The Effects of Storage at Low Temperature on The Viability of Lactobacillus casei and The Stability of Antibacterial Activity in Green Grass Jelly Synbiotic Drinks

By Samsul Rizal; Suharyono; Fibra Nurainy; Julfi Restu Amelia

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ABSTRACT

Synbiotic drinks from green grass jelly have shown antibacterial activity against pathogenic bacteria. Synbiotic drinks are usually stored at low temperature to maintain their characteristics. The aim of this study was to determine the effect of storage at a low temperature of 10^{o}C on the viability of lactic acid bacteria and the stability of the antibacterial activity in synbiotic drinks made of green grass jelly. Antibacterial activity of green grass jelly synbiotic drink was conducted against pathogenic bacteria (*Staphylococcus aureus*, *Salmonella sp*., *Bacillus cereus*, and *Escherichia coli*). The products were stored for 28 days in 10^{o}C temperature. Observations on the antibacterial activity, pH value, total acid, and total lactic acid bacteria were carried out every 7 days. Antibacterial activity was evaluated using agar well diffusion method. The results showed that storage at low temperature (10 ± 2^{o}C) for 28 days decreased the antibacterial activity and pH value but sharply increased total lactic acid bacteria (at 0 to 7 days of storage) in green grass jelly synbiotic drinks. *Salmonella sp*. showed the highest inhibition caused by the antibacterial agents in green grass jelly synbiotic drinks while the lowest inhibition was found on *Staphylococcus aureus*. During storage at low temperature, green grass jelly synbiotic drinks had a total of lactic acid bacteria that ranged from 9.54 to 10.12 (Log CFU/mL) or equal to $3.5x10^{9}$-$1.3x10^{10}$ CFU/mL; a total of lactic acid that ranged from 0.42% to 0.87%; and pH values that ranged from 3.80 to 4.10.

Keywords: Antibacterial activity, green grass jelly, pathogenic bacteria, synbiotic,

INTRODUCTION

The balance of microorganisms in human intestines could influence human health. An increase in the number of pathogenic bacteria in the human intestinal tract disturbs its balance; thus, the human body becomes less resistant to intestinal infection.

Consuming drinks fermented by lactic acid bacteria is known to suppress the growth of pathogenic bacteria. These drinks mainly affect the development of microbiota that inhabits organisms by ensuring the right balance between pathogens and bacteria needed for the normal functioning of the organism (Oelschlaeger, 2010). Another function of these drinks is to counteract the activity of pathogenic intestinal microbiota, which comes from contaminated food and environment (Markowiak and Sli˙zewska, 2017). Probiotic microbes work by the manipulation mechanism of the intestinal microbial community, suppression of pathogens, immunomodulation, and differentiation of epithelial cells and fortification of the intestinal barrier (Thomas and Versalovic, 2010). The use of probiotics with the right dose is known to reduce and treat diarrhea (Aleta et al. 2020). In addition to probiotics, synbiotics also exhibit similar activity. Suharyono et al. (2012) and Rizal et al. (2019) proposed the idea of a green grass jelly drink fermented by lactic acid bacteria as a synbiotic drink.

Simply put, synbiotic is defined as a mixture of probiotic and prebiotic. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al. 2014). Food and Agriculture Organization of the United Nations et al. (2006) has defined probiotics as “live micro-organisms which when administered in adequate amounts confer a health benefit on the host.” Prebiotic is defined as non-digestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of one or a limited number of probiotic in the colon (Manning and Gibson, 2004). Green grass jelly (*Premna oblongifolia* Merr) is an Indonesian food plant with a high dietary fiber content (Nurdin et al. 2017) with potential to be prebiotic.

Green grass jelly synbiotic drink is a type of drink that contains lactic acid bacteria as probiotics, green grass jelly powder as a prebiotic, and skim milk and glucose as sources of nutrients for the growth of lactic acid bacteria (Rizal et al. 2019). According to Rizal et al. (2019), this green grass jelly synbiotic drink contained antibacterial compounds, mainly lactic acid; thus, it showed antibacterial activity against pathogenic bacteria, such as *Staphylococcus aureus* and *Eschericiacoli*. To prevent changes in properties, fermented drinks should be stored in cold conditions at 10 ± 2^{o}C. However, studies on the effect of storage at low temperature (10 ± 2^{o}C) for green grass jelly synbiotic drinks on the viability of lactic bacteria and the stability of antibacterial activity were still limited. Research by Mirdalisa et al. (2016) showed that milk fermented by *Lactobacillus casei* showed a decrease in antimicrobial activity during storage at 5^{o}C for up to 45 days. Qu et al. (2010) also showed that at low temperature, the antibacterial activity of Silver nanoparticles (SNPs) had decreased. Therefore, this study aimed to determine the effect of storage at low temperature on the viability of *Lactobacillus casei* and the stability of the antibacterial activity against pathogenic bacteria in green grass jelly synbiotic drinks.

MATERIALS AND METHODS

Materials

The main ingredients used in this study were grass jelly leaves from grass jelly plants (*Premna oblongifolia* Merr.), pure culture inoculums of lactic acid bacteria (*Lactobacillus casei*), pure culture of targeted bacteria (*Salmonella sp.*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*), skim milk, and sucrose. The chemicals used were citric acid, MRS Broth, MRS Agar, Merck brand Nutrient Broth, Nutrient Agar brand Oxoid, distilled water, NaCl 0.85%, alcohol, NaOH 0.1N, phenolphthalein indicator, and other analytical materials.

Methods

The research was carried out in several stages. It started by making green grass jelly powder which was then extracted to be green grass jelly extract. The next step was producing green grass jelly synbiotic drink by mixing green grass jelly extract, skim milk, glucose, and water in a container. It was then fermented with *L. casei* for 48 hours at 37°C. The resulting green grass jelly synbiotic drinks were then stored at a low temperature of 10 ± 2°C for 28 days and observed every 7 days starting at 0 day.

Production of Green Grass Jelly Powder

Green grass jelly powder was produced according to a method developed by Nurdin, et al. (2004). After discarding the damaged and unusable parts, green grass jelly leaves were washed with clean water then drained. The cleaned green grass jelly leaves were slashed into small parts of 3 x 1,5 cm^2 and dehydratedin an oven at 50ºC for 24 hours. The dehydrated leaves were then grounded with a blender, packed in a plastic bag, and stored at room temperature.

Extraction of Green Grass Jelly Powder

The extraction of green grass jelly powder was done according to a method developed by Nurdin et al. (2004). It started by adding 25 grams of green grass jelly powder into 500 mL of hot water (100°C) added with 0,1% (b/v) of citric acid. It was then homogenized with stirrer 'VWR Hot Plate" at full speed for 15 minutes. The mixture was strained to produce thick liquid of green grass jelly extract. This extract was poured into a large tray and dehydrated in an oven at 50°C for 48 hours. The resulting material was then grounded with a blender and strained through an 80-mash filter.

StarterPreparation

The *Lactobacillus casei* starter culture was prepared using a method developed by Rizal, et al. (2016). Pure culture of *Lactobacillus casei* in ampoules was transferred to an Erlenmeyer flask containing 50 mL of MRS Broth medium and then incubated for 48 hours at 37°C. Two drops of the incubated MRS Broth medium were inoculated into 10 mL solution of 10% (w/v) skim milk that had been sterilized at 121ºC for 15 minutes. After 48 hours of incubation at 37ºC, the assay that was the mother culture was inoculated again in 0.5% (v/v) into the same medium for another 48 hours of incubation at 37ºC to obtain intermediate culture. As much as 0.5% (v /v) of the intermediate culture was then inoculated into the same medium with the addition of 3% glucose and incubated for 24 hours at 37ºC. The resulting culture was the starter used for the production of green grass jelly synbiotic drinks.

Production of Green Grass Jelly Synbiotic Drinks

Production of green grass jelly synbiotic drinks was done according to the procedure recommended by Nurdin, et al. (2004) with a few modifications. It started by adding 2% (w/v) of skim milk and 2% (w/v) of sucrose into an Erlenmeyer flask containing 100 mL of distilled water and 0.5% (w/v) of green grass jelly extract. This mixture was homogenized using a stirrer for 2 minutes, then pasteurized at 80-85°C for 15 minutes. Finally, it was cooled down to 37°C, inoculated with 4% (v/v) of the *Lactobacillus casei* starter, and incubated at 37°C for 48 hours to produce green grass jelly synbiotic drinks. The drinks were then stored at a low temperature of 10 ± 2°C for 28 days. Antibacterial activity, total lactic acid bacteria, pH value, and total lactic acid of the products were evaluated at day 0, 7, 14, 21, and 28 of storage.

Preparation of Targeted Cultures and Evaluation of Antibacterial Activity

Each targeted bacteria (*Staphylococcus aureus*, *Salmonella* sp., *Bacillus cereus*, and *Escherichia coli*) was transferred from stock culture into flasks containing 10 mL of Nutrient Broth and incubated for 24 hours at 37°C. Each 20 µL of the targeted culture was then seeded into Erlenmeyer flask containing 20 mL of sterilized NA, homogenized, poured onto a petri dish, and let solidified. Agar wells were created on the seeds using sterilized Agar Borer. Each60 µL of green grass jelly synbiotic drink was inoculated into the well. Antibacterial activity of green grass jelly synbiotic drinks was determined by agar well diffusion method (Murhadi, 2002) with some modifications. Antibacterial activity was determined by the measurement of zone of inhibition (ZOI) around the wells after 24 hours of incubation at 37°C. The diameter of the resulting zone was measured in millimeters. The experiment was performed in triplicates with every reading taken by two observers and the mean was calculated.

RESULTS AND DISCUSSION

Total Lactic Acid Bacteria (*Lactobacillus casei)*

Figure 1 shows the effect of storage in low temperature (10 ± 2°C) on the total lactic acid bacteria in green grass jelly synbiotic drinks. According to the figure, the average total *L. casei* in the product stored at low temperature (10 ± 2°C) for 0, 7, 14, 21, and 28 days were 10.10, 9.89, 9.58, 9.59, and 9.51(Log CFU/mL) or equal with $1.3x10^{10}$CFU/mL, $1.3x10^{10}$ CFU/mL, $4.7x10^{9}$ CFU/mL, $3.5x10^{9}$ CFU/mL, and $3.5x10^{9}$ CFU/mL respectively. This indicated that total lactic acid bacteria in green grass jelly synbiotic drinks was relatively stable during storage at low temperature (10 ± 2°C) for 28 days; thus, it showed that storing green grass jelly synbiotic drinks at low temperature did not affect the viabilityof lactic acid bacteria in the drinks. This was relatively close to the results of a research by Rizal (2009) which showed that storing of *Turi* milk yogurt in low temperature for 12 days had no significant effect in total lactic acid bacteria. In cold storage, lactic acid bacteria were alive but did not appear to perform significant metabolism activities. According to Pineiro and Stanton (2007), an important criteria for probiotics included that they should not only be able to survive through the digestive tract by showing tolerance of acid and bile, but also have the ability to multiply in the intestines. The ability of lactic acid bacteria to live in green grass jelly synbiotic drinks with low pH in cold condition is important to its potential as a probiotic drink.

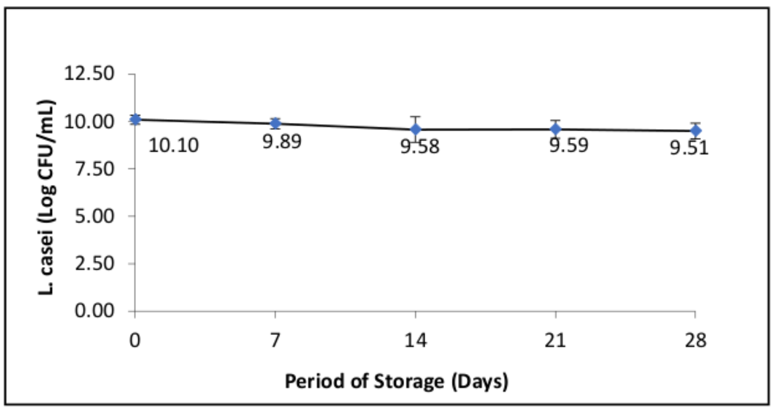

Figure 1. Total *L. casei* in green grass jelly synbiotic drinks during storage in low temperature (10 ± 2ºC)

This slight decrease might be caused by a declinein pH value and an increase in total lactic acid of the product during storage period. According to Wee et al. (2006), lactic acid accumulation inhibited the growth of lactic acid bacteria due to the changes in pH value to acidic conditions. Overall, this result was similar to a research by Sutikno et al. (2013) that revealed that sugar concentration decreased the total lactic acid bacteria in *Turi* fermented milk after being stored at a low temperature for 12 days.

Total Lactic Acid

Lactic acid is the main compound resulted from a fermentation process involving lactic acid bacteria (Villalobos et al. 2020; Huang et al. 2020). As shown in Figure 2, total lactic acid in green grass jelly synbiotic drinks stored in a low temperature of 10 ± 2°C on day 0, 7, 14, 21, and 28 were 0.48%, 0.81%, 0.92%, 0.90%, and 0.87% respectively.

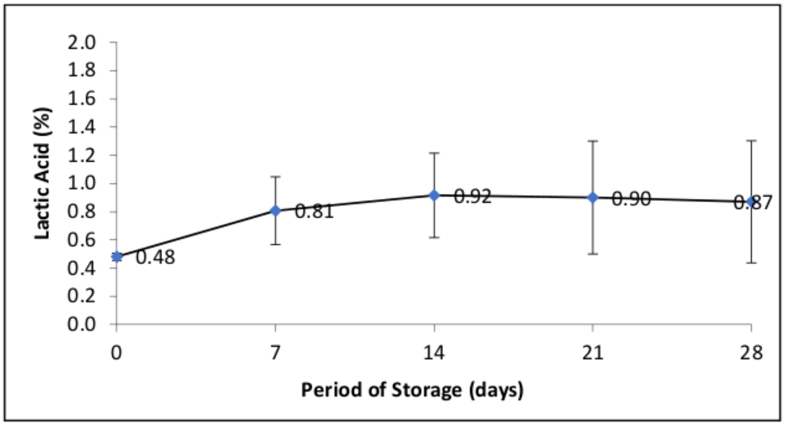

Figure 2. Total lactic acid in green grass jelly synbiotic drinks during storage in low temperature (10 ± 2ºC)

According to Figure 2, storage time in low temperature increased total lactic acid in the product. There was a sharp increase in total lactic acid from day 0 at 0.42% to day 7 at 0.74 %. From day 7 to day 28, total lactic acid count increased gradually from 0.74% to 0.87% at the end of storage period. This result was in line with the result from a research by Rizal et al. (2009) in which during storage at low temperature (10 ± 2°C) for 12 days, the total lactic acid in *Turi* milk yogurt increased slightly from 0.73% on day 0 to 0.81% on day 12. Nurainy et al. (2017) also stated that there was an increase in total lactic acid in a synbiotic guava juice for 28 days of cold storage.

The increase of total lactic acid in green grass jelly synbiotic drinks during storage was caused by the production of lactic acid by lactic acid bacteria even though there was not a significant growth of lactic acid bacteria during that time. Charlier et al. (2009) stated that lactic acid was the main result of sugar metabolism by lactic acid bacteria. The presence of sucrose and skim milk in the product also caused the increase of total lactic acid. Abdel-Rahman et al. (2013) stated that carbohydrates were metabolized by lactic acid bacteria to produce lactic acid as their main metabolic end-product. Additionally, sucrose was used as a source of energy for *Lactobacillus casei* to turn the lactose in skim milk into lactic acid.

pH Value (Level of Acidity)

Acidity (pH value) is an important indicator of the quality of fermented drinks as it is closely related to taste and texture (Li et al. 2017). Appropriate level of acidity will give the product a unique taste and inhibit the growth of pathogenic bacteria from food (Mufandaedza et al. 2006). As shown in Figure 3, the average pH value of green grass jelly synbiotic drinks stored at low temperature (10 ± 2ºC) on day 0, 7, 14, 21, dan 28 were 4.10, 4.01, 3.92, 3.85, and 3.80 respectively.

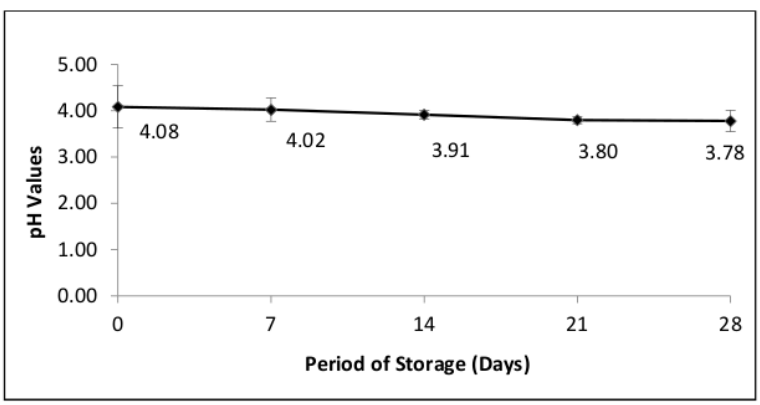

Figure 3. pH value of green grass jelly synbiotic drinks during storage in low temperature (10 ± 2ºC)

According to Figure 3, pH value of green grass jellysynbioticdrinks during storageat low temperature dropped slightly but was relatively stable. This indicated that storinggreen grass jellysynbiotic drink in a lowtemperature of 10 ± 2ºCdid not affect its pH value. Despite showing only slight changes, these pH values were low enough to inhibit the growth of preference bacteria. A slight decrease in pH was due to accumulation of lactic acid in the product from lactic acid bacteria metabolism. As stated by Huang (2020), the accumulation of lactic acid during fermentation of goat milk by the metabolism of lactic acid bacteria caused the pH value to decrease while the acidity that could be titrated increases (Huang et al. 2020). Charlie et al. (2009) added that lactic acid bacteria were a ubiquitous and heterogeneous species with a common feature of lactic acid production as a main result of sugar metabolism which lead to acidification of the environment up to pH 3.5.

Antibacterial Activities

Antibacterial activity of synbiotic green grass jelly drink is indicated by diameter of inhibition zone; the higher the diameter of the inhibition zone is, the higher the antibacterial activity of the products. Antibacterial activity of green grass jelly synbiotic drinks against targeted diarrhea-causing pathogenic bacteria during storage at low temperature (10 ± 2°C) is illustrated in Figure 4. According to Figure 4, the antibacterial activity of green grass jelly synbiotic drinks against all targeted bacteria decreased during 28 days of storage at low temperature (10 ± 2°C). These results were similar to the results of a research by Mirdalisa et al. (2016) that showed decrease in antimicrobial activity of fermented milk during storage at a low temperature of 5°C for 45 days. This decrease might be caused by the lack of growth in lactic acid bacteria during storage at low temperature as shown in Figure 1. In their research, Sathe et al. (2007) evaluated that *Lactobacillus plantarum* at 30°C showed maximum antimicrobial activity at the end of the exponential growth phase which then decreased in the stationary phase after 48 hours of growth.

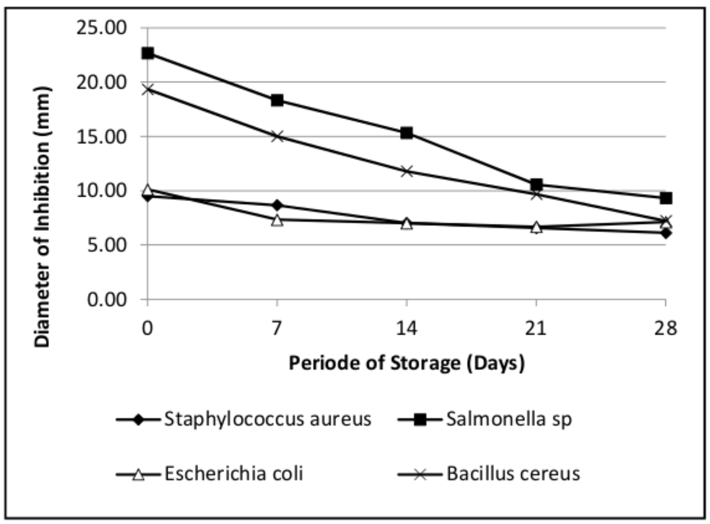

Figure 4. Antibacterial activities of green grass jelly synbiotic drinks against pathogenic bacteria during storage at low temperature (10 ± 2°C)

Overall, the reduction in the diameter of the inhibition zones of all targeted bacteria indicated the decrease of antibacterial activities. Against *Escherichia coli* and *Staphylococcus aureus*, the antibacterial activities showed similarity and they were relatively stable throughout the storage period. Antibacterial activities against *Salmonella sp*. and *Bacillus cereus* were similar to each other and with significant decrease. The diameter of *Salmonella sp*. inhibition zone decreased sharply from 22.67 mm at day 0 to 9.33 mm at day 28 of storage, which amounted to 13.34 mm decrease. The diameter of *Bacillus cereus* inhibition zone dropped significantly from 19.33 mm at day 0 to7.22 mm at day 28m which amounted to 12.11 mm decrease. However, the product still showed significant antibacterial activity because their diameter of inhibition zones was more than 5 mm. These results were in line with several researches that stated probiotics might effectively inhibit the growth of pathogenic bacteria, such as *Eschericia coli* (Chingwaru and Vidmar, 2017), *Staphylococcus aureus* (Sikorska and Smoragiewicz, 2013), and *Salmonella sp*. (Carter et al., 2017). Liptáková et al. (2017) also stated that lactic acid bacteria and their metabolites were able to slow or inhibit the growth of undesirable bacteria. Due to these facts, probiotics had been proposed as preventative and therapeutic measures to restore the composition and function of healthy intestinal microbiomes (Hemarajata and Versalovic, 2013).

Figure 4 also shows the comparison of resistance of targeted bacteria against antibacterial agents in green grass jelly synbiotic drinks. According to Figure4, *Salmonella sp.* was the most sensitive bacteria to the antibacterial activity of the green grass jelly synbiotic drinks, followed respectively by *Bacillus cereus, Escherichia coli,* and *Staphylococcus aureus*. The difference of antibacterial activity might be influenced by peptidoglycan in the cell walls of bacteria.

CONCLUSIONS

Antibacterial activity, pH, and total lactic acid bacteria in green grass jelly synbiotic drinks decreased gradually during storage at low temperature (10 ± 2ºC) for 28 days, but the total lactic acid increased gradually. Among the targeted pathogenic bacteria, *Salmonella sp*. was the most sensitive against the antibacterial activity of green grass jelly synbiotic drinks, while *Staphylococcus aureus* was the most resistant one. Green grass jelly synbiotic drinks did not change significantly during storage at low temperature (10 ± 2ºC) for 28 days.

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