from Pampangan buffalo. The morphological characteristic of isolates was determined through Gram staining which resulted that 21 isolates were Gram positive bacteria and 9 isolates were Gram negative. Isolates with Gram negative (-) were not used as those weren't LAB isolates. According to the shape test result of 21 isolates, only 6 isolates had round shape (28.6%) and 15 isolates had rod shape (71.4%) (Table 1). This result was also in agreement with Rizqiati *et al.* (2015a) who reported that among of 96 pure isolates from river buffalo milk of North Sumatera, 84 isolates were Gram positive and 12 isolates were Gram negative. Shape result test showed that among of 84 isolates, 19 isolates had round shape (22.6%) and 65 isolates had rod shape (77.4%). According to Axelsson *et al.* (2004), LAB is considered as Gram positive with rod or round shape and catalase negative.

**Table 1: Morphological characteristic of LAB isolated from Pampangan buffalo milk.**

| No. | Origin | Isolate code | Gram staining | Shape |
|---|---|---|---|---|
| 1. | Rambutan District | SKP1 | Positive (+) | Rod |
| 2. | Rambutan District | SKP2 | Positive (+) | Rod |
| 3. | Rambutan District | SKP3 | Positive (+) | Rod |
| 4. | Rambutan District | SKP4 | Positive (+) | Round |
| 5. | Rambutan District | SKP5 | Positive (+) | Round |
| 6. | Rambutan District | SKP6 | Positive (+) | Round |
| 7. | Rambutan District | SKP7 | Positive (+) | Round |
| 8. | Rambutan District | SKP8 | Positive (+) | Round |
| 9. | Rambutan District | SKP9 | Positive (+) | Rod |
| 10. | Rambutan District | SKP10 | Positive (+) | Rod |
| 11. | Pampangan District | SKP11 | Positive (+) | Round |
| 12. | Pampangan District | SKP12 | Positive (+) | Rod |
| 13. | Pampangan District | SKP13 | Positive (+) | Rod |
| 14. | Pampangan District | SKP14 | Positive (+) | Round |
| 15. | Pampangan District | SKP15 | Positive (+) | Rod |
| 16. | Pampangan District | SKP16 | Positive (+) | Rod |
| 17. | Pampangan District | SKP17 | Positive (+) | Rod |
| 18. | Pampangan District | SKP18 | Positive (+) | Round |
| 19. | Pampangan District | SKP19 | Positive (+) | Rod |
| 20. | Pampangan District | SKP20 | Positive (+) | Rod |
| 21. | Pampangan District | SKP21 | Positive (+) | Rod |

**Physiological and Biochemical Characteristic for LAB Identification**

The classification of LAB species based on physiological characteristic consisted of tolerance on temperature, salinity level and pH. Meanwhile, biochemical characteristic based on

catalase test consisted of ability to produce $CO_2$, dextran and $NH_3$. Table 2 shows LAB species classification of Pampangan buffalo milk based on physiological and biochemical characteristic test.

**Table 2: Classification of LAB species from Pampangan buffalo milk based on physiological and biochemical characteristic.**

| Isolate code | Cata lase | Temperature ($^oC$) | | | Salinity content (%) | | | pH | | | $CO_2$ | Dextran | $NH_3$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 37 | 45 | 0 | 4 | 6.5 | 4.5 | 7.0 | 9.6 | | | |
| SKP1 | - | - | + | - | + | + | + | - | + | + | + | - | - |
| SKP2 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP3 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP4 | - | + | + | + | + | + | + | + | + | + | + | - | + |
| SKP5 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP6 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP7 | - | - | + | - | + | + | + | - | + | + | - | - | - |
| SKP8 | + | - | + | + | + | + | + | + | + | + | + | - | - |
| SKP9 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP10 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP11 | - | + | + | - | + | + | + | + | + | + | + | - | - |
| SKP12 | - | - | + | + | + | + | + | - | + | + | - | - | - |
| SKP13 | - | + | + | - | + | + | + | - | + | + | - | - | - |
| SKP14 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP15 | - | - | + | - | + | + | + | + | + | + | + | - | - |
| SKP16 | - | - | + | + | + | + | + | + | + | + | + | - | - |
| SKP17 | - | - | + | + | + | + | + | - | + | + | - | - | - |
| SKP18 | - | - | + | - | + | + | + | + | + | + | - | - | + |
| SKP19 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP20 | - | + | + | + | + | + | + | + | + | + | - | - | - |
| SKP21 | - | + | + | + | + | + | + | + | + | + | - | - | - |

Physiological characteristic test consisted of survivability test on temperature and pH. All LAB isolates (21 isolates) grew at temperature of 37$^o$C (100%), 15 isolates grew at temperature of 45$^o$C (71,4%) and only 13 isolates (61,3%) grew at temperature 10$^o$C. LAB isolated from cheese tested on temperature and saline condition showed that all LAB *lactococci* types could grow at 10, 30 and 40$^o$C and saline content 1, 2 and 4 % (Ayad *et al.* 2006).

Survivability test of LAB on high temperature showed that among of 21 isolates, there were 15 isolates (71.4%) could survive at high temperature and only 6 isolates (28.6%) couldn't survive at high temperature. Survivability test of LAB isolated from North Sumatera river buffalo milk against high temperature showed that 92.8% could survive at high temperature

and 7.1% couldn't survive (Rizqiati et al. 2015a). Elgandi et al. (2008) reported survivability test of 14 isolates from fresh milk which can grow at temperature of 45$^{o}$C. According to El Soda et al. (2003), the thermophilic *lactobacilli* and *cocci* can grow at temperature 45$^{o}$C but not at 10$^{o}$C. Mesophilic group can grow at temperature 10$^{o}$C but not at 45$^{o}$C. The mesophilic *lactococci* can grow at temperature 10$^{o}$C but not at 45$^{o}$C. *Eneterococci* can grow at temperature 45 and 10$^{o}$C.

LAB tolerance test on saline condition at 4,0 dan 6,5 % showed that all LAB isolates could grow well. Ayad et al (2006) reported that *Lc. subsp cremoris* strain had better tolerance compared with *lactococci* strain in a 4,0 and 6,5 % saline content.

Survivability test on pH of 21 isolates showed that 16 isolates (76.2%) could grow well at pH 4.5 and 5 isolates (23.8%) couldn't survive at this condition. According to survivability test of LAB isolated from North Sumatera river buffalo milk at pH 4.5, Rizqiati et al. (2015) reported that 89.7% of LAB isolates can grow at pH 4.5 and 10.3% can't grow at this condition. Fowoyo & Ogunbanwo (2010) reported that fewer population of LAB isolates can grow at pH 2. Some of lactic acid bacteria types which can survive at pH 2 have higher potency to survive at more acid environment and can produce higher organic acid. This organic acid can be used to improve flavor, texture and taste of fermented products.

Based on the biochemical characteristic test of 21 LAB isolated from indigenous Pampangan buffalo milk showed that 6 isolates (28.6%) could produce $CO_2$ (heterofermentative) and 15 isolates (71.4%) couldn't produce $CO_2$ (homofermentative). According to the $CO_2$ production ability test from glucose of LAB isolated from indigenous North Sumatera river buffalo milk, Rizqiati et al. (2015) reported that 30% is heterofermentative and 70% is homofermentative. Similar result was also reported by Abdulah & Oman (2010) who said that more homofermentative LAB is found in cow milk, cheese and fermented milk. Based on the fermentation pattern, Axelsson (2004) stated that LAB consists of three groups namely obligate homofermentative, facultative heterofermentative and obligate heterofermentative.
Catalase test of 21 isolates showed that 20 isolates (95.2%) did not produce $O_2$ gas bubble which then characterized as negative catalase bacteria and 1 isolate (4.8%) produced $O_2$ gas bubble which then characterized as positive catalase. Based on the catalase test of LAB isolated from North Sumatera river buffalo, Rizqiati et al. (2015a) reported that 74.3% was negative catalase bacteria and 25.7% was positive catalase bacteria. Catalase test was carried out to determine the presence of catalase enzyme occurred at bacteria starter culture. Based

on dextran production test from sucrose on 21 isolates, it showed that all isolates didn't produce dextran. This result was a little bit different with those found by Rizqiati et al. (2015a) who carried out dextran production test of North Sumatera river buffalo milk. It is reported that 7.7% isolates positively produces dextran and 92.3% doesn't produce dextran. LAB isolates which not produce dextran can be said that those isolates is not included in *Leuconostoc* group. One of *Leuconostoc* characteristic is to visualize dextran production as mucoid. Dextran is defined as water soluble polyglucosan caride which formed from α1-6 glyosidic with 0-20% proportion (Sarwat et al. 2008).

Based on $NH_3$ production from arginine of 21 isolates, 2 isolates (9%) positively produced $NH_3$ and 19 isolates (91%) didn't produce $NH_3$. Isolates with $NH_3$ production was not selected to be used for next stage as $NH_3$ production could influence the flavor of processed product. This finding was in agreement with Rizqiati et al. (2015a) who reported that 14.6% isolates positively produce NH3 and 85.4% doesn't produce NH3. Tserovska et al. (2002) mentioned that 60% LAB from cheese and milk is able to produce $NH_3$ from arginine.

**LAB Identification through API test CHL 50**

LAB species identification through API Test CHL 50 was carried out at 10 LAB isolates i.e. SKP2, SKP3, SKP5, SKP6, SKP9, SKP10, SKP14, SKP19, SKP20 and SKP21. The testing result found 5 LAB species (Table 3) including *L. brevis* (1 isolate), *L. paracasei* (4 isolates), *L. pentosus* (1 isolate), *L. plantarum* (2 isolates) and *Lactococus lactis* (2 isolates).

**Table 3: LAB species identification as probiotic candidate using API test CHL 50.**

| NO | ISOLATE CODE | SPECIES NAME | % ID |
|---|---|---|---|
| 1 | SKP2 | *Lactobacillus brevis* | 99,9 |
| 2 | SKP3 | *Lactobacillus paracasei ssp paracasei* | 93,7 |
| 3 | SKP5 | *Lactococcus lactis spp lactis* | 93,3 |
| 4 | SKP6 | *Lactobacillus pentosus* | 99,9 |
| 5 | SKP9 | *Lactobacillus paracasei ssp paracasei* | 97,7 |
| 6 | SKP10 | *Lactobacillus plantarum* | 99,9 |
| 7 | SKP14 | *Lactococcus lactis spp lactis* | 99,9 |
| 8 | SKP19 | *Lactobacillus paracasei ssp paracasei* | 97,7 |
| 9 | SKP20 | *Lactobacillus paracasei ssp paracasei* | 95,6 |
| 10 | SKP21 | *Lactobacillus plantarum* | 99,9 |

SKP2 isolate was identified as *Lactobacillus brevis* with resemblance level 99.90%. Isolate SKP3, SKP9, SKP19 and SKP20 were identified as *Lactobacillus paracasei* with resemblance level 93.70%; 97.70%, 97, 70% and 95.60%, respectively. Isolate SKP6 was identified as *Lactobacillus pentosus* with resemblance level 99.90%, isolate SKP10 and SKP

21 were identified as *Lactobacillus plantarum* with resemblance level 99.90%. Isolate SKP5 and SKP14 were identified as *Lactobacillus lactis* with resemblance level 93.30% and 99.9%, respectively.

Species identification testing result from North Sumatera river buffalo milk using API test CHL 50 showed that there are four LAB species such as *Lactobacillus plantarum, L. brevis, L. pentosus* and *Lactococus lactis* (Rizqiati *et al*. 2015). LAB species identification test of India buffalo milk shows that six LAB species are identified i.e. *L. bulgaricus, L. plantarum, L. lactis, L. acidophilus, L. brevis* and *L. rhamnosus* (Tambekar et al. 2009; Singh et al. 2009; Azis et al. 2009; Syafakatullah et al. 2014). LAB species identification test of buffalo whey results six LAB species i.e. *L. brevis, L. plantarum, L. casei, L. paracasei, Lactococcus lactis, Leuconostoc mesenteroides* (Surono 2003). Sunaryanto and Marwoto (2012) are able to identify one LAB species i.e. *Lactobacillus plantarum*.

## CONCLUSION

This research was able to isolate and identify 21 LAB isolated from Pampangan buffalo milk. The morphological characteristic was Gram positive, rod and round shape. Based on the physiological and biochemical characteristic, there were 10 isolates which was able to survive at pH 4.5, temperature 45$^{o}$C, negative catalase, not produce $CO_2$, $NH_3$ and dextran i.e. SKP2, SKP3, SKP5, SKP6, SKP9, SKP10, SKP14, SKP19, SKP20 and SKP21 Those 10 isolates were then selected for next stage namely probiotic characteristic test. LAB species identification using API Test CHL 50 resulted five LAB species i.e. *L. brevis, L. paracasei, L. pentosus, L. plantarum* and *Lactococus lactis*.

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