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Characteristics and antimicrobial activity of *dangke* whey fermentation with sugar addition

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Abstract

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This research was purposed to analyze the chemical and biological characteristics and antimicrobial activity of fermented whey made from *dangke* whey as affected by cane sugar. Whey was prepared by mixing *dangke* whey with sugar at three concentrations (9, 12 and 15% w/v). Tapioca was added (about 0.7% w/v) to increase the viscosity of the fermented whey. *Lactobacillus plantarum* FNCC 0047 was added (about 3% v/v). Increasing the sugar concentration in the *dangke* whey affected the solid content of the mixture and the lactose, sucrose, starch and protein content at the end of fermentation was also affected. The use of sugar concentrations above 9% caused a decrease in total plate count of *L. plantarum* FNCC 0047 and lactic acid content. Changes in lactic acid at the end caused changes in the pH. The addition of above 9% sugar caused a decrease in antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. The use of sugar up to a concentration of 9% in the production generated the desired characteristics in terms of nutrition and antimicrobial activity.

Keywords: antimicrobial activity; biological characteristics; chemical characteristics; *dangke* whey; fermentation; sugar
Abbreviations: ATCC – American Type Culture Collection; MRS – De Man, Rogosa and Sharpe; MRSA – De Man, Rogosa and Sharpe Agar; FNCC – Food and Nutrition Culture Collection; LBS – Lactobacillus Selection Agar; pH – Power of hydrogene; CFU – Colony Forming Unit; NA – Nutrient Agar; °C – Degree of Celcius; µL – Microliter; v/v – Volume per volume; w/v – Weight per volume; ml – Mililiter; mmol – Milimolar; % – Percentage; mm – Milimeter; SE – Standard of error; ± – Approximately; g – Gram

Introduction

Dangke whey is a by-product of *dangke* production. *Dangke* is processed without fermentation but involves the coagulation of milk protein using papain enzyme (Mukhlisah et al., 2017). The production of cheese from 10 liters of milk results in approximately 6-9 liters of whey, depending on the type of cheese (Almeida et al, 2008). *Dangke* whey has not been fully utilized to date. Whey contains about 55% of the total nutrients from milk, such as lactose, soluble proteins, fats, water-soluble vitamins and mineral salts (Vinderola et al., 2000). Lactose in *dangke* whey can be used as a raw material for fermented products. Lactose can be utilized by bacteria to produce both probiotic and non-probiotic products as nutrients for growth (Panesar et al., 2007; Bovo et al., 2014). Whey also has bioactive components (immunoglobulin, lysozyme, and lactoferrin) which can inhibit the growth of some pathogenic bacteria (Ramos et al., 2012).

The most widely used probiotics include lactic acid

bacteria, specifically *Lactobacillus* and *Bifidobacterium* species (Rocha et al., 2014). *Lactobacillus plantarum* is commonly used in the manufacture of fermented milk (Vasiee et al., 2014). *L. plantarum* can inhibit pathogenic bacteria that are harmful to human health (Kaushik et al., 2009; Gaudana et al., 2010; Setyawardani et al., 2014). The application of *L. plantarum* in the manufacture of fermented beverages can increase the value of *dangke* whey.

Fermentation products can be treated with sweeteners such as sugar, fructose, glucose, sucralose or glycerol (Thaweboon et al., 2011). Sweetener commonly used for milk fermentation products is sugar. Sugars can be sourced from sugar cane and contain sucrose; their presence is responsible for consumer acceptance and the characteristics of fermented beverages (Widyastuti et al., 2014). The high sugar content may adversely affect the growth of lactic acid bacteria. Strains of bacteria commonly used in the fermentation process may show a different tolerance to sugar (Thaweboon et al., 2011). In the production of fermented products from milk, too high concentrations of sugar before inoculation or incubation periods lead to unfavorable effects on the fermentation process. This occurs because of changes in the osmotic pressure of milk. Factors that affect the activity of *Lactobacillus* in the fermentation products include strains of probiotic bacteria, concentrations of inoculant, incubation temperature, duration of fermentation, storage conditions, pH, sugar concentration (osmotic pressure), solids content of the milk, the interaction of species used, the factors supporting and inhibiting the growth, storage temperature, and nutrient availability (Vasiee et al., 2014). Therefore, sugar addition must be determined precisely to obtain the desired effects and avoid any negative ones. This study aimed to analyze the antimicrobial and characteristics of a fermented beverage made from *dangke* whey with various concentrations of sugar added.

Materials and Methods

Materials used in the study were *dangke* whey (*dangke* by-product), tapioca (rose brand®, Indonesia), sugar (gulaku®, Indonesia), distilled water, and buffer at pH of 4 or 7. Bacterial media were purchased from Oxoid, UK, while other analytical chemicals were obtained from Sigma, USA. The bacteria used as a starter were *L. plantarum* FNCC 0027, *E. coli* FNCC 0091, and *S. aureus* FNCC 0047, obtained from the Center for the Study of Food and Nutrition, Gadjah Mada University, Yogyakarta.

Maintenance of starter cultures. The bacterium *L. plantarum* FNCC 0027 (dried culture) was obtained in dry form. Dried cultures were activated with 3 consecutive transfers in MRS broth (24 h incubation at 37°C) before use (Sieladie et al., 2011). The total bacteria of activated culture were determined before further use. The active culture in MRS broth was stored in a refrigerator (5°C) until use. The culture was propagated every 2 weeks. Sub-culture in the form of the frozen solution was stored in Eppendorf tubes at -20°C. The sub-culture consisted of a starter culture suspended in MRS broth and glycerol (1:1). The frozen culture was thawed in tap water for 10 minutes before use. The thawed culture can be directly inoculated into media (2% v/v) and incubated at 37°C (Olson & Aryana, 2008).

Production of fermented whey. *Dangke* whey was mixed with starch at a concentration of 0.7%, and the initial volume was determined (initial volume before heating). The whey mixture was then heated and sugar was added (9, 12 or 15%) while stirring for 5 minutes at 70°C (dissolved completely). Heated whey was added to distilled water to obtain the initial volume. Then, the whey was pasteurized at 80°C for 30 minutes (Alakali et al., 2008, with modifications), cooled and inoculated with 5% of bacterial starter, before being incubated at 37°C for 18 hours (based on optimum growth).

Lactic acid bacteria population (pour plate method). Samples (1 ml) were added to 9 ml of sterile water. This solution was further diluted stepwise yielding a diluted solution with a concentration of 10^{-1} to 10^{-8} times lower than the stock solution. Each dilution 10^{-6} to 10^{-8} (1 ml) was poured into a Petri dish (Duplo) and mixed with 15 mL of deMan, Rogosa, and Sharp agar (MRSA) media. The samples were homogenized and allowed to solidify. Each Petri dish was then incubated at 37°C for 24–48 hours (Othman et al., 2012).

Maintenance of bacterial culture for antimicrobial test. *Escherichia coli* FNCC 0091 and *S. aureus* FNCC 0047 were maintained in Tryptone Soy Broth (Oxoid, UK) media. All indicator strains were subcultured twice prior to use in each experiment to obtain active cultures. Additionally, a sub-culture was prepared in Eppendorf tubes and stored at -20°C, grown in Tryptone Soy broth media, inoculated to use 2% (v/v), and incubated at 37°C (Kar & Misra, 1999).

Total antimicrobial activity of fermented whey. The total antimicrobial activity of fermented whey was assessed using well diffusion method (Seydim & Sarikus, 2006, with modifications) at different concentrations. *E. coli* and *S. aureus* were used as representatives of pathogenic bacteria. The cultures were propagated every two weeks. The stock culture was diluted to achieve a popula-

tion of 10^6 CFU/mL in nutrient agar, denoted as ready-to-use cultures. Then, 1 mL of each culture was poured into the Petri dish, followed by the addition of 25 mL of Nutrient Agar (NA). After solidification of the medium, 2 wells were created in the middle of each Petri dish by using a stainless steel ring with a 9.6 mm diameter. Approximately 200 µL of fermented whey sample with different concentrations of sugar (0, 9, 12 and 15%) was poured into the good hole and kept at 10°C for 30 minutes before being incubated at 37°C for 24 hours. The inhibition zone, which was a clear area around the well, was measured by using a caliper three times in three different places and the result was averaged. The test was measured three times in duplicate replications.

Determination of pH and titratable acidity. The pH of fermented beverages was measured using a pH meter (Hanna). Titratable acidity was measured by a titration method. The lactic acid percentage was calculated using the following formula (Othman et al., 2012):

$$Lactic\ acid\ (\%) = \frac{V \times 0.01 \times 90.08 \times 100}{10g},$$

where: V is volume of titration.

Proximate analysis. Proximate analysis was performed to determine protein and fat in fermented whey and the analysis were conducted according to AOAC procedures (AOAC, 2005).

Total solids. By directly forced air oven drying, the analysis was performed to determine total solids. Total solids were determined by weighing fermented whey, drying, and then weighing the fermented whey residue. Experimental samples were dried for 4 h at 100±1°C in the oven. The total solids content of fermented beverages was the weight of dried fermented beverages residue expressed as % of original fermented beverages test portion weight (AOAC, 2005).

Lactose. The Munson-Walker gravimetric analysis was performed to determine lactose according to AOAC procedures (AOAC, 2005).

Sucrose. IDF_ISO_AOAC Method analysis was performed to determine sugar. The percentage of sugar was calculated using the following formula, with corrected direct invert readings S={100(-a-b)/[142.35-(t/2)]}×(26/w); where S = sugar in the test sample; a = the corrected direct polarization; t = temperature of the polarized solution; and w = weight test portion taken. The analysis was conducted according to AOAC procedures (AOAC, 2005).

Starch. Starch content was determined using direct acid hydrolysis. The method first determined the weight of glucose. Starch by weight was the weight of the obtained glucose multiplied by 0.9 (AOAC, 2005).

Statistical analysis. Data were analyzed according to the one-way ANOVA procedure using SPSS 16.0. All samples were analyzed in duplicate, and all experiments were conducted in five replicates. The data were presented as means and standard deviations. The significance of their variance was verified using Duncan's Multiple Range Test (Nahartyo, 2016).

Results

Physicochemical characteristics of fermentation media and whey. Different concentrations of sugar in the manufacture of fermentation media only affected total solid and sucrose levels ($p < 0.05$). Our experiments revealed that increasing the sugar concentration in the fermentation medium led to changes in the solids. The results shows that the incorporation of 9, 12 and 15% of sugar (sweeteners) besides results in sugar contents of only 6.40%, 9.48%, and 12.35%, respectively (Fig. 1).

Lactose, sugar, starch, and protein present in the fermentation media declined at the end of fermentation. Their reduction was dissimilar, as a result of different initial sugar concentrations ($p < 0.05$). However, the fat contained in each media with the presence of sugar was not significant ($p > 0.05$) (Fig. 2).

L. plantarum FNCC 0047 ***cell counts and viability.*** A decline in the amount of *L. plantarum* in the fermented whey was a result of an increase in the sugar concentration. The activity of *L. plantarum* FNCC 0047 was dependent on growth medium. The most desirable conditions for *L. plantarum* growth were media containing 9% sugar and media without sugar addition. In media with more than 12% sugar, a decrease in *L. plantarum* FNCC 0047

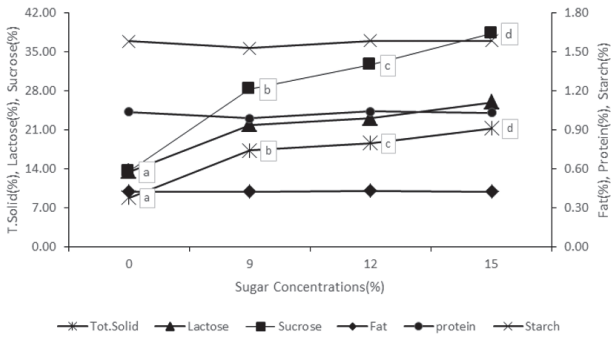

Fig. 1. Physicochemical characteristics of fermentation media

Different letters in the same line indicate significant difference ($p < 0.05$)

was observed ($p < 0.05$), as excessive sugar concentrations led to changes in osmotic pressure and low water activity (aw) (Fig. 3).

Lactic acid levels in whey media with 9% sugar were significantly higher ($p < 0.05$) than in the absence of sugar and with other sugar levels (Fig. 4). Increasing sugar content until optimum amount led to improve production of lactic acid in highest point. Furthermore, increased sugar content in excess amount (12 and 15 % sugar) would inhibit lactic acid production (Fig. 4).

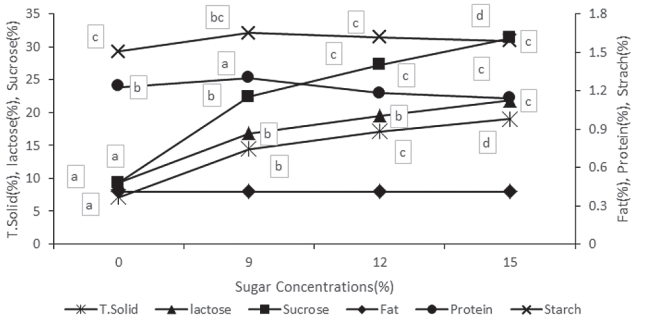

Fig. 2. Physicochemical characteristics of fermented whey
Different letters in the same line indicate significant difference ($p < 0.05$)

Changes in the pH value of fermented whey are caused by changes in the amount of lactic acid during fermentation. The pH is negatively correlated with the lactic acid level in the fermented whey. The pH value of the whey medium treated with 9% of sugar was lower than that in the absence of sugar and with other sugar treatments ($P < 0.05$) (Fig. 5).

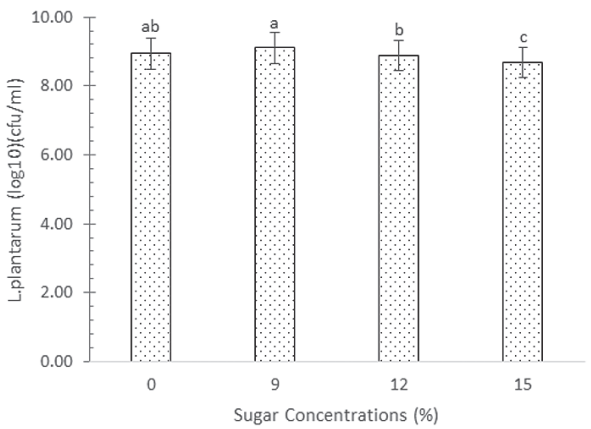

Fig. 3. *Lactobacillus plantarum* (log10 CFU/ml) of whey fermented with different concentrations of sugar
Different letters in the same bar indicate significant difference ($p < 0.05$)

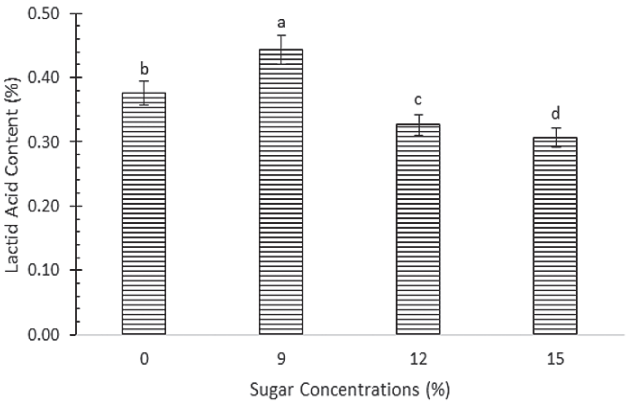

Fig. 4. Lactic acid content of whey fermented with different concentrations of sugar
Different letters in the same bar indicate significant difference ($p < 0.05$)

Antibacterial activity on whey fermentation against gram positive and negative bacteria. Antibacterial activity against *E. coli* FNCC 0091 (Gram-negative) and *S. aureus* FNCC 0047 (Gram-positive bacteria) were marked by presence of inhibition zone of bacteria. Inhibition zone against *E. coli* FNCC 0091 in the absence of sugar was greater than that of *S. aureus* FNCC 0047. Overall, increased of sugar concentration caused inhibiton zone was smaller. But, inhibition zone of *E. coli* between 9 and 12 % sugar were not different significantly (Fig. 6).

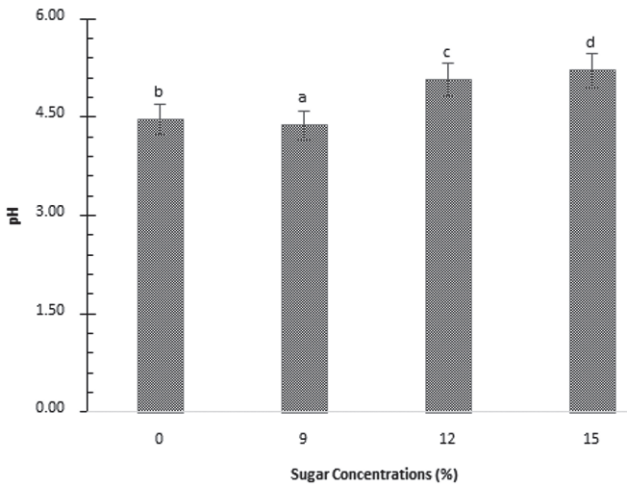

Fig. 5. The pH value of whey fermented with different concentrations of sugar
Different letters in the same bar indicate significant difference ($p < 0.05$)

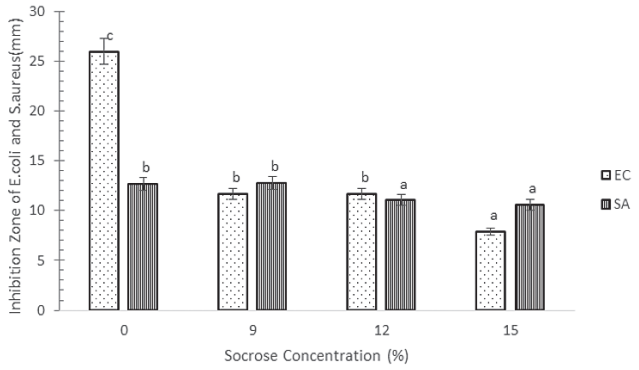

Fig. 6. Inhibition zone (mm) in fermented whey against *E. coli* FNCC 0091 and *S. aureus* FNCC 0047 with different concentrations of sugar. Data are expressed as mean of inhibition zone diameter (mm) ± SE

Different letters in the same bar denote statistical significance ($p < 0.05$)

Discussion

Incorporation of sweetener beside result in sugar content indicates that the sugar did not completely contain sucrose. The content of fat, protein, lactose, and tapioca was from *dangke* whey. The composition of the fermentation medium was affected by the content of the raw material and the addition of other ,ingredients in the media (Ayar & Burucu. 2013). *Dangke* whey was the main ingredient of fermentation media in this research. The fermentation medium was used as a growth medium for *L. plantarum* FNCC 0027.

Previous studies have reported that mixed components are determinant factors for physicochemical characteristics. Acelora contained total solids (14.8-15%), protein (0.34-0.52%) and fat (0.12-0.14%) in the mixture of whey butter, cheese, and juice (Cruz et al., 2009).

L. plantarum utilized various carbohydrates for fermentation, and also utilized essential amino acids and vitamins for growth. *L. plantarum* FNCC 0027 required a variety of carbohydrates and protein as an energy source. These materials were initially reformed into simpler components (Wheater, 1955). Some *Lactobacillus* have the ability to break down corn starch at a concentration of 0.1-0.2 mg/ml (Lee et al., 2001). Microorganisms metabolized complex carbohydrates into monosaccharides. The formed monosaccharides were then involved in glycolysis to form pyruvic acid. In the presence of lactate dehydrogenase, pyruvic acid received electron pairs. A pair of electrons was released by the oxidation of glyceraldehyde 3-phosphate acid via glycolysis. The outcome of this process is reduced to lactic acid. Finally, the resulted metabolite was responsible for characteristics of the fermentation product (Panesar et al., 2007).

The proportion of carbohydrate used by *L. plantarum* FNCC 0027 varies during fermentation and depends on the presence of more simple components. However, physiological characteristics, both fermentation media and whey, show a decrease in all components of carbohydrate at the end of fermentation. Activity of lactic acid and the number of bacteria from the largest to the smallest; a row occurred in LBS media with added glucose, fructose, sucrose, lactose, and galactose (Srinivas et al., 1990).

L. plantarum FNCC 0047 was used in the production of fermented whey. Such bacteria were also utilized in the preparation of fermented milk, vegetables, and meat products (sausages) (Sawitzki et al., 2009). *L. plantarum* FNCC 0047 was classified as homofermentative. The enzymatic activity of these bacteria during fermentation metabolized complex nutritional components into simpler components. Content of lactic acid in fermented milk products was affected by the amount and type of bacteria and their ability to break down the carbohydrate component. Increased levels of lactic acid led to a decrease in the pH of the product (Tamime, 2002).

L. plantanarum need sugar in optimum amount to improve their activity and growth. Addition of sugar in the fermentation product concentrations above 7% (w/v) prior to fermentation should be considered, promoting an inhibitory effect on microorganisms (Tamime, 2002). Excessive sucrose levels (more than 10%) prior to inoculation or incubation periods demonstrated unfavorable effects on fermentation conditions due to alterations in the osmotic pressure of milk and lower water activity. Environmental conditions that are similar to previous conditions promote the proper adaptive activity of microorganisms. Changes in environmental conditions led to the adjustment of microorganisms (Tamime & Robinson, 1999).

Factors affecting the activity of *Lactobacillus* in yogurt, among other strains of probiotic bacteria, were nutrient availability, level of inoculation, incubation temperature, time of fermentation, storage conditions, pH, concentration of sugar (osmotic pressure), content of milk solids, and temperature storage (Vasiee et al., 2014).

Lactic acid in fermented whey was a product of the breakdown of nutritional components in the media. Lactic acid bacteria metabolized complex carbohydrates into simple carbohydrates. The end product of carbohydrate metabolism is lactic acid.

The lactic acid content of this study is higher than that of the study using *L. acidophilus* CRL 636, with media whey protein concentrates (Pescuma et al., 2010). Lactic acid content at fermentation times of 12 and 24 hours were 32 mmol/ml (0.17%) and 40 mmol/ml (0.22%), respectively.

Increased acidity basically enhanced the concentration of hydrogen ions, leading to a reduction of pH value (Panesar et al., 2007). Whey fermentation in this study (18 hours of incubation) resulted in an average pH value of 4.61. This finding was dissimilar to a previous report which using whey protein concentrate (fermented using *L. acidophilus* CRL 636 and incubated for 12 and 24 hours), where the pH value was 5.5 and 4.8 (Pescuma et al., 2010). The pH of soygurt added lactose (incubated for 18 hours) using *S. thermophilus* and *L. bulgaricus* was around 3.96. These discrepant data may result from differences in material or growth media, type and strain of bacteria, the concentration of inoculum, incubation time, and the availability of nutrients (Yusmarini & Efendi, 2004). Factors that influencing the activity of *Lactobacillus* in yogurt included the strain of probiotic bacteria, nutrient availability, concentration of inoculation, incubation temperature, fermentation time, storage conditions, pH, concentration of sugar (osmotic pressure), solids content of the milk, and storage temperature (Vasiee et al., 2014).

Zone of inhibition was observed in previous report, against Gram-positive and negative bacteria, including S. *aureus*, *L. monocytogenes, Salmonella typhmurium, Shigella flexneri, E. coli, Klebsiella pneumoniae, Bacillus cereus, Pseudomonas aeruginosa, and Enterobacter* spp. *Brucella abortus* (Mkrtchyan et al., 2010). Additionally, inhibitory effect of pure isolates *L. plantarum* L. p9 and *L. plantarum* C5CC5276 against *E. coli*, resulting in inhibitory zones of 16 and 20 mm, respectively (Kaushik et al., 2009).

The inhibition zone against *E. coli* FNCC 009 and S. aureus FNCC 0047 was the result of the antibacterial activity of fermented whey, which may result from primary metabolites such as lactic acid. Lactic acid is the end-product of carbohydrate metabolism of *L. plantarum* FNCC 0047 in whey beverage products. Lactic acid attenuated the pH values and inhibited microbial growth. Primary metabolites are chemical compounds produced by microbes, and are needed for growth. One of the primary metabolites was lactic acid. Mostly, pH condition for the optimum growth of microorganisms ranged from 6.6 to 7.5 (neutral) (McNeil & Harvey, 2008). Food acidification, at least, may provide double antimicrobial properties: effect on pH and the nature of the typical inhibition of the acids formed (Rocha et al., 2015).

An inhibition zone against *E. coli* FNCC 0091 and *S. aureus* FNCC 0047 also resulted from the presence of bacteriocin, a type of secondary metabolite. These secondary metabolites are compounds that are synthesized by microbes but not the basic physiological needs. *L. plantarum* FNCC 0027 is a probiotic bacteria and is Gram-positive (Daems et al., 2016). Bacteriocins from Gram-positive bacteria contain 30 to 60 amino acids, with activities varying from a narrow to a broad spectrum. Bacteriocin can inhibit Gram positive and negative bacteria (Cotelo et al., 2013). Research on the inhibition of bacteriocins from *L. plantarum* Lp9 and *L. plantarum* C5CC5276 was reported previously (Kaushik et al., 2009). The antibacterial properties not only inhibit *E. coli* but also retard the growth of *B. cereus*, *Listeria monocytogenes*, *Salmonella typhi* and *S. aureus*. *L. plantarum* ATCC 8014 can inhibit *Shigella dysentery*, *S. aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 5668, *Salmonella typhi* and *E. coli* S5 (Gaudana et al., 2010).

Bioactive components contained in the whey, including lysozyme, lactoperoxidase, and lactoferrin among others, also contribute to the inhibition of some bacterial pathogens. Lactoferrin was an iron-binding glycoprotein. Most microorganisms need iron for growth. The inhibitory activity of bacteria by lactoferrin is through the limitation of iron for growth. A plausible mechanism of bacterial inactivation by lysozymes is disruption of the glycosidic bond formation between the two components of the peptidoglycan found in the cell walls of bacteria. Bacteria were inhibited by lactoperoxidase through the oxidation of sulfhydryl groups of the cell membrane. Lactoperoxidase generally shows bacteriostatic properties against Gram positive and Gram negative bacteria in the form of bacteriolysis (Murata et al., 2013).

Conclusion

The application of various sugar concentrations affected the chemical and biological characteristics and antibacterial activity of fermented whey. Whey fermented with sugar concentrations above 9% reduced antibacterial activity against *E. coli* and *S. aureus* and altered their chemical and biological characteristics.

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