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Fermentation time optimization: Unleashing quality with microbial consortia in whey

Sri Mulyani, Rafli Z. Kamil*, Brigitta I. Permata, Setya B. M. Abduh, and Ahmad N. Al-Baarri

Food Technology Study Program, Department of Agriculture, Faculty of Animal and Agricultural Sciences, Diponegoro University, Semarang, Indonesia
*Corresponding author: rafli.zulfa@live.undip.ac.id

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Abstract

During cheese production, whey is often produced as a by-product with various nutrients content and can cause damage to the environment when not processed appropriately. To reduce its environmental impacts, it is important to develop various methods for processing, such as lactic acid bacterial (LAB) fermentation by using the nutrients content. Therefore, this study aims to use a mixed LAB culture containing *Lactobacillus casei* FNCC 0090, *Lactobacillus bulgaricus* FNCC 0041, and *Streptococcus thermophilus* 0040 for the production of whey beverages. The experiment was carried out with various incubation times of 0, 2, 4, 6, and 8 hours to determine the effect on the products. The variables examined included total dissolved solids (TDS), viscosity, pH, salinity, whiteness index, and total LAB count using the pour-plated method. Subsequently, a sensory evaluation was conducted to evaluate consumer acceptance. The results showed that LAB count ranged from 7.37 to 8.72 log CFU/ml, pH values 4.85 to 5.59, TDS 9.05 to 7.18 °Brix, and sensory scores 2.82 to 2.92. The increase in LAB count correlated with decreased pH, TDS, and salinity, significantly contributing to increased viscosity. However, the incubation period did not affect the whiteness index of whey beverages. Based on the results, a 4-hour incubation period yielded the most favorable sensory outcomes, meeting both quality standards and production efficiency criteria.

Keywords: Fermentation, Lactic acid bacteria, Whey cheese, Whey product innovation

1. Introduction

Whey is the primary by-product of the cheese processing industry that is often manufactured in large quantities worldwide, with an annual production of 160 million tons [1]. During cheese processing, approximately 10 liters of fresh milk produce 1 kg of cheese and 8-9 liters of whey [2]. However, due to its high Biological Oxygen Demand (BOD) (30.000−50.000 mg/liter) and Chemical Oxygen Demand (COD) (60.000−80.000 mg/L) levels, the by-product is considered a pollutant, exceeding the permissible safe limit and presenting significant environmental discharge challenges [3]. Given these environmental concerns, it is important to develop an alternative reutilization process. Several studies have shown that whey contains various nutritious components, including simple sugar (lactose), protein, vitamins, minerals, and low-fat content. This shows that it can be processed as nutritious food, such as beverages due to the biological and functional protein.

According to previous studies, whey can be produced into various beverages, including mixed whey (with fruits or vegetable juices), dairy type, thirst-quenching carbonated, and thick beverages (fermented or unfermented) [4]. Despite this potential, it has been reported to possess certain limitations, including low total solid content affecting the textural and mouthfeel of the final product, unappealing taste with excessive acidity, and unpleasant animal aroma. Therefore, further processing is needed to improve its characteristics, specifically the sensory attributes, such as taste, aroma, and viscosity. In this context, another method that can be used to improve the acceptance of raw whey is fermentation using lactic acid bacteria (LAB). Several studies have shown that the nutrient content of whey is often used by LAB for their growth. These microbes produce metabolites that can enhance the characteristics

and functionality of beverages. LAB can also metabolize whey protein into peptides that have functional properties, such as antimicrobial, antihypertensive, immunomodulator, antioxidant, and other bioactive effects.

In line with several studies, LAB fermentation is an economical alternative for whey processing because it improves product flavor and increases consumer acceptance. Building on this idea, the lactose and other nutrient content are often used as a fermentation medium. Previous reports have shown that the production of fermented whey is typically carried out with both single and mixed cultures, but mixed culture produces acid faster [5]. In this present study, several bacteria strains, including *L. casei, L. bulgaricus*, and *S. thermophilus* were combined to develop fermented beverages with the desired characteristics, including high probiotic properties, high organic content, and pleasant taste [6]. *L. bulgaricus* has been reported to interact in mutualistic symbiosis with *S. thermophilus*, where *S. thermophilus* functions dominantly in the beginning and creates an optimum acidic condition for the growth of *L. bulgaricus*, thereby improving the flavor profile of the mixed culture [7,8]. During the process, fermentation period is an essential variable for the determination of products quality, such as physical, chemical, microbiological, and organoleptic characteristics. For example, several reports showed that the use of *Bifidobacterium longum* and *L. acidophilus* produced the best preference scores with a fermentation period of 36 h [2]. In addition, a long fermentation process duration was deemed inefficient for industrial purposes. This indicates the pressing need to explore the optimum period using other LAB strains while maintaining the expected product quality. Therefore, this study aims to determine the optimum fermentation period to produce fermented whey with optimal quality in terms of total LAB, pH value, total dissolved solids (TDS), and hedonic quality.

2. Materials and methods

2.1. Preparation of materials

Whey was obtained as a by-product from a cheese manufacturer located in Klaten, Central Java, Indonesia, during the initial stage of processing. To ensure sample homogeneity, whey was collected from each batch of mozzarella cheese production (2-3 batches) and its characteristics were analyzed. The initial characteristics of the fresh samples (before pasteurization), included total LAB at 1.50 x 10^7 CFU/mL, pH 5.65, TDS at 5.10 °Brix, and salinity at 2.22 ng/L.

Bacteria cultures, including *L. casei* FNCC 0090, *L. bulgaricus* FNCC 0041, and *S. thermophilus* FNCC 0040 were obtained from the Center for Food and Nutrition Studies Universitas Gajah Mada. Other materials were sourced from various providers, such as skim milk powder (Lactona, Yogyakarta) from Indoprima, Indonesia, de Man Rogosa Sharpe Agar (MRSA), and de Man Rogosa Sharpe Broth (MRSB) from Merck, Germany, technical alcohol 96%, sterile NaCl solution 0.85%, prepared from sodium chloride bacteriological (Oxoid), aquadest processed from Unit Pelaksana Teknis (UPT) Laboratorium Terpadu, and High Fructose Syrup (HFS) 55% from Indopangan (Indonesia).

2.2 Whey fermentation procedure

2.2.1 Culture stock preparation

Each bacteria, *L. casei* FNCC 0090, *L. bulgaricus* FNCC 0041, and *S. thermophilus* FNCC 0040 was grown in MRSB at 37°C for 24 h. Following the propagation, the resulting cell pellets were obtained through centrifugation at 4,000 rpm for 15 minutes. Subsequently, the pellets were thoroughly washed with 0.85% sterile NaCl, and subjected to centrifugation under the same condition, a process that was repeated twice. After the final washing step, the cell pellets were combined with 5 mL of a cryoprotectant solution, which consisted of 10% (w/v) skim milk and 1% (w/v) sucrose. These prepared culture stocks were then stored at -40°C and reactivated before use.

2.2.2 Production of fermentation starter

The *L. casei* culture was transferred using an inoculation loop into 15 mL centrifuge tubes containing 5 mL pre-sterilized MRSB media at 121°C and 2 atm for 15 min. The culture in a centrifugation tube was then incubated at 37 °C for 24 h to activate the culture, as showed by the cloudy medium. The same preparation protocol was followed to activate *L. bulgaricus* FNCC 0041 and *S. thermophillus* FNCC 0040. A total of 3% (v/v) of these previously activated cultures were inoculated into Erlenmeyer flasks containing a pre-sterilized solution of 10% (w/v) skim milk and 1% (w/v) sucrose. Subsequently, the mixture was incubated at 37°C for 24 h. The initial population of each bacterium was *L. casei* at 1.83 x 10^9 CFU/mL, *L. bulgaricus* at 2.70 x 10^9 CFU/mL, and *S. thermophilus at* 1.19 x 10^8 CFU/mL.

2.2.3 Production of whey fermentation

Whey was pasteurized at 75°C for 15 seconds and then cooled down to room temperature until it reached 42°C. A total of 4% (v/v) of HFS, 2.5% (v/v) *L. casei* FNCC 0090, 1.25% (v/v) *L. bulgaricus* FNCC 0041, and 1.25% (v/v) starter *S. thermophilus* were added to the pasteurized whey and thoroughly mixed. The mixture was then incubated at 42°C, at various fermentation durations, including 0, 2, 4, 6, and 8 h. When the fermentation period was achieved, the pre-inoculated whey was cooled to room temperature, transferred into sterilized, tightly closed bottles, and stored at cold temperatures (4-5°C) to stop the fermentation process.

2.3. Sensory Tests

Hedonic testing was conducted to evaluate the acceptance of fermented whey products, comprising 40 semi-trained panelists, primarily students, who assessed their level of preference based on parameters, such as taste, aroma, color, and overall impression. Each panelist was required to taste all fermented products and evaluate each using a scoring method. For accurate assessments, cleansing the mouth with water and white bread was required, to neutralize the lingering taste from previous samples before evaluating the sensory attributes of the different fermented products. The hedonic test used 4 scores where 1 = strongly dislike, 2 = dislike, 3 = like, and 4 = strongly like.

2.4 Fermented whey characterization

2.4.1 Bacteria enumeration

The growth of LAB after different fermentation periods was determined using the pour-plated method in an De Man Rogosa Sharpe (MRS) agar medium. A serial dilution was performed by taking 1 mL of samples and transferring into 9 mL of sterilized 0.85% NaCl solution. At the appropriate dilution series, 1 mL of the diluted samples was inoculated into a Petri dish and covered with approximately 10 mL of unsolidified MRS agar medium. The MRS agar medium was allowed to cool and incubated upside down at 37°C for 48 h. Following the incubation period, the colonies were enumerated, and the LAB was represented as log CFU/mL.

2.4.2 Acidity evaluation

Acidity of fermented whey was evaluated by measuring the pH value using an electrode of portable pH/ORP/Conductivity/DO Meter (Danoplus, Taiwan). The probe was calibrated with several buffer solutions at pH 4, 6, and 8 before use. After calibration, the probe was immersed in 20 mL of fermented whey until a constant value was showed. Each measurement was conducted with 4 replications, and each sample change was rinsed with tap water at the pH cathode.

2.4.3 Total dissolved solids (TDS)

In this study, the TDS of fermented whey was measured using a calibrated hand-refractometer. Each whey sample was carefully dripped into the prism of the refractometer and then directed against the light source to measure the TDS level. Subsequently, each sample change in the test was rinsed on the prism using aquadest.

2.4.4 Viscosity

The viscosity of the samples was measured using an Ostwald viscometer by placing in an Ostwald pipe and the viscosity value was calculated using the following equation.

$$\rho \text{ sample} = m' - mv$$

$$\text{Viscosity} = \frac{\rho \text{ sample x t sample x } \eta \text{ water (0,1)}}{\rho \text{ water x t water}}$$

2.4.5 Salinity

Salinity test was conducted using a portable pH/ORP/Conductivity/DO Meter (Danoplus, Taiwan), dipped into 20 mL of fermented whey until a constant salinity value was shown.

2.4.6 Whiteness Index

Measurements were conducted using a colorimeter (AMT501, United States) with the sensor positioned close to the sample, and the results were analyzed on the display. The analysis was presented in the form of L* (brightness color index), a* (reddish to greenish index), and b* (yellowish to bluish index). The whiteness index was calculated using the following equation.

$$\text{Whiteness Index} = 100 - \sqrt{(100 - L*)^2 + a*^2 + b*^2}$$

2.5 Data analysis

Parametric data were analyzed using Analysis of Variance (ANOVA) with a significance level of 5%, and Duncan's Multiple Range Test (DMRT). Hedonic data were analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney test with a significance level of 5%. All statistical analyses were performed using SPSS 26.0 for Windows.

3. Results

3.1 Sensory acceptance of fermented whey

Table 1 Sensory evaluation of fermented whey.

Parameter	0 h	2 h	4 h	6 h	8 h
Taste	2.45 ± 0.93^{a}	2.48 ± 0.88^{a}	2.80 ± 0.85^{ab}	2.98 ± 0.89^{b}	2.80 ± 1.02^{ab}
Aroma	2.65 ± 0.83^{a}	2.70 ± 0.82^{a}	3.20 ± 0.69^{b}	2.80 ± 0.82^{a}	2.98 ± 0.86^{ab}
Viscosity^{ns}	2.45 ± 0.78	2.65 ± 0.62	2.73 ± 0.72	2.93 ± 0.73	2.68 ± 0.83
Color ^{ns}	3.10 ± 0.74	2.98 ± 0.58	2.90 ± 0.63	3.03 ± 0.70	3.30 ± 0.56
Overall	2.82 ± 0.83^{a}	2.82 ± 0.83^{ab}	2.92 ± 0.80^{b}	2.91 ± 0.80^{b}	2.86 ± 0.84^{ab}

a-b: Values with different superscripts show a significant difference ($p<0.05$). ^{ns} showed no significant difference. Hedonic test scale: 1 = strongly dislike; 2 = dislike; 3 = like; and 4 = very like.

The hedonic evaluation of fermented whey was divided into 5 parameters, namely taste, aroma, viscosity, color, and overalls, as shown in Table 1. Figure 1(A)-1(E) showed the appearance of fermented whey beverages at different fermentation periods, which are 0, 2, 4, 6, and 8 h, respectively. According to Kruskal-Wallis' analysis in Table 1, variations in fermentation time had no significant effect ($p>0.05$) on the panelists' preference for products color and viscosity. However, fermentation time significantly affected ($p<0.05$) the taste, aroma, and overall acceptance. The highest scores were 6 h and 4 h of fermentation for taste and aroma, respectively. In addition, 4 h of fermentation was the highest score for overall acceptance.

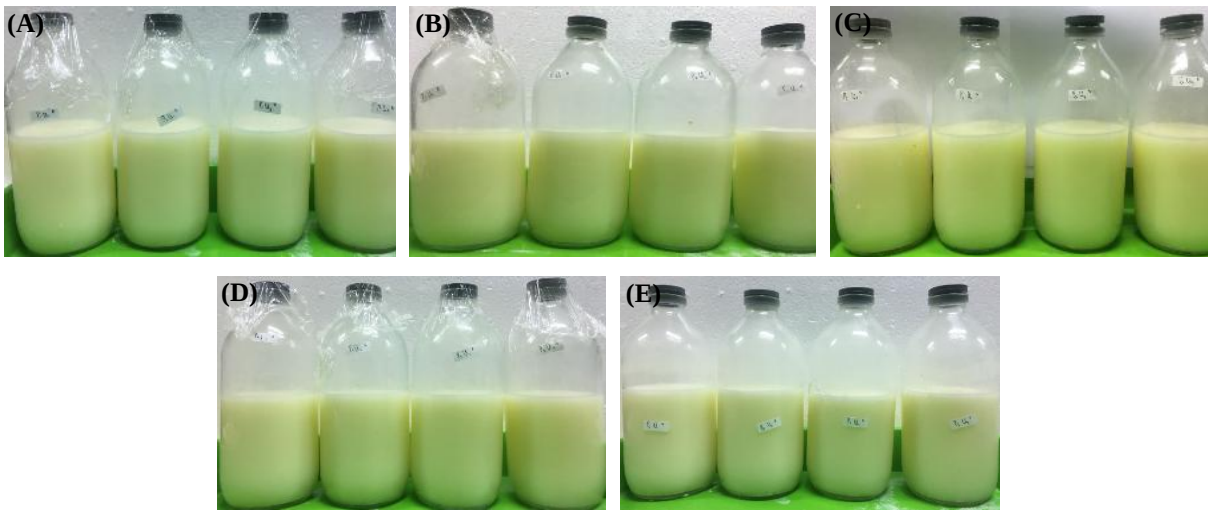

Figure 1 Visualization of fermented whey with various fermentation duration (A) 0 h, (B) 2 h, (C) 4 h, (D) 6 h, (E) 8 h.

3.2 Total lactic acid bacteria of fermented whey

Table 2 Total LAB of fermented whey.

Fermentation time (h)	Total LAB (log CFU/mL)
0	7.37 ± 0.38^{a}
2	8.31 ± 0.16^{b}
4	8.32 ± 0.15^{b}
6	8.57 ± 0.45^{b}
8	8.72 ± 0.50^{b}

a-b: Values with different superscripts showed a significant difference ($p<0.05$).

The growth and quantity of LAB were crucial indicators of quality of fermented beverages. The results of the analysis of lactic acid in whey fermentation bacteria testing data with varying fermentation periods were presented in Table 2. Based on statistical calculations with ANOVA, the differences in fermentation time had a significant effect ($p<0.05$) on the total LAB of whey beverages fermented with cultures of *L. casei*, *L. bulgaricus*, and *S. thermophilus*. The results showed an increase in total LAB compared to the control (0 h of fermentation), suggesting that its activity was occurring and metabolizing the nutrient in whey. However, there was no significant difference in total LAB after 2 h of fermentation.

3.3 Characteristics of fermented whey

Table 3 Physical characteristics of fermented whey.

Parameter	0 h	2 h	4 h	6 h	8 h
pH value	5.59 ± 0.11^{d}	5.54 ± 0.08^{c}	5.39 ± 0.08^{b}	5.13 ± 0.18^{ab}	4.85 ± 0.06^{a}
TDS (°Brix)	9.05 ± 0.06^{e}	8.78 ± 0.05^{d}	8.43 ± 0.05^{c}	7.95 ± 0.06^{b}	7.18 ± 0.10^{a}
Viscosity (cP)	0.09 ± 0.01^{a}	0.11 ± 0.01^{b}	0.11 ± 0.01^{b}	0.13 ± 0.01^{b}	0.15 ± 0.01^{c}
Salinity (ng/L)	2.72 ± 0.06^{a}	2.50 ± 0.21^{b}	2.31 ± 0.10^{c}	2.20 ± 0.08^{c}	2.17 ± 0.23^{c}
Whiteness Index	63.05 ± 1.61	63.69 ± 1.21	64.00 ± 2.58	64.57 ± 2.16	66.68 ± 0.71

a-e: Values with different superscripts show a significant difference ($p<0.05$).

The results of the analysis of physical characteristics (pH value, TDS, viscosity, salinity, and whiteness index) of whey beverages with varying fermentation time treatment were presented in Table 3. Statistical calculations with variance analysis (ANOVA), showed that the difference in whey fermentation time significantly affected ($p<0.05$) the pH value, TDS, viscosity, and salinity. Specifically, the pH value ranged from 4.85 ± 0.06 to 5.59 ± 0.11, TDS ranged from 7.18 ± 0.10 to 9.05 ± 0.06 °Brix, viscosity ranged from 0.09 ± 0.01 to 0.15 ± 0.01 cP, salinity ranged from 2.17 ± 0.23 to 2.72 ± 0.06 ng/L, and whiteness index ranged from 63.05 ± 1.61 to 66.68 ± 0.71.

4. Discussion

The idea of fermenting whey as a by-product was to improve its sensory characteristics. In this study, different fermentation times were used to enhance the taste, aroma, and overall acceptance of fermented whey. The most preferred taste results of fermented whey products were obtained at P3 (2.98 ± 0.89), which had a sour taste. Unfermented whey retained a sweetness influenced by the taste of the initial raw material and the addition of HFS. Besides accelerating fermentation process, the addition of HFS in the manufacturing of fermented beverages could affect the taste, structure, and viscosity of product [9]. In this study, HFS was used to improve the taste and as a carbon source for LAB growth.

The sour taste produced by products was influenced by the length of fermentation time using LAB strains, which could produce lactic acid, enhancing product's taste, increasing its freshness, and masking unwanted aroma [10]. LAB convert sugar into lactic acid and other metabolites, such as acetaldehyde, during the initial step of fermentation. The use of mixed starters in the form of *L. casei*, *L. bulgaricus*, and *S. thermophilus* also contributed to product's taste. The total lactic acid produced was influenced by the activity of the starter cultures. Homofermentative bacteria could produce 85−90% lactic acid, creating acidic conditions in product [11]. The most preferred aroma of fermented whey products was obtained at P2 (3.20 ± 0.69), which had a distinctive sour fermentation aroma. The resulting products exhibited a characteristic whey aroma combined with a strong sour fermentation scent. In addition, the aroma of fermented beverages arose due to the presence of volatile and carbonyl compounds either in the raw materials or formed during fermentation process, such as acetaldehyde, acetic acid, diacetyl, and other acids [12]. These volatile compounds determine the type and intensity of the aroma produced by products [13].

The viscosity of products was influenced by the total value of dissolved solids, with casein clumping playing a significant role in changing the viscosity of the resulting products. Consequently, the viscosity increased with the length of fermentation time. The proteolysis process influenced the texture and changes in calcium concentration, supported by a decrease in pH, which contributed to the formation of a perishable matrix [14]. Lactic acid produced from fermentation influenced casein micelle aggregation and interactions between casein

micelles. The interaction between casein micelles will form a strong and smooth gel where the strength of the casein gel is influenced by pH, calcium concentration, and temperature [15].

The color of fermented whey produced was yellowish-white, with whey probiotics exhibiting a greenish-yellow white pigment derived from lactoflavin and carotene pigments, which produced the yellow color [16]. Based on the study conducted by Mani et al [17], it was stated that there was no significant color change in milk fermentation products using LAB starter variations. The most preferred overall fermented whey results were obtained at P2 (2.92 ± 0.80), with panelists' assessment of overall attributes based on all products quality. The overall results were derived from a combination of panelists' favorability assessment of taste, aroma, viscosity, and color for fermented whey products. Overall attributes showed quality factors that were useful for determining consumer acceptability of products [18].

The increase of LAB during the first 2 h of fermentation showed that it could still process whey used in this study for fermentation. The initial whey pH, TDS, and salinity did not hinder the growth of LAB, although there was no significant increment of LAB after 2 h of fermentation, which ranged from 8.31 ± 0.16 to 8.72 ± 0.50 log CFU/mL. The final number of LAB complied with the minimum viable cell in fermented products, which was 6 log CFU/g, making it a potential probiotic [19]. Whey contained lactose that could break into lactic acid and simple sugars such as glucose by LAB. HFS in whey served as an added sugar that LAB metabolized, speeding up fermentation process. The longer the fermentation time, the greater the amount of sugar that LAB metabolized for growth.

The results of cellular metabolism influenced the increase in total LAB during fermentation process, with the heightened activity of *L. casei* being influenced by the availability of nutrients in products. Agustine et al [20] stated that there was an increase in the total starter bacteria of *L. casei* because of sugar-rearranged metabolism. Due to the mutualism symbiosis, the use of *L. bulgaricus* and *S. thermophilus* as a starter for fermented beverages was effective. In this context, *S. thermophilus* facilitated the growth of other starters by proliferating and synthesizing formic acid [21]. The growth of *L. bulgaricu* was enhanced by the low pH value and acidic condition provided by *S. thermophilus* through the breakdown of lactose into lactic acid and formic acid. Several amino acids were synthesized by *L. bulgaricus*, such as glycine, valine, histidine, glutamic acid, leucine, isoleucine, and glycine. In this context, glycine and histidine have been reported to have the potential to stimulate the growth of *S. thermophilus*.

In line with Chavan et al [22], fermented whey products generally have an acidic pH ranging from 4.8−4.5. The β-galactosidase enzyme and lactate dehydrogenase in LAB will convert lactose into lactic acid during fermentation. Lactose entered LAB through permease then the enzyme β-galactosidase broke the existing glycoside bonds, leading to lactose breakdown into glucose and galactose into lactic acid [23]. LAB growth and activity affected the decrease in pH value in products, showing their proliferation during fermentation process. The raw sample whey had a pH of 5.65. *S. thermophilus* bacteria predominantly grow at the beginning of fermentation process and produce lactic acid and formic acid, optimally thriving in an environment with a pH of 6 to 6.5) [24]. The organic acids produced by *S. thermophilus* accumulated until the sample conditions became acidic, stimulating the growth of *L. bulgaricus*, which could grow optimally in an environment with a pH of 4.6−5.4 [25]. These acidic environmental conditions were produced in fermented whey with P2 (pH 5.39), P3 (pH 5.13), and P4 (pH 4.85) treatments.

TDS showed residual sugar that had not been broken down by LAB into lactic acid, as well as fermented metabolites in the form of organic acids and proteins present in products [26]. The addition of HFS increased the total value in fermented whey sample. The decrease in the total amount of dissolved solids was directly proportional to the decrease in pH value [27]. Compared to other fermented beverages, fermented whey exhibited a low viscosity value caused by whey having a low TDS of around 6%. Several factors affected the viscosity of fermented beverages, including TDS, the ability of LAB to produce acid during fermentation, and the protein content of raw materials. The longer fermentation, the viscosity of whey increased as more lactic acid was formed. The accumulation of lactic acid led to a more acidic atmosphere in whey, causing the pH to decrease until the viscosity of products increased. When acidity level of fermented whey reached the iso-electric point of the protein, it coagulates [28].

Salinity served as an indication of the dissolved salt content in water, with whey salinity arising from salt addition in the cheese-making process. This addition aimed to impart a distinctive flavor, improve the texture and appearance of the cheese, control fermentation process, and inhibit spoilage microbes. According to Anggraeni et al [29], LAB grow more optimally and produce higher lactic acid in environments with low salt concentrations. Longer fermentation time caused the salinity value of whey to decrease due to the decomposition of salt into Na^+ and Cl^- ions [30]. The growth of salt-resistant bacteria could affect quality of fermented products.

5. Conclusion

In conclusion, the longer fermentation time increased the total LAB but decreased pH and TDS. The best product in overall preference was obtained at fermentation time of 4 h with a total LAB that met quality standards of fermented milk beverages.

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7. Conflict of Interest

The authors declared that there is no conflict of interest.

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