

## Viability and Quality of Fermented Milk Made Using Local and Commercial Starters during Fermentation and Cold Storage

U. Pato<sup>1\*</sup>, V. S. Johan<sup>1</sup>, A. R. Raidinawan<sup>1</sup>, A. A. Ginting<sup>1</sup>, and I. Jaswir<sup>2</sup>

### ABSTRACT

Recent trends in food consumption and lifestyle show an increased demand for foods that are not only tasty and nutritious, but also provide additional benefits related to health, i.e. functional foods. One example of functional food is probiotic fermented milk produced with lactic acid bacteria. This has been shown to be beneficial to human gut health. The present study aimed to study the viability of *Lactobacillus Casei* subsp. *casei* R-68 (LCR-68) and *Lactobacillus Casei* strain Shirota (LCS) during fermentation and cold storage, as well as the quality of fermented milk produced from both strains. The research was conducted using a Completely Random Design. The data obtained was analyzed using ANOVA and DNMRT. The *t*-test was used to compare the growth and viability of LCR-68 and LCS. Fermentation time significantly affected the pH value, total lactic acid, total LAB and protein content, but did not significantly affect the fat and ash content of the fermented milk product. The best probiotic fermented milk in terms of viability and quality was produced via fermentation for 15 hours using strain LCR-68 as a starter. LCR-68 and LCS cultured in skimmed milk showed slightly different growth patterns. However, both strains showed similar viability. The total LAB after cold storage for a month was 6.64 and 6.68 log CFU mL<sup>-1</sup> in the LCR-68 and LCS fermented milk, respectively. According to the results, LCR-68 can be used as a starter for making probiotic fermented milk.

**Keywords:** *Lactobacillus casei* strain Shirota, *Lactobacillus casei* subsp. *casei* R-68, Probiotic fermented milk.

### INTRODUCTION

Probiotics are referred to as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (FAO/WHO, 2010). It is well-known that probiotics have many health benefits such as antimicrobial activity, diarrhea alleviation, anti-carcinogenic properties, improving lactose intolerance, strengthening the immune system (Cenci *et al.*, 2002; Shah, 2007; FAO/WHO, 2010; Pato *et al.*, 2017; Mahmoudi *et al.*, 2019a),

the ability to survive and adhere the gastrointestinal tract (Pato, 2003; Mahmoudi *et al.*, 2019a; Mahmoudi *et al.*, 2019b) and as a therapy against cold and flu pathogens (Leyer *et al.*, 2009).

The demand for healthy foods has been one of the most important trends of food consumption in recent years (Bigliardi and Galati, 2013). Consumers are increasingly aware of their own health as well as the social and environmental impact of their food consumption (Falguera *et al.*, 2012). Fermented milk is believed to have a good

<sup>1</sup>Department of Agricultural Technology, Faculty of Agriculture, Universitas Riau, Kampus Bina Widya KM 12.5 Simpang Baru, Pekanbaru 28293, Indonesia.

\*Corresponding author; e-mail: usmanpato@yahoo.com

<sup>2</sup>International Institute for Halal Research and Training, International Islamic University Malaysia, 53100 Jalan Gombak, Selangor, Malaysia.



nutrition level, in addition to properties that are beneficial for human health, especially the digestive system, because it contains probiotics that can nourish the human digestive tract. The majority of commercial probiotics are *Lactobacillus* and *bifidobacteria* species used in products such as yogurt, fermented milk and frozen desserts (Tamin and Robinson, 2001; Shah, 2007). According to Maulidya (2007), fermented products that use skimmed milk as a starting ingredient are advantageous. This is because it is easier for digestion and contains a very low amount of fat, allowing the product to be stored longer compared to products made from whole milk.

One of the requirements for Lactic Acid Bacteria (LAB) being used as a probiotic is viability during the processing and storage stages. Storage time and temperature affect bacterial survival (Anggraini, 2016). No general agreement has been reached on the recommended intake level. The suggested levels range from  $10^6$  CFU mL<sup>-1</sup> (Kurman and Rasic, 1991) to over  $10^7$  and  $10^8$  CFU mL<sup>-1</sup> (Korbekandi *et al.*, 2011). However, it is generally recommended that the probiotic culture must be present in the product at a minimum number of  $10^7$  CFU mL<sup>-1</sup> (Ishibashi and Shimamura, 1993). These suggestions have been made to compensate for the possible decline in the concentration of the probiotic organisms during the processing and storage of the product, as well as throughout the passage through the upper and lower parts of the gastrointestinal tract.

One example of a fermented milk product sold commercially is Yakult. Yakult uses *Lactobacillus Casei* strain Shirota (LCS) as a starter. LCS has various beneficial health effects in humans (Spanhaak *et al.*, 1998; Nagao *et al.*, 2000; Matsumoto *et al.*, 2006; Sutula *et al.*, 2013). The shelf life of LCS is about 40 days with  $10^6$  CFU mL<sup>-1</sup> total LAB during cold storage (Anonymous, 2015). Meanwhile, in West Sumatra, Indonesia, dadih is a traditional fermented food made from buffalo milk that is sold and consumed locally. Among the LAB isolates derived

from dadih curd (Hosono *et al.*, 1989) with the potential to be used as a probiotic, there is *Lactobacillus casei* subsp. *casei* R-68 (LCR-68) (Hosono *et al.*, 1990; Pato, 2003, Pato and Hosono, 2004; Pato *et al.*, 2017). Currently, there are no reports on the viability of LCR-68 during fermentation and cold storage. Therefore, the present study aimed to compare the viability of commercial strain LCS against local strain LCR-68 during cold storage, and the quality of the fermented milks produced from these strains.

## MATERIALS AND METHODS

### Research Method

The study was conducted experimentally. *Lactobacillus casei* subsp. *casei* R-68 (LCR-68) was isolated from dadih, traditional fermented buffalo milk, and identified by Hosono *et al.* (1989), *Lactobacillus Casei* strain Shirota (LCS) was obtained from the Indonesian Yakult Company. The viability of LCR-68 and LCS and the biochemical changes during fermentation were compared. Fermentation time varied between 6 to 26 hours at 37°C followed by cold storage at  $\pm 3-5^\circ\text{C}$ . The cultures were observed every week starting weeks 0 to 6.

### Preparation of Active Culture

Active culture was prepared according to Setioningsih *et al.* (2004), whereby 100 mL pure cultures of LCR-68 or LCS were inoculated into test tubes containing 5 mL of sterile MRS broth. The medium was stirred using an automatic mixer until homogeneous, then incubated at 37°C for 24 hours.

### Preparation of Starter

The starter was prepared following methods described by Setioningsih *et al.*

(2004) using LCR-68 and LCS (Yakult). First, a 15% skimmed milk medium was prepared and stirred evenly using a mixer until homogeneous. Then, the medium was sterilized at 115 °C for 10 minutes. After the medium reached a temperature of  $\pm 40^{\circ}\text{C}$ , the medium was inoculated with active 5% culture, then incubated at 37°C for 12 hours (starter I). Next, 5% of starter I was inoculated into 250 mL of 15% skimmed milk medium. This was incubated at 37°C for 12 hours to obtain starter II. Starter II was used to prepare the probiotic fermented milk.

### Preparation of Probiotic Fermented Milk

Probiotic fermented milk was prepared according to Ginting (2016). First, the medium was prepared as follows: skimmed milk (157.5 g), CMC (0.525 g) and sucrose (52.5 g) were weighed accurately, and water was added to the mix to a volume of 1,050 mL. The mixture was stirred using a mixer. The homogeneous medium was distributed into 42 plastic bottles each of 25 mL, and pasteurized at 85°C for 15 minutes. The medium was then cooled to 40°C and inoculated with 5% LCR-68 starter II in 21 bottles, and LCS starter II in 21 bottles. The bottles were then incubated at 37°C for 6 to 22 hours to obtain the final probiotic fermented milk product. Fermentation times were optimized to produce the probiotic fermented milk for the study.

### Cold Storage Treatment of Probiotic Fermented Milk

Cold storage of probiotic fermented milk was carried out according to methods described by Usman and Hosono (1999). Probiotic fermented milks made using LCR-68 or LCS were stored at  $\pm 3-5^{\circ}\text{C}$  for six weeks, and observed every week starting from weeks 0 (first day) to 6.

### Parameters Observed

The parameters measured in probiotic fermented milk were moisture, fat, protein, ash, carbohydrate, lactose, sucrose, and reducing sugar content as well as pH, total lactic acid, and LAB viability. Proximate analysis as well as lactose, sucrose, and reducing sugar contents were performed according to AOAC (2012). LAB viability was calculated according to the method described by Fardiaz (1998). pH was measured using a pH meter, and total lactic acid was determined by alkalimetric titration using 0.1N NaOH.

### Data Analysis

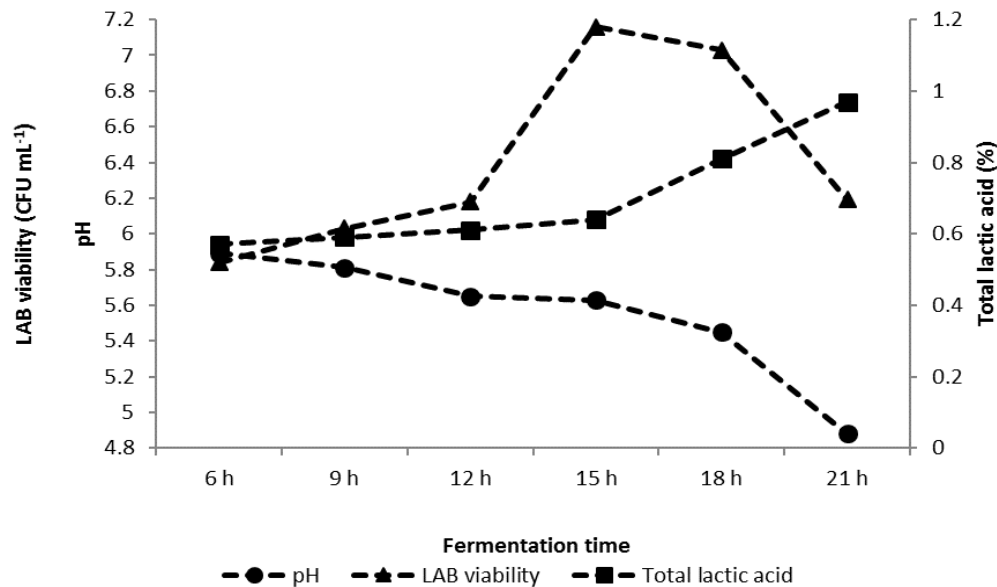
The data were analyzed using analysis of variance (ANOVA). Data with F count equal to or greater than F table were further tested using the Duncan New Multiple Range Test (DNMRT) at 5% level to determine the differences between treatments. Data resulting from the comparison tests were further analyzed by *t*-test.

## RESULTS AND DISCUSSION

### LCR-68 Fermented Milk Quality Parameters

The results show that fermentation time significantly affected pH, total lactic acid and LAB viability in LCR-68 fermented milk (Figure 1).

Figure 1 shows that longer fermentation times decreased pH values significantly, and conversely increased total lactic acid. The longer the fermentation process, the more simple sugars (especially lactose and glucose) were broken down by LCR-68 into lactic acid. Similar results were reported in a traditional fermented milk product from Ghana called *Nunu* (Akabanda *et al.*, 2010) and in fermented sheep's milk (Lasik *et al.*, 2011).



**Figure 1.** pH, total lactic acid, and LAB viability during fermentation using starter *Lactobacillus Casei* subsp. *casei* R-68 (LCR-68).

Total lactic acid content of the probiotic fermented milks produced using different fermentation times in this study ranged from 0.57 to 0.97%. All the treatments produced fermented milks that met yogurt quality standards for total lactic acid content, which is between 0.5 to 2.0%; the minimum Codex standard for total lactic acid content is 0.3% and Yakult quality standards is 0.5%.

Longer fermentation times resulted in higher LAB viability. After fermentation for 15 to 18 hours, LAB viability increased significantly. After 21 hours of fermentation, LAB viability decreased significantly. Increased fermentation times led to an increased release of metabolic compounds into the fermented milk in the form of lactic acid. Increased lactic acid in the media inhibits the growth of LCR-68. The LAB viability of the probiotic fermented milk under treatments T4 and T5 (see Table 1) met the LAB viability of the yogurt quality standard equal to a minimum of  $10^7$  CFU g<sup>-1</sup> (SNI 2981: 2009). It also met the Codex standards for fermented milk (Codex Stan 243-2003) and Yakult quality standards, while the commercial fermented milk products use LCS as a starter culture.

Analysis of the quality parameters of the fermented milk indicated that fermentation time significantly affected protein and carbohydrate levels, but did not significantly affect moisture, fat and ash contents of LCR-68 fermented milk (Table 1).

The data in Table 1 show that long fermentation times did not significantly affect moisture, fat and ash contents of fermented milk. Similar amounts of water, sucrose and skim milk were used as starting ingredients in the preparation of the fermented milk: 15% skimmed milk and 1% sucrose were used in the media. Across different fermentation times, it is possible that LCR-68 does not metabolize fat in the medium as an energy source during growth. Moreover, the moisture and ash components remained relatively constant in the media due to the constant water and mineral requirements by LAB during fermentation.

The data in Table 1 shows that the longer fermentation times increased the protein level in the fermented milk. Fermentation for 18 and 21 hours showed significantly increased protein levels. The increase in protein level can be attributed to the increase in the number of LCR-68, where protein

**Table 1.** Quality parameters of probiotic fermented milk made using starter *Lactobacillus casei* subsp. *casei* R-68 (LCR-68) during the fermentation process.

Fermentation time (h)	Moisture (%)	Fat (%)	Protein (%)	Ash (%)	Carbo-hydrate (%)
T1 (Fermentation time for 6 h)	85.22 a <sup>a</sup>	0.33 a	2.92 b	0.42 a	11.10 a
T2 (Fermentation time for 10 h)	85.14 a	0.35 a	3.21 ab	0.44 a	10.51 ab
T3 (Fermentation time for 14 h)	85.51 a	0.34 a	3.50 ab	0.46 a	10.25 abc
T4 (Fermentation time for 18 h)	85.05 a	0.34 a	3.79 ab	0.47 a	10.35 abc
T5 (Fermentation time for 22 h)	85.51 a	0.31 a	4.09 a	0.50 a	9.480 c
T6 (Fermentation time for 26 h)	85.36 a	0.30 a	4.09 a	0.51 a	9.750 bc
Commercial Yakult	81.90	0.10	1.20	0.30	16.50

<sup>a</sup> Means followed by the lowercase letters in the same column indicate significant difference ( $P < 0.05$ ).

makes up a large part of the LAB cells. LAB cells containing protein were found in the cell wall, cell membrane, ribosome and cytoplasm (Moat *et al.*, 2002; Hu *et al.*, 2011). Thus, the higher the number of LABs, the higher level of protein obtained in the cocoghurt. Imam *et al.* (2015) reported an increase in protein levels in cocoghurt during fermentation from 3 to 15 hours. Conversely, longer fermentation times lowered the carbohydrate level of the fermented milk. The decrease in carbohydrate level is due to the metabolism of the lactose and sucrose sugars in the fermentation medium as a conversion into lactic acid by the LCR-68. Strain LCR-68 mainly metabolizes lactose and sucrose to obtain energy and to form lactic acid as a by-product (Pato *et al.*, 2017). This metabolic process is further indicated by a decrease in pH value and an increase in the total amount of lactic acid produced (Figure 1).

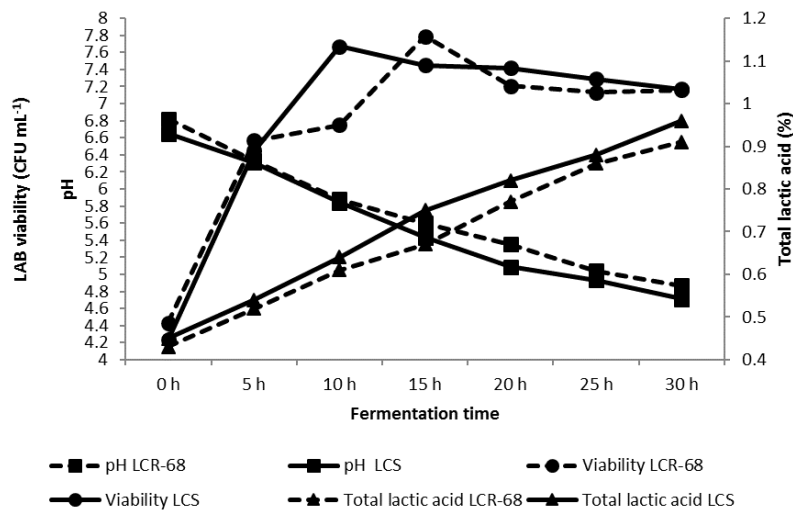
The moisture content of the fermented milks in the present study was slightly higher (85.14-85.51%) than that in commercial Yakult (81.9%). Fat content was also slightly higher (0.30-0.35%) than in Yakult (0.1%). Protein content in LCR-68 and LCS probiotic fermented milk (2.92-4.09%) was higher than that in Yakult (1.2%). Overall, protein levels in the final fermented milks met the standard protein content of at least 2.7% following the SNI and Codex standard. Meanwhile, ash content in the probiotic fermented milks (0.42-0.51%) was slightly higher than that in Yakult (0.3%). However, carbohydrate levels in Yakult were found to be higher

than in our fermented milks following all treatment times. Yakult carbohydrate levels at 16.5% were much higher than those measured in our LCR-68 fermented milk (9.48-11.10%). The higher carbohydrate levels in Yakult could be attributed to the addition of sucrose syrup to the medium during the manufacturing process to produce a sweet-tasting product that is more preferred by consumers (Anonymous, 2015).

Based on the results of the first part of this study, it was found that a 15-hour fermentation time produced the best LCR-68 fermented milk in terms of quality and LAB viability. Therefore, part two of the study was carried out to compare the viability between strains LCR-68 and LCS over time. The results are shown in Figure 2. The longer the fermentation process, the more significant the decrease in pH value in both fermented milks (strain LCR-68 and strain LCS). Decreasing pH values in both types of fermented milk showed relatively similar patterns with no significant differences according to the *t*-test. The decrease in pH value in both probiotic fermented milks is a result of the metabolism of lactose and sucrose into lactic acid.

### Comparison of LCR-68 and LCS Fermented Milk

Longer fermentation times significantly increased total lactic acid levels produced in both LCR-68 and LCS probiotic fermented milk. The increase in total lactic acid



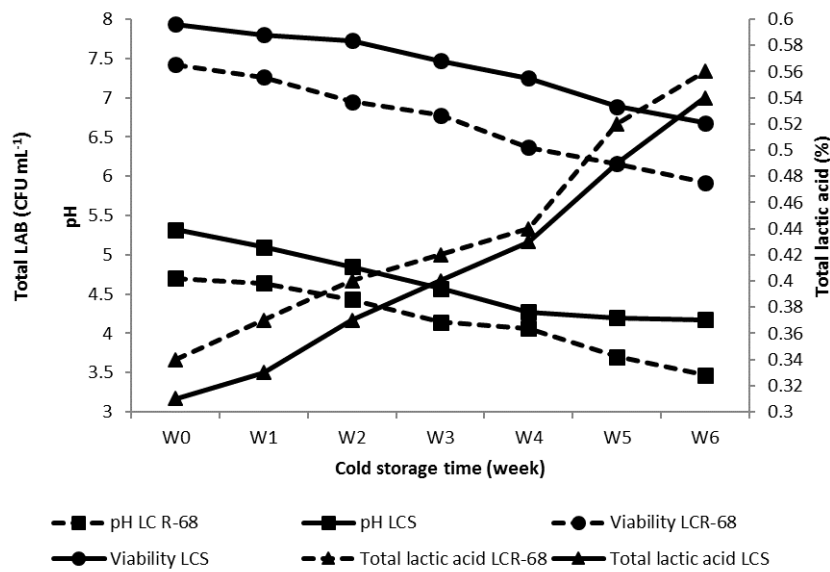
**Figure 2.** Comparison of pH, total lactic acid and LAB viability between LCR-68 and LCS during fermentation.

produced in both types of fermented milk showed relatively similar patterns. The amount of lactic acid produced by strain LCS was higher than that of strain LCR-68, but total lactic acid at similar fermentation times showed no significant difference, according to the *t*-test. Longer fermentation times increased the number of LAB significantly in both LCR-68 and LCS probiotic fermented milk. The increase was due to the availability of nutritional sources derived from skimmed milk and sucrose contained in the medium. Sucrose and lactose were used as energy sources for the relatively rapid growth of LAB at 10-hour fermentation time for strain LCS and 15 hours for LCR-68. In the production of kefir, during the first 24 hours of fermentation, the lactose content decreased from a mean value of 4.92 (w/w) to 4.02% (w/w); the concentration of L(+)-lactic acid increased from 0.01% to 0.76% (w/w) and the pH decreased to 4.24 over the same period. After 24 hours of fermentation, the changes in the levels of lactose and L(+)-lactic acid, and in pH, occurred more slowly (Fontan *et al.*, 2006). Decrease in pH from 6.09 to 4.85 and lactose content from 45.80 to 32.40 mg mL<sup>-1</sup> occurred at the end of 6 to 24 hours fermentation time in Brazilian kefir (Leite *et al.*, 2013).

LAB in both types of fermented milk increased, despite showing a somewhat different pattern (Figure 2). Both strains had the same growth pattern when fermented for five hours. After five hours, LCS continued growth in the logarithmic phase until 10 hours of fermentation before entering the stationary phase. On the other hand, LCR-68 growth remained in the logarithmic phase for up to 15 hours, before entering the stationary phase at 20 to 30 hours of fermentation. The *t*-test indicates significant differences between both strains after fermentation of 10 to 15 hours. At the end of single starter fermentation, LCR-68 and LCS were present at levels of 7.16 and 7.17 log units, respectively. In contrast, fermentation of kefir using mixed starters of lactic acid bacteria, acetic acid bacteria, and yeast presented lactic acid bacteria at 10 log units (Leite *et al.*, 2013).

#### Viability of LCR-68 and LCS in Cold Storage

Yakult and probiotic fermented milk are generally distributed and sold in cold conditions. Therefore, it is necessary to study the viability of probiotics during cold storage.



**Figure 3.** LAB viability, pH and total lactic acid in probiotic fermented milk during cold storage.

Lactic acid bacteria viability in both strains LCR-68 and LCS decreased with length of cold storage. Total LCR-68 in fermented milk at weeks 0 to 6 decreased 20.21% from 7.42 to 5.92 log CFU mL<sup>-1</sup> (down 1.50 log CFU mL<sup>-1</sup>), whereas total LCS from weeks 0 to 6 decreased 15.90% from 7.94 to 6.68 log CFU mL<sup>-1</sup> (down 1.26 log CFU mL<sup>-1</sup>). Under the same conditions, LCR-68 showed greater decrease in LAB compared to LCS. A similar decrease in lactose levels in the fermented milk was observed; lactose levels in LCR-68 (42.27%) decreased more than LCS (35.0%) (Figure 3). According to Anggraini, 2016, the slow growth of LAB in fermented milk is caused by the limited amount of lactose in fermented milk. As a result, activity of LAB was inhibited, resulting in decreased total LAB during cold storage. In contrast, the total lactic acid bacteria in Brazilian kefir remained constant during cold storage (Leita *et al.*, 2013).

There are four phases of LAB growth: the lag phase (adaptation), the logarithmic (exponential) phase, the stationary phase, and the death phase (Volk and Wheeler, 1993). The data in Figure 3 shows a decrease in the number of both LAB strains from weeks 0 to 6. The total decrease in LAB is caused by a lack of additional nutrients for LAB growth

during the cold storage process. The cells run out of energy, including reserved energy in their cells. After going through the logarithmic and stationary phases, the cells undergo the decline or death phase, which is characteristic of the low levels of LAB in the fermented milk after several weeks (Maulidya, 2007). Mulyani *et al.* (2008) reported that probiotic ice cream stored for 10, 20, and 30 days showed a decrease in the LAB viability from  $1.5 \times 10^{10}$  to  $1.3 \times 10^9$  CFU mL<sup>-1</sup>, and then to  $1.1 \times 10^8$  CFU mL<sup>-1</sup>, respectively. Soyghurt probiotics used as praline chocolate fillers stored for four weeks showed a decrease in total LAB from weeks 0 to 4, i.e. 10.18, 10.15, 10.25, 10.34 and 9.97 CFU mL<sup>-1</sup>, respectively (Rangkuti *et al.*, 2013). The number of Lactobacilli and Streptococcus slightly decreased during cold storage at 5°C for 15 days for both ewe and cow milk yoghurts (Karami, 2018). The minimum number of probiotic strains in fermented milk products or food products is 10<sup>8</sup> CFU mL<sup>-1</sup>. Meanwhile, the recommended daily intake for probiotics is around 10<sup>8</sup> CFU mL<sup>-1</sup> (Shah, 2007). Based on these results, LCR-68 fermented milk meets the criteria up to the fifth week; while LCS fermented milk meets the criteria up until the sixth week. At least 100 mL intake per day is required in



order for the fermented milk probiotics to benefit the human digestive tract.

Total lactic acid in the fermented milk during cold storage increased with length of storage time. Total lactic acid in LCR-68 fermented milk increased from weeks 0 to 6 from 0.34 to 0.56% (64.7% increase), while total lactic acid in LCS fermented milk from weeks 0 to 6, increased from 0.31 to 0.54% (74.2% increase) (Figure 3). The increase in total lactic acid in LCS fermented milk was greater than LCR-68. Lactic acid levels in fermented milks increase with increased storage time, as reported by Asaminew and Eyassu (2011). During cold storage, the fermentation process continues, whereby LAB metabolizes lactose and sucrose into lactic acid. An increase in total lactic acid correlates with an increase in total dissociated and non-dissociated acids (Usmiati *et al.*, 2011). Lactic acid levels in yogurt that is accepted from the sensory standpoint ranges from 0.8 to 2.0%. The production of lactic acid in fermentation is closely related to the number of *Lactobacillus casei* in fermented products. In a study by Rangkuti *et al.* (2013), probiotic soyghurt used as praline chocolate filler had a shelf life storage period of four weeks. Total lactic acid in the soyghurt increased from week 0 by 0.32 to 0.55% at week 4. Another study by Ayuti *et al.* (2016) reports that *Lactobacillus casei* grown at 4-10°C over 90 days showed an increase in total lactic acid from 0.89% on the first day to 1.35% on Day 90.

pH during cold storage decreased with length of storage time (Figure 3). The pH of LCR-68 fermented milk from weeks 0 to 6 decreased from 4.70 to 3.47 (26.17% decrease), while pH in LCS fermented milk from weeks 0 to 6 showed a decrease from 5.32 to 4.17 (21.61% decrease). pH in both types of fermented milk decreased with increased storage time. LCR-68 fermented milk showed greater decrease in pH in cold storage than LCS. The fermentation process continues slowly in cold temperatures; sugars such as lactose and sucrose in fermented milk, which act as sources of

energy, continue to be converted by LAB into organic acids, especially lactic acid. Lactic acid is dissociated into  $H^+$  and  $CH_3CHOHCOO^-$ . High levels of lactic acid allows the release of high levels of  $H^+$  ions into the medium, causing the pH of the fermented milk to drop (Khotimah and Kusnadi, 2014; Mirdalisa *et al.*, 2016). The results of this study are in line with the findings of Usmiati *et al.* (2011) where Dadih fermented for 21 days showed a decrease in pH from 4.51 to 3.95 and Leita *et al.* (2013) where Brazilian kefir fermented for 28 days in cold storage resulted in a decrease in pH from 4.75 to 4.32. Hence, storage time affects the pH in probiotic fermented milk. A decrease in pH in fermented milks coincides with lowered amounts of sucrose and lactose during cold storage (Figure 4).

Lactose and sucrose are used as an energy source by lactic acid bacteria; they are converted into lactic acid in the fermented milk (Figure 4). Lactose levels decreased during cold storage with increased length of storage time. Lactose levels in LCR-68 fermented milk decreased from weeks 0 to 6 from 4.92 to 2.84% (42.27% decrease), while lactose levels in LCS fermented milk from weeks 0 to 6 decreased from 5.03 to 3.27% (35% decrease). The rate of decrease in lactose levels in LCR-68 fermented milk was greater than that of LCS fermented milk during cold storage. LCR-68 metabolizes lactose at a faster rate than LCS, as indicated by the lower amount of lactose remaining in LCS fermented milk at the end of storage time. Lactose is one of the simple sugars used by LAB for metabolic activity (Pramono *et al.*, 2011). The more sugars metabolized, the more lactic acid produced, resulting in reduced pH of the fermented milk. Decrease in lactose levels during cold storage was also reported in soyoghurt (Muawanah, 2007) and in Brazilian kefir (Leita *et al.*, 2013).

Sucrose levels during cold storage decreased with length of storage time (Figure 4). Sucrose levels in LCR-68 fermented milk from weeks 0 to 6 decreased from 4.70 to 0.67%, (85.75%



decrease over six weeks or 14.30% per week), while sucrose levels in LCS fermented milk from weeks 0 to 6 decreased from 4.80 to 0.84% (81.45% decrease over six weeks, or an average reduction of 14.57% per week). The rate of decrease in sucrose levels in fermented milk during cold storage was greater in LCR-68 than in LCS. According to Sobowale *et al.*, 2011, sucrose is needed as an energy source to maintain cell survival in cold conditions. Therefore, longer storage periods led to higher levels of sucrose decline. Lactic acid bacteria use sugar as an energy source for growth, and produce metabolites in the form of lactic acid during fermentation (Salminen *et al.*, 1998). Similar decrease in sucrose levels from 1.56 to 1.28% was also reported in soy milk yoghurt by Muawanah (2007).

Reducing sugars are monosaccharides and disaccharides with reducing groups, such as glucose, fructose, galactose, maltose, and lactose. Reducing sugar levels in fermented milk during cold storage decreased with increase in length of storage time (Figure 4). Reducing sugar levels in LCR-68 fermented milk from weeks 0 to 6 decreased from 34.36 to 25.50% (25.78% decrease), while reducing sugar levels in LCS fermented milk from weeks 0 to 6 decreased from 34.51 to 25.32% (26.62% decrease). LCR-68 fermented milk showed lower decrease in reducing sugar levels compared to LCS fermented milk during cold storage. During fermentation, disaccharides are broken down into monosaccharides. Lactose in skimmed milk is broken down into glucose and galactose, and sucrose is converted into glucose and fructose. Reducing sugar levels correlate with total lactic acid (Figure 3). Higher levels of total lactic acid in fermented milk coincide with lower reducing sugar content. This is because, during fermentation, LAB uses simple sugars as an energy source for growth, and produces end metabolites in the form of lactic acid sugars to produce lactic acid (Salminen *et al.*, 1998). In a study by Fithri *et al.* (2008) on fermented soy milk, reducing sugar levels decreased from 2.70 to 0.75%. The drop in reducing sugar levels was caused by utilization of the sugars by *L. casei* strains as an energy

source for cell growth and the formation of metabolites such as lactic acid.

## CONCLUSIONS

This study established that fermentation time significantly affected pH value, total lactic acid, total LAB, and protein content, but did not significantly influence fat and ash content in probiotic fermented milk produced with *Lactobacillus Casei* subsp. *casei* strain R-68 (LCR-68) and *Lactobacillus Casei* strain Shirota (LCS). High quality probiotic fermented milk was produced using strain LCR-68 as starter with 15-hours fermentation time. LCR-68 and LCS cultured in skimmed milk showed slightly different growth patterns, but similar viability in cold storage for six weeks. The quality parameters of LCR-68 fermented milk at weeks 0 and 6 contained total LAB of 7.42-5.92 log CFU mL<sup>-1</sup>, total lactic acid 0.34-0.56%, pH 4.70-3.47, lactose 4.92-2.84%, sucrose 4.70-0.67%, and reducing sugar 34.36-25.50%. Whereas for LCS fermented milk, total LAB was 7.94-6.68 log CFU mL<sup>-1</sup>, total lactic acid 0.31-0.54%, pH 5.32-4.17, lactose 5.03-3.27%, sucrose 4.80-0.84%, and reducing sugar 34.51-25.32%. In conclusion, LCR-68 can be used as a starter for making probiotic fermented milk.

## ACKNOWLEDGEMENTS

We sincerely thank the Institute for Research and Community Service, Universitas Riau, and the Ministry for Research, Technology, and Higher Education of the Republic of Indonesia for providing the research grant for this study.

## REFERENCES

1. Anggraini, M. 2016. Effect of Carboxi Methyl Cellulose (CMC) Concentration and Duration of Storage in Cold Temperatures on Stability and Characteristics of Pineapple Probiotic Drinks. Thesis, Faculty of Agriculture. University of Lampung. Lampung.



2. Akabanda, F., Owusu-Kwarteng, J., Glover, R. L. K., Tano-Debrah, K. 2010. Microbiological Characteristics of Ghanaian Traditional Fermented Milk Product, *Nunu. Nat. Sci.*, **8**:178–187.
3. Anonymous. 2015. *Yakult*. <http://id.m.wikipedia.org>. Accessed on 17 June 2019.
4. AOAC. 2012. *Official Methods of Analysis of the Association of Official Analytical Chemists*. AOAC Inc., Washington.
5. Asaminew, T. and Eyassu, S. 2011. Microbial Quality of Raw Cow's Milk Collected from Farmers and Cooperatives in North Sumatra and Mecha District, Ethiopia. *Agric. Biol. J. N. Am.*, **2**: 29-33.
6. Ayuti, S. R., Nurliana, Yurliasni, Sugito and Darmawi. 2016. Dynamics of Growth of *Lactobacillus casei* and Characteristics of Fermented Milk Based on Temperature and Storage Time. *J. Kesehatan Masyarakat Veteriner Agripet.*, **16**(1): 23-30.
7. Bigliardi, B. and Galati, F. 2013. Innovation Trends in the Food Industry: The Case of Functional Foods. *Trends Food Sci. Technol.*, **31**(2):118–129.
8. Cenci, G., Rossi, J., Trotta, F. and Caldini, G. 2002. Lactic Acid Bacteria Isolated from Dairy Products Inhibits Genotoxic Effect of 4-Nitroquinoline-1-Oxide in SOS-chromotest. *Syst. Appl. Microbiol.*, **25**: 483-90.
9. Ertanto, T., Widarso, T. D., Ekafitri, R., Faradilla, R. H. F. and Mujiono, M. 2009. Physical, Chemical, and Microbiological Properties of Probiotic Product Based on Coconut Milk (Cocogurt) during Storage. *J. Biosci. Bioengin.*, **108**, S135–S146.
1. FAO/WHO. 2001. *Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria*. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, October, 1-4, Cordoba, Argentina.
2. FAO/WHO. 2010. *Codex Standard for Fermented Milks*. 2nd Edition, Codex Stan 243-2003.
3. Falguera, V., Aliguer, N. and Falguera, M. 2012. An Integrated Approach to Current Trends in Food Consumption: Moving Toward Functional and Organic Products? *Food Control*, **26**(2):274-281.
4. Fardiaz, S. 1998. *Food Microbiology Analysis*. Bogor Agricultural Institute, Collaboration with PAU between the University of Food and Nutrition, PT. Raja Grafindo Persada, Bogor.
5. Fithri, C. N., Kusnadi, J. and Chrisnasari, R. 2008. Viability and Detection of Sublethal Probiotic Bacteria in the Instant Fermented Soy Milk Using Freeze Drying Method. *J. Teknol. Pert.*, **9**(1): 40-51.
6. Fontan, M. C. G., Martinez, J., Franco, I. and Carballo, J. 2006. Microbiological and Chemical Changes during the Manufacture of Kefir Made from Cows' Milk, Using a Commercial Starter Culture. *Intern. Dairy J.*, **16**(7): 762-767.
7. Ginting, A. A. 2016. Viability of *Lactobacillus casei* subsp. *casei* R-68 and the Quality of Fermented Milk during Fermentation. Thesis, Faculty of Agriculture. Universitas Riau, Pekanbaru.
8. Hosono, A., Wardoyo, R. and Otani, H. 1989. Microbial Flora in “dadih”, a Traditional Fermented Milk in Indonesia. *Lebens-Wissen. und-Technol.*, **22**: 20-24.
9. Hosono, A., Tanabe T. and H. Otani. 1990. Bindingproperties of Lactic Acid Bacteria Isolated Fromkefir Milk with Mutagenic Amino Acid Pyrolysates. *Milchwissenschaft*, **45**: 647-651.
10. Hu, S., Kong, J., Sun, Z., Han, L., Kong, W. and Yang, P. 2011. Heterologous Protein Display on the Cell Surface of Lactic Acid Bacteria Mediated by the S-Layer Protein. *Microb. Cell Fact.*, **10**(86): 1-13.
11. Imam, M.N., Pato, U. and Hamzah, F. 2015. Duration of Fermentation on the Quality of Cocoghurt Using *Enterococcus faecalis* UP-11 Isolated from Tempoyak. *JOM Faperta.*, **2**(2): 1-11.
12. Ishibashi, N. and Shimamura, S. 1993. Bifidobacteria: Research and Development in Japan. *Food Technol.*, **47**: 126-135.
13. Karami, M. 2018. Investigation of Physicochemical, Microbiological, and Rheological Properties and Volatile Compounds of Ewe and Cow Milk Yoghurt. *J. Agr. Sci. Tech.*, **20**: 1149-1160.
14. Khotimah, K. and Kusnadi, J. 2014. Antibacterial Activity of Probiotic Dates (*Phoenix dactylifera* L.) Using *Lactobacillus plantarum* and *Lactobacillus casei*. *J. Pangan Agroind.*, **2**(3): 110-120.
15. Korbekandi, H., Mortazapian, A. M. and Iravani, S. 2011. Technology and Stability of Probiotic in Fermented Milks Containing Probiotics and Prebiotics. In: “*Probiotic and Prebiotic Foods: Technology, Stability and*

- Benefits to the Human Health*" Chapter 7. (Eds.): Shah, N. P., da Cruz, A. G., Faria, J. A. F., Nova Science Publishers, Inc. USA.
16. Kurman, J. A. and Rasic, J. L. 1991. The Health Potential of Products Containing Bifidobacteria. In: "Therapeutic properties of functional milks" (Ed.): Robinson, R. K. ISBN-13: 978-1851665525, Elsevier Applied Science, London, UK., PP.117-157.
  17. Lasik, L., Pikul, J., Danków, R., Cais-Sokolińska, D. 2011. The Fermentation Dynamics of Sheep Milk with Increased Proportion of Whey Proteins. *Acta. Sci. Pol. Technol. Aliment.*, **10(2)**: 155-163.
  18. Leite, A. M. O., Leite D. C. A., Del Aquila E. M., Alvares T. S., Peixoto R. S., Miguel M. A. L., Silva, J. T. and Paschoalin, V. M. F. 2013. Microbiological and Chemical Characteristics of Brazilian Kefir during Fermentation and Storage Processes. *Intern. Dairy J.*, **96(7)**: 4149-4159.
  19. Leyer, G. J., Li, S., Mubshaer, M. E., Reifer, C. and Ouwehan, A. C. 2009. Probiotics Effects on Cold and Influenza-Like Incidence and Duration in Children. *Pediatrics.*, **124**: 172-178.
  20. Mahmoudi, M., Khomeiri, M., Saeidi, M., Kashaninejad, M. and Davoodi, H. 2019a. Study of Potential Probiotic Properties of Lactic Acid Bacteria Isolated from Raw and Traditional Fermented Camel Milk. *J. Agr. Sci. Tech.*, **21(5)**: 1161-1172.
  21. Mahmoudi, I., Moussa, O. B., Khaldi, T. E., Kebouchi, M., Soligot-Hognon, C., Leroux, Y. and Hassouna, M. 2019b. Adhesion Properties of Probiotic *Lactobacillus* Strains Isolated from Tunisian Sheep and Goat Milk. *J. Agr. Sci. Tech.*, **21(3)**: 587-600.
  22. Matsumoto, K., Takada, T., Shimizu, K., Kado, Y., Kawakami, K., Makino, I., et al. 2006. The Effects of a Probiotic Milk Product Containing *Lactobacillus casei* Strain Shirota on the Defecation Frequency and the Intestinal Microflora of Sub-Optimal Health State Volunteers: A Randomized Placebo-Controlled Cross-Over Study. *Biosci. Microflora.*, **25**: 39-48.
  23. Maulidya, A. 2007. Yogurt Making Study of Corn Milk as a Probiotic Drink Using a Culture Mixture of *Lacobacillus delbruekii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus casei* subsp. *rhamnosus*. Thesis, Faculty of Agricultural Technology. Bogor Agricultural University. Bogor.
  24. Mirdalisa, C. A., Zakaria, Y. and Nurliana. 2016. Effect of Temperature and Shelf Life on the Antimicrobial Activity of Fermented Milk with *Lacobacillus casei*. *J. Kesehatan Masyarakat Vet. Agripet.*, **16(1)**: 49-55.
  25. Moat, A.G., Foster, J. W. and Spector, M. P. 2002. Cell Structure and Function. Chapter 7. In: "Microbial Physiology". Fourth Edition. Wiley-Liss, Inc. Wilmington, USA.
  26. Mulyani, S., Legowo, A. M. and Mahanani, A. A. 2008. Viability of Lactic Acid Bacteria, Acidity, and Melting Time of Probiotic Ice Cream Using the Starter of *Lactobacillus casei* and *Bifidobacterium bifidum*. *J. Indon. Tropic. Anim. Agric.*, **33(2)**: 121-125.
  27. Muawanah, A. 2007. Effect of Incubation Length and Starter Type Variation on Sugar, Lactic Acid, Total Acid, and pH of Soybean Milk Yogurt. *J. Valensi.*, **1(1)**: 1-6.
  28. Nagao, F., Nakayama, M., Muto, T., Okumura, K. 2000. Effects of a Fermented Milk Drink Containing *Lactobacillus casei* Strain Shirota on the Immune System in Healthy Human Subjects. *Biosci. Biotechnol. Biochem.*, **64**: 2706-2708.
  29. Pato, U. 2003. The Potential of Lactic Acid Bacteria Isolated from *Dadih* to Reduce the Risk of Cancer. *J. Natur. Indon.*, **5(2)**: 162-166.
  30. Pato, U., Johan, V. S., Khairunnisa, F. and Hasibuan, R. D. H. 2017. Antibiotic Resistance and Antibacterial Activity of *Dadih* Originated *Lactobacillus casei* subsp. *casei* R-68 against Food Borne Pathogens. *Asian J. Microbiol. Biotech. Environ. Sci.*, **19(3)**: 577-587.
  31. Pramono, Y. B., Nurwantoro, Masykuri and Bambang, D. 2011. *Characteristics of Microbiological, Chemical, Physical, and sensoric Yogurt with Addition of Red Sweet Potatoes*. Research Report, Faculty of Animal Husbandry, Diponegoro University. Semarang.
  32. Rangkuti, S. S., Rossi, E. and Efendi, R. 2013. *Shelf Life of Probiotic Soyghurt as a Filler for Chocolate Praline*. <http://repository.unri.ac.id:80/handle/123456789/4160>. Accessed on 29 April 2018.
  33. Salminen, S., Deighton, M., Benno, Y. and Gorbach, S. 1998. Lactic Acid Bacteria. In: "Health and Disease", (Eds.): Salminen, S. and von Wright A., New York, PP. 211-253.
  34. Setioningsih, E., Setyaningsih, R. and Susilowati A. 2004. Making Probiotic Drinks from Soy Milk with *Lactobacillus casei*,



- Lactobacillus plantarum*, and *Lactobacillus acidophilus* Inoculums. *Bioteknologi*, **1(1)**: 1-6.
35. Shah, N. P. 2007. Functional Cultures and Health Benefits. *Dairy J.*, **17**: 1262-1277.
36. Spanhaak, S., Havenaar, R. and Schaafsma, G. 1998. The Effect of Consumption of Milk Fermented by *Lactobacillus casei* Strain Shirota on the Intestinal Microflora and Immune Parameters in Humans. *Eur. J. Clin. Nutr.*, **52**: 899-907.
37. Sutula T., Cascino G., Cavazos J., Parada I., Ramirez L. 1989. Mossy Fiber Synaptic Reorganization in the Epileptic Human Temporal Lobe. *Ann. Neurol.*, **26**: 321-330.
38. Tamime, A. Y. and Robinson, R. K. 2007. Quality Control in Yoghurt Manufacture. In: "Tamime and Robinson's Yoghurt" 3rd Edition, Science and Technology, Woodhead Publishing, 808PP.
39. Usman and Hosono, A. 1999. Viability of *Lactobacillus gasseri* and its Cholesterol-Binding and Antimutagenic Activities during Subsequent Refrigerated Storage in Nonfermented Milk. *J. Dairy Sci.*, **82(12)**: 2536-2542.
40. Usmiati, S., Broto, W. and Setiyanto, H. 2011. Characteristics of Cow Milk Curd Using a Starter of Probiotic Bacteria. *J. Ilmu. Ternak. Vet.*, **16(2)**: 140-152.
41. Volk, W. A. and Wheeler, M. F. 1993. Basic Microbiology, 5thEd. Erlangga, Jakarta.

## زیستایی و کیفیت شیر تخمیری تولید شده با استفاده از مایه میکروبی محلی و تجارتي در طی تخمیر و در سرد خانه

ی. پاتو، و. س. جوهان، ا. ر. رایدیناوان، ا. ا. گینتینگ، و. ی. جاسویر

### چکیده

روند های اخیر در مصرف مواد غذایی و سبک زندگی، افزایش تقاضا برای غذاهایی را نشان میدهد که فقط خوش مزه و مغذی نیستند بلکه منافع دیگری نیز برای سلامت انسان دارند: یعنی مواد غذایی زیست فعال (functional food). یک نمونه از مواد غذایی زیست فعال، شیر تخمیری پروبیوتیک تولید شده با باکتری های لاکتیک اسید است. چنین نشان داده شده که این ماده برای سلامت معده مفید است. هدف این پژوهش بررسی زیستایی *Lactobacillus casei* subsp. *casei* R-68 و *Lactobacillus casei* strain Shirota (LCS و LCR-68) در طی تخمیر و در سردخانه بود و نیز بررسی کیفیت شیر تخمیری تولید شده با هر دو سویه. این پژوهش با استفاده از طرح کاملا تصادفی انجام شد. داده های به دست آمده با استفاده از ANOVA و DNMRت تجزیه تحلیل شد. نیز از آزمون t برای مقایسه رشد و زیستایی LCS و LCR-68 استفاده شد. نتایج نشان داد که طول زمان تخمیر بر pH، لاکتیک اسید کل، LAB کل، و محتوای پروتئین به طور معناداری تاثیر داشت ولی اثرش بر چربی و خاکستر شیر تخمیری تولید شده معنی دار نبود. از نظر زیستایی و کیفیت، بهترین شیر تخمیری پروبیوتیک با ۱۵ ساعت تخمیر و استفاده از سویه LCR-68 به عنوان مایه میکروبی تولید شد. الگو های رشد LCS و LCR-68 کشت شده در شیر پس چرخ (skimmed)

مilk) اندکی تفاوت داشت. با اینهمه، هر دو سویه زیستایی مشابهی داشتند. بعد از یک ماه در سردخانه، LAB کل، در شیر تخمیری LCR-68 و LCS به ترتیب برابر  $6/64 \log \text{CFU mL}^{-1}$  و  $6/68$  بود. بر پایه این نتایج، LCR-68 را می توان به عنوان مایه میکروبی برای تهیه شیر تخمیری پروبیوتیک استفاده کرد.