BIODIVERSITAS
Volume 23, Number 8, August 2022
Pages: 4329-4335

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230858

Physicochemical and microbiological fermented buffalo milk produced by probiotic *Lactiplantibacillus pentosus* HBUAS53657 and sweet orange juice (*Citrus nobilis*)

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Manuscript received: 22 June 2022. Revision accepted: 7 August 2022.

Abstract. *Susmiati, Melia S, Purwati E, Alzahra H. 2022. Physicochemical and microbiological fermented buffalo milk produced by probiotic* Lactiplantibacillus pentosus *HBUAS53657 and sweet orange juice (*Citrus nobilis*). Biodiversitas 23: 4329-4335.* Fermented milk is a type of dairy product that has been fermented with lactic acid bacteria. Changes in starter lactic acid bacteria and fruits can improve fermented dairy's physicochemical quality and total lactic acid bacteria (LAB) colonies. This study investigates how changes in the concentration of starter *Lactiplantibacillus pentosus* HBUAS53657 and sweet orange juice concentration affect protein, fat, moisture, total titratable acidity, pH value, and total lactic acid bacteria (LAB) colonies in buffalo fermented milk. This study used randomized block design (RBD) experiment, with factor A being starter *L. pentosus* strain HBUAS53657 4%, 5% and 6% (A1, A2 and A3) and factor B being sweet orange juice 10%, 15% and 20% (B1, B2 and B3) with three replications as a treatment. The data were statistically processed using analysis of variance, and the smallest significant difference test was $p<0.05$. The results showed that the protein content ranged from 5.81-6.33%, fat content 6.14-6.35%, water content 81.63- 85.78%, pH 3.57-4.23, TTA value 1.30-1.85 and total LAB colonies from 4.67×10^9 to 9.0×10^9 CFU/mL. Fermented milk products with 6% starter *L. pentosus* HBUAS53657 and 20% sweet orange juice produced the best results. This product is suitable for consumption as a probiotic beverage.

Keywords: *Lactiplantibacillus pentosus* strain HBUAS53657, microbiological, sweet orange juice, physicochemical, probiotic

INTRODUCTION

Fermented milk is one of the suitable carriers for probiotics, which are used to promote health (Khorshidian et al. 2020). Probiotics are live microorganisms that, when administered in sufficient quantities, confer a health advantage on the host (Hill et al. 2014; Triana and Yulinery 2015). Various probiotics such as *Lactobacillus rhamnosus* GG, *L. reuteri, Bifidobacteria*, and multiple *L. casei* have been used widely used in fermented milk. It has been examined for its health advantages and the usage of *L. plantarum* strains, which have been demonstrated to have strong adaptability and adhesion abilities in the gastrointestinal tract and might improve host health (Garcia-Gonzalez et al. 2021). *L. pentosus* has been shown to have probiotic characteristics (Guantario et al. 2018). Red ginger fermented milk was made with *Pediococcus acidilactici* BK01, a probiotic isolated from Bekasam (Melia et al. 2022). *Lactobacillus* is a broad genus widely used in industrial and biological applications. *Lactiplantibacillus pentosus*, formerly known as *L. pentosus* (Zheng et al. 2020), is a species isolated from fermentation products and the gut microbiota of mammals. Several *L. pentosus* strains show primary health-promoting functions, like immunomodulatory and antiproliferative properties, and are regarded as potential probiotic strains (Stergiou et al. 2021). The potentially probiotic strain *Lactiplantibacillus pentosus* HBUAS53657, which was isolated from curd, was employed as a starter in the production of fermented buffalo milk. *Lactiplantibacillus pentosus* HBUAS53657 is a probiotic strain mostly found in curd originating from Tanjung Bonai Tanah Datar, West Sumatra. This curd contains good quality nutrients, that content different group of probiotic microorganisms (Alzahra et al. 2021).

Milk is a product from livestock with good nutritional value for the body but is susceptible to damage if not handled and processed correctly. Therefore, milk is processed by fermenting milk with the addition of lactic acid bacteria (LAB) so that dairy products have a longer shelf life and provide better benefits when consumed by humans (Nout 2009). Furthermore, fermented milk has a high sensory acceptance (Sakandar and Zhang 2021). Milk from cows, goats, sheep, and buffalo can be utilized to make fermented milk (Melia et al. 2020). Regarding its significant components, buffalo milk contains more nutrients than cow milk (Becskei et al. 2020). The high value of energy, protein and calcium and Zn can meet the health needs of children during their growth period so that they can overcome stunting problems and act as an anti-inflammatory (Licata et al. 2012). Buffalo milk contains more vitamins and minerals than cow milk, with high in calcium, phosphor, and vitamin and is a source of casein-derived peptides, which can improve bone health and reduce the risk of osteoporosis ([USDA] United States Department of Agriculture 2011; Basilicata et al. 2018). Exploring some of the benefits of buffalo milk production to develop into functional food through enhancing its efficacy and sensory and health benefits could be an exciting alternative for further research (Vargas-Ramella et al. 2021).

The ability of the strain to survive the gastrointestinal tract is crucial to the role of probiotics. One method for preserving the presence of microorganisms in the intestine is to supplement growth factors such as prebiotics. Prebiotics are non-living food components that modulate the microbiota for increased health benefits for the host (Pineiro et al. 2008). Many studies have shown the beneficial effects of using a combination of probiotics and prebiotics or what is known as "synbiotics" (Markowiak and Śliżewska 2017; Terpou et al. 2019). Previous research has discovered that incorporating probiotics into various fruit juices can be used to create functional drinks (Pimentel et al. 2015). An antioxidant, orange juice, is added to fermented buffalo milk to improve its quality and make it a functional food. Sweet orange (*Citrus nobilis* L.) is a medicinal plant that contains antioxidant compounds that can be used to prevent and treat various diseases. Chemical compounds found in sweet orange include flavonoids, tannins, phenols, terpenoids, vitamin C, calcium, potassium, thiamine, niacin, and magnesium (Etebu and Nwauzoma 2014). Orange juice is a suitable carrier for probiotic culture and shows the presence of beneficial compounds, vitamins, minerals, fiber and antioxidants and enjoyable taste in all age groups (Ding and Shah 2008). The research also discovered an effect of almond yogurt formulation and orange juice addition on the organoleptic quality of yogurt in the categories of color, taste, and viscosity (Lubis and Anjani 2016). Therefore, in this study, the addition of sweet orange juice by 10%, 15% and 20% and varying concentrations of starter *L. pentosus* HBUAS53657 have been used. This study investigated the physicochemical and microbiological of fermented milk produced by *L. pentosus* HBUAS53657 and sweet orange juice.

MATERIALS AND METHODS

Samples collection

Starter *L. pentosus* HBUAS53657 was isolated from dadih (Minangkabau traditional food made from buffalo milk naturally fermented in closed bamboo) and sweet orange (*Citrus nobilis* L.) from Jorong Tanjung Modang Nagari Tanjung Bonai, North Lintau Buo District, Tanah Datar Regency, West Sumatra. The samples for this study were buffalo milk with the addition of the starter *L. pentosus* HBUAS53657 4%, 5% and 6% (A1, A2 and A3) and sweet orange juice 10%, 15% and 20% (B1, B2 and B3). This experimental method was conducted at the Laboratory of Animal Products Technology, Faculty of Animal Husbandry, Andalas University, Padang, Indonesia.

Protein content

Protein levels were determined using the Kjeldahl technique according to the Association of Official Analytical Chemists (2005) (AOAC 2005). The sample was weighed to one gram and placed in a Kjeldahl flask. The flask is heated with one gram of selenium and one mL of H_2SO_4. The process of destruction continues until the solution is clear or colouless. Volumetric flask (500 mL) was filled halfway with the solution, then dilute to the line mark with distilled water. Then, in a distillation flask, mixed 25 mL of the sample solution, 25 mL of 30% NaOH, and 150 mL of distilled water. The solution was heated (2/3 distilled) until all of the nitrogen in the flask's liquid was collected by 0.05N H_2SO_4, which was then combined with three drops of methyl red in an Erlenmeyer first. In Erlenmeyer, the distillate was titrated with 0.01N NaOH (Z mL). As a blank, 25 mL of 0.05N H_2SO_4 and three drops of methyl red indicator were added to another Erlenmeyer and titrated with 0.1N NaOH until the color changed from pink to yellow (Y mL).

Fat content

The pumpkin fat oven was used for 30 min at a temperature of 100-105°C to evaluate fat content using the AOAC method (2005). The fat flask should then be dried in a desiccator and weighed (A). The wrapped sample was dried in a 105°C oven for 4 hours before being weighed with an analytical balance (as weight B). The material was placed in a fat flask and extracted for 5-6 hours, or until the fat solvent has descended into the flask and is translucent. The fat solvent that was utilized is collected and distilled. Following that, the sample was dried in an oven for 1 h at 100-105°C, and weighed as weight C. The weight difference between dried and extracted dried samples was used to calculate the percentage of total fat content. It was then split by the sample weight.

Moisture content

The dish was baked for one hour at 110°C to test the water content using the AOAC method (2005). The sample was weighed after cooling in a desiccator to remove moisture (A). A sample weighing up to five grams is placed in a dry cup (B) and baked for 8 h at 105°C. The sample was weighed after cooling for 30 min in a desiccator (C). This step is repeated until the weight remains constant.

pH value

Using a pH meter and calibrated with a buffer solution with pH values of 4 and 7, the degree of acidity of the sample was assessed using the AOAC method (2005). A five-gram sample was mixed with 10 mL of distilled water for five minutes before being placed in a measuring cup. The pH value is determined by dipping the pH meter approximately 2-4 cm into the sample and reading the scale shown by the pointer.

Total titratable acidity value

The determination of the total titratable acidity test was carried out based on the AOAC method (2005). Fermented milk is poured into an Erlenmeyer flask containing 5 mL and three drops of phenolphthalein. Each Erlenmeyer was titrated with 0.1N NaOH until a consistent pink color was formed when stirred. The amount of NaOH solution used was recorded, and the total acidity of fermented milk was calculated.

Enumeration of lactic acid bacteria

The total number of LAB colonies was determined using the method of Purwati et al. (2005). One gram of sample was dissolved in 9 mL De Man, Rogosa and Sharpe (MRS) broth solution and vortexed until uniformally mixed. The dilution results were collected in a 100 μL Eppendorf tube containing 900 μL MRS Broth solution and vortexed until homogeneously mixed. Until 10^{-6}, all serial dilutions were performed with the same solution. On a Petri dish containing MRS Agar, 100 µL of the 10^{-6} dilution was spread and levelled with a sterile hockey stick. After storing the inoculum in an anaerobic jar, which was incubated in an incubator for 48 h at 37°C. The Quebec colony counter was used to keep track of the expanding colonies. The results were given in log CFU/mL.

Data analysis

All the tests were carried out three times, and the data were presented as mean values for the measurements. A complete factorial design was used to compare mean values using Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Protein content

Protein content in buffalo fermented milk with different concentrations of starter *L. pentosus* HBUAS53657 and different concentrations of sweet orange juice showed no interaction with the protein content (Table 1).

Fat content

The fat content was significantly influenced by the combination of treatment with starter *L. pentosus* HBUAS53657 and varied concentrations of sweet orange juice ($p<0.05$). The A2B1 treatment had the highest fat content at 6.35%, whereas the A3B3 treatment had the lowest fat content at 6.14%. Fermented milk fat content ranged from 6.14 to 6.35% on average. The greater starter *L. pentosus* HBUAS53657 concentration indicates a lower fat content in fermented milk (Table 1). Lactic acid bacteria use fat as an energy source, decreasing fat content. Sweet orange fiber has been shown to reduce ice cream fat by 50% while increasing beneficial ingredients such as fiber and carotenoids (Crizel et al. 2014). According to Nofrianti et al. (2013), LAB produce lipase enzymes that hydrolyze fat. Fat content was influenced by total LAB and fatty acid additives (Manurung and Rusmarilin 2014).

In addition, the higher the concentration of sweet orange juice indicates a decrease in fat content in fermented milk. It is because citrus fruits contain simple carbohydrates and complex carbohydrates. Simple carbohydrates in citrus fruits are in the form of fructose, glucose and sucrose. According to Kartikasari and Nisa (2014), foods containing sugar will support the metabolism of simple microorganisms such as proteins, fats, vitamins, nucleic acids and minerals, all of which are essential for the synthesis of cell constituent chemicals. Furthermore, increasing the amount of water in the body by drinking sweet orange juice also helps the growth of bacteria (Febrihantana et al. 2013). The presence of water in the growth media could help the process of nutrient diffusion, allowing for optimal bacterial growth and metabolism.

According to studies, some fruit and vegetable fiber can be used as a functional food by lowering fat content and increasing bioactive chemical content. Improved binding and water retention, thickening, and gelling are all features of fruit and vegetable fibers. Fiber from fruits and vegetables can help microorganisms survive longer and boost the amount of conjugated fatty acids in dairy products (Salehi 2021).

Moisture content

The varying concentrations of sweet orange juice and concentration starters of *L. pentosus* HBUAS53657 had a significant effect ($p < 0.05$) on water content, with water content values ranging from 81.79 to 85.59%. The highest water content was treated with 20% sweet orange juice concentration (B3) with an average value of 85.59%, and the lowest was 10% sweet orange juice concentration (B1) considerably (Table 1). The increase in water content corresponded to the rise in the concentration of sweet orange juice.

pH value

In the combination of treatment with different concentrations of starter *L. pentosus* HBUAS53657 and varying amounts of sweet orange juice, the pH value of buffalo fermented milk showed an interaction with the pH value.

The combination of treatment with the addition of starter *L. pentosus* HBUAS53657 and sweet orange juice showed a significant interaction ($p<0.05$) on the pH value. The highest pH value was found in fermented milk treatment A1B1 (*L. pentosus* HBUAS53657 4% and sweet orange juice 10% with a pH value of 4.23, while the lowest was found in treatment A3B3 (*L. pentosus* HBUAS53657, 6% and sweet orange juice 20% with a pH value of 3.57. The average pH value ranged from 3.57 to 4.23 (Table 1). The addition of sweet orange juice causes a decrease in pH and an increase in acidity (Ning et al. 2021; Yasmin et al. 2022). Sweet orange juice has an acidic taste with a pH of 4.00, which causes a reduction in pH. In line with the study, the pH value of fermented milk fell as the red dragon fruit skin concentration increased (Dianasari et al. 2020).

Increased bacterial metabolic activity will ferment lactose and produce lactic acid, acetaldehyde, and diacetyl (Gaspar et al. 2013). This is also due to the fermentation process caused by lactic acid bacteria, which produces organic acids. More lactic acid bacteria caused the decrease in pH in fermented milk utilizing the monosaccharides in citrus fruit juices, making the environment acidic (pH drops). Furthermore, higher bacterial growth caused a decrease in pH, which was linked to an increase in lactic acid, ethanol, and carbon dioxide owing to lactic acid fermentation. Proper acidity also imparts flavor to the product while inhibiting the growth of spoilage microbes and food-borne illnesses (Mufandaedza et al. 2006).

Titratable acidity

The titratable acidity (TTA) value of buffalo fermented milk in the combination treatment with different concentrations of starter *L. pentosus strain* HBUAS53657 and different concentrations of sweet orange juice showed an interaction with the TTA value. The interaction of treatment with varied starter concentrations of *L. pentosus* HBUAS53657 and the concentration of sweet orange juice on the titratable acidity value was significant (P<0.05). Treatment A3B3 (*L. pentosus* HBUAS53657 6% and sweet orange juice 20%) had the greatest titratable acidity value of 1.85, whereas treatment A1B1 (*L. pentosus* HBUAS53657 4% and sweet orange juice 10%) had the lowest at 1.30. The range of titratable acidity was between 1.30 and 1.85 (Table 1).

The greater the starter concentration of *L. pentosus* HBUAS53657 and the higher the fruit juice concentration, the higher the acidity in the fermented milk, as shown in Table 2. This study is supported by the study of Arioui et al. (2017) and Guven and Karaca (2002); an increase in acidity is directly proportional to the rise in fruit concentration. The starter culture activity of fermented milk converts lactose to lactic acid, whereas date syrup contains simple carbohydrates that might induce bacterial action (Shahein et al. 2022).

Table 1. Physicochemical and microbiological characteristics of buffalo fermented milk

Factors A (Starter concentration / *L. pentosus* HBUAS53657)	**Factors B (Sweet orange juice concentration)**			**Mean ± SD**
	B1 (10%)	**B2 (15%)**	**B3 (20%)**	
Protein content				
A1 (4%)	6.10	5.91	5.82	5.94 ± 0.14^{b}
A2 (5%)	6.13	6.01	5.81	5.98 ± 0.16^{b}
A3 (6%)	6.33	5.99	6.22	6.18 ± 0.18^{a}
Mean ± SD	6.19 ± 0.13^{a}	5.97 ± 0.05^{b}	5.95 ± 0.23^{b}	
Fat content				
A1 (4%)	6.33^{bc}	6.30^{bc}	6.26^{b}	6.30 ± 0.04^{ab}
A2 (5%)	6.35^{c}	6.32^{bc}	6.29^{bc}	6.32 ± 0.03^{b}
A3 (6%)	6.30^{bc}	6.34^{c}	6.14^{a}	6.26 ± 0.11^{a}
Mean ± SD	6.33 ± 0.03^{b}	6.32 ± 0.02^{b}	6.23 ± 0.08^{a}	
Water content				
A1 (4%)	81.76	83.52	85.78	83.68±2.02
A2 (5%)	81.99	83.11	85.65	83.59±1.87
A3 (6%)	81.63	83.85	85.34	83.61±1.87
Mean ± SD	81.79 ± 0.19^{b}	83.49 ± 0.37^{ab}	85.59 ± 0.22^{a}	
pH				
A1 (4%)	4.23^{f}	4.17^{f}	4.07^{e}	4.16 ± 0.12^{c}
A2 (5%)	4.03^{de}	3.93^{bcd}	3.97^{cde}	3.98 ± 0.16^{b}
A3 (6%)	3.87^{bc}	3.83^{b}	3.57^{a}	3.76 ± 0.16^{a}
Mean ± SD	4.04 ± 0.18^{b}	3.98 ± 0.17^{b}	3.87 ± 0.26^{a}	
Titratable acidity				
A1 (4%)	1.30^{f}	1.37^{e}	1.45^{d}	1.37 ± 0.08^{c}
A2 (5%)	1.42^{de}	1.54^{c}	1.62^{b}	1.53 ± 0.10^{b}
A3 (6%)	1.53^{c}	1.66^{b}	1.85^{a}	1.68 ± 0.16^{a}
Mean ± SD	1.42 ± 0.12^{c}	1.52 ± 0.15^{b}	1.64 ± 0.20^{a}	
Total lactic acid bacteria ($\times 10^{9}$ CFU/mL)				
A1 (4%)	4.667^{d}	6.00^{c}	6.87^{bc}	5.84 ± 1.10^{a}
A2 (5%)	6.37^{bc}	7.30^{b}	8.57^{a}	7.41 ± 1.10^{b}
A3 (6%)	9.00^{a}	8.93^{a}	8.47^{a}	8.80 ± 0.29^{c}
Mean ± SD	6.68 ± 2.18^{c}	7.41 ± 1.47^{b}	7.97 ± 0.95^{a}	

Note: The mean of superscripts with lowercase letters shows a significant difference (P<0.05)

Likewise, in a study, increased acidity in ice cream was associated with probiotic culture and increased fruit concentration. During fermentation, the pH dropped from 6.8 to 4.1, and the acidity risen from 0 to 1.2%. This setting is also helpful for eradicating *Listeria* in buffalo curd. The essential pH value for *Listeria* inactivation is 5.5. *Listeria* in buffalo milk can be effectively eliminated through fermentation (Jayamanne and Samarajeewa 2011). Lactic acid is produced by LAB, which is excreted out of cells that collect on the substrate so that it can increase the acidity level in yogurt. Lactic acid is produced when the LAB breaks down lactose sugar and other carbohydrates into lactic acid. Furthermore, the vitamin C content of citrus fruits increases the total quantity of acid in fermented milk. The titratable acidity value of fermented dairy remains within the 2003 CODEX norm of at least 0.6% and the 2009 Indonesia National Standard (SNI) standard of at least 0.5-2%.

Total lactic acid bacterial colonies

The total LAB of fermented milk interacted with the whole LAB of fermented milk when treated with different starter concentrations of *L. pentosus* HBUAS53657 and different concentrations of sweet orange juice.

The addition of different starter concentrations of *L. pentosus* HBUAS53657 and the concentration of sweet orange juice resulted in a significant interaction ($P<0.05$) on the total LAB of fermented milk. Treatment A3B1 (*L. pentosus* HBUAS53657 6% and sweet orange juice 10%) had the highest total lactic acid bacteria of fermented dairy, with 9.0×10^9 CFU/mL, while treatment A1B1 (*L. pentosus* HBUAS53657, 4% and sweet orange juice 10%) had the lowest, with 4.67×10^9 CFU/mL. On average, lactic acid bacteria in fermented milk ranged from 4.67×10^9 to 9.0×10^9 CFU/mL. The changes in the total number of lactic acid bacteria after adding sweet sweet oranges to fermented milk has been reported (Table 1).

The bacterial cells could grow to the maximum number in the media, which is influenced by the availability of nutrients in the media. The addition of citrus fruits provides excess nutrients for LAB growth. LAB in yogurt drinks with the addition of sweet orange juice can utilize glucose in citrus fruit for its growth (Espirito-Santo et al. 2015). The addition of sweet orange juice significantly affected the colonies of fermented milk's total lactic acid bacteria. According to Multari et al. (2020), *L. plantarum, L. rhamnosus, L. paracasei,* and *L. brevis* could ferment citrus fruit juices. Sweet orange juice is proven to be the best choice for *Lactobacillus* spp. fermentation and can boost phenolic levels. The *Lactobacillus* spp. is also the most widely used probiotic in fermented vegetable drinks (Shori 2016). As a result, the sweet orange juice milk drink's composition offers an ideal environment for the growth of lactic acid bacteria. The survivability of probiotics in meals is affected by the food matrix's consistency, the food matrix's acidity, and the interaction between the probiotic and starter culture (Mishra and Mishra 2013). *Pediococcus acidilactici* BK01 was inoculated into fermented whey to obtain 8.87×10^9 LAB (Melia et al. 2021). Probiotics help prevent, treat, and improve health (Siciliano et al. 2021). To ensure the benefits, the content of sufficient amounts of probiotics, when consumed must be examined (Sakandar and Zhang 2021). Probiotics with a therapeutic effect must contain at least 10^6-10^7 CFU/g of probiotic bacteria (Tavakoli et al. 2019) to show a therapeutic impact on a daily intake of 100 g or 100 mL of probiotic food (Flach et al. 2018)

In conclusion, the health benefits of milk are boosted by fermentation. According to the findings of the study, protein content ranged from 5.81 to 6%, fat content from 6.14 to 6.3%, water content from 81.63 to 85.78%, pH value 3.57-4.23, titratable acidity value 1.30-1.85, and total LAB colonies from 4.67×10^9 to 9.0×10^9 CFU/mL. The fermentation of milk produces the best results in fermented milk products with starter additions of up to 6% and sweet orange juice additions of up to 20%. This product qualifies as a probiotic drink that can be advised to promote health.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Faculty of Nursing, Andalas University, Padang, Indonesia for funding this research with contract number 055a/SPK/PNBP/FKep/Unand-2021 to Dr. Susmiati.

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