The Effect of Inulin and Sucrose Addition on the Number of Colonies *L. acidophilus* and *B. bifidum* in the Soyghurt After the Freeze-Drying Process

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Abstract. The freeze-dried soyghurt generally will be last much longer. However, the reduction of *L. acidophilus* and *B. bifidum* (LAB) colonies during the freeze-drying process could be occurred and it could be minimized by adding inulin as a prebiotic and sucrose as a cryoprotector. This research aimed to determine whether or not the colonies number in freeze-dried soyghurt with the SNI and to analyze the effect of inulin and sucrose addition to the growth of the LAB in the freeze-dried soyghurt. This experimental laboratory study with pretest- posttest design consists of 1 negative control group, 1 group of soyghurt, and 4 groups of soyghurt with the addition of 10% sucrose and 0.5%, 1%, 3%, 5% inulin. The bacterial growth was calculated using the TPC method. All data were tested using the Saphiro Wilk test, followed by the Kruskal Wallis and Mann Whitney Post Hoc test. The conclusion of this research was that the number of colonies in each experimental groups decreased after the freeze-dried process and only the group of soyghurt without any additions did not meet the standard of SNI. Statistical tests showed a significant distinction between groups, giving a conclusion that the addition of inulin and sucrose gave a better incretion of the LAB significantly. 10% sucrose and 3% inulin was the best combination which has 2.34 × 1029 CFU/ml as the highest TPC. It means that the higher of sucrose and inulin addition to the soyghurt, does not guarantee the number of LAB colonies inside will also be higher.

Keywords: B. bifidum · Inulin · L. acidophilus · Soyghurt · Sucrose

1 Introduction

The freeze-dried yoghurt generally will be last for 6 months to 2 years [1]. One of the most commonly used methods to make yogurt last much longer is the freeze-drying

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process or lyophilization [2]. However, this process can cause damage to bacterial cells so that the number of LAB colonies can decrease [3, 4]. The number of bacterial colonies in yogurt products recommended by the Indonesian National Standard (SNI) is 10^6 to 10^8 CFU/ml [5]. To overcome this, inulin as a prebiotic and sucrose as cryoprotectant as well as prebiotics need to be added [4, 6]. According to research, an inulin concentration of 0.5% is the most effective concentration [7]. While the maximum concentration of sucrose is best used is 15% [8]. Because the research will be conducted this time using two types of prebiotics, the concentration of sucrose selected is 10% so that the osmotic pressure balance of bacterial cells can be maintained [3, 4].

The study aimed to determine the appropriate number of BAL colonies in soyghurt after the dry freeze process with SNI and analyze the effect of the addition of inulin and sucrose on soyghurt after the dry freeze process on the growth of *L. acidophilus* and *B. bifidum*. The hypothesis of this study amounts to two points, namely 1) there is a significant difference between the group that contains only soyghurt and the group added inulin and sucrose (H1); 2) The higher the concentration of inulin and sucrose added, the higher the number of BAL colonies in it (H2).

2 Materials and Methods

The subjects of the study were *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium bifidum* ATCC 29521. The study was conducted at the Microbiology Laboratory of the Faculty of Medicine, Jenderal Achmad Yani University from August 2021 to January 2022. The research design used is *pretest-posttest design*. There were 6 groups tested, consisting of 1 negative control group, 1 soyghurt group without any additions, and 4 groups of soyghurt with sucrose 10% and inulin 0.5%, 1%, 3%, 5% which were successively named KK (−). K1, K2, K3, K4, and K5. The ingredients used in the study were soy milk, *Lactobacillus acidophilus* ATCC 4356, *Bifidobacterium bifidum* ATCC 29521, MRSA, MRSB, NaCl physiological, violet crystal solution, sodium bicarbonate solution, PVP-iodine solution, safranin O solution, 96% acetone-alcohol solution, and emersi oil.

After all the tools are sterilized using autoclaves at a pressure of 1 atm and a temperature of 121 °C for 15 min, the reidentification of *L. acidophilus* and *B. bifidum* bacteria is carried out. Both bacteria are inoculated on the surface of MRSA and incusted at 37 °C in a CO_2 incubator with CO_2 levels of 7.5% for 2 × 24 h which are then identified macroscopically by looking at the shape of the colony on the surface of MRSA as well as microscopically with Gram staining. Furthermore, the two bacteria are dissolved separately into the physiological NaCl until a turbidity of 0.5 McFarland is achieved which means the number of bacteria in it has reached 10^7 CFU/ml. The starter culture of each bacterium is then mixed with a ratio of 1:1. Soy milk that has been made from U.S. soybeans poured as much as 225 ml into each bottle of the trial group that has been labeled the group name. Furthermore, inulin and sucrose are poured into K2 to K5 bottles according to the specified concentration and the entire group is sterilized with autoclaves. Mixed starter cultures that are ready to be mixed into soy milk in K1 to K5 with a ratio of 1:9 or as much as 25 ml and incredied at 37 °C in a CO_2 incubator with CO_2 levels of 7.5% for 1 × 24 h. Once the soyghurt is ready, each group is diluted

repeatedly and calculated the number of colonies by the *Total Plate Count* method and the repetition of calculations duplo. A total of 60 ml from each group of soyghurt is then made dry frozen preparations. Dried frozen soyghurt from each group was taken 0.03 g to mix with a physiological NaCl of 1.5 ml so that multilevel dilution could be done. Furthermore, the number of colonies on the dried frozen soyghurt is recalculated by the same method.

The number of colonies that have been obtained is analyzed using the SPSS statistics program version 25. All data is tested for normality with *Saphiro Wilk* test. Then continued with *the Kruskal Wallis* test and *the Post Hoc Mann Whitney* test so that differences between groups can be assessed for their significance.

3 Results and Discussion

3.1 Reidentification of L. acidophilus

Bacterial reidentification is done macroscopically and microscopically. Macroscopically, the *L. acidophilus* colony in Fig. 1 looks milky white and round with uneven colony boundaries and a slightly convex surface. When compared to the parameters next to it, it can be seen that the colonies formed in this study are larger in diameter which is likely to occur because between inoculation strokes are too tight. Microscopically, in Fig. 2 can be seen the presence of a picture of bacteria in the form of a rod (basil) and purple that gives the meaning that the bacteria are Gram positive bacteria. The picture is in accordance with the parameters in addition to the results of research and literature that mentions that *L. acidophilus* is a rod-shaped gram positive bacteria that is anaerobic and does not form endospores [9].

3.2 Reidentification of B. bifidum

The results of the reidentification *of B. bifidum* ATCC 29521 are macroscopic can be seen in Fig. 3. Colony *B. bifidum* looks colorless and its shape follows the direction of the ose scratch. The colony is strictly limited to the surface of the colony that is slightly raised. The picture of the colony is similar to the parameters of *colony B. bifidum.* According to Li et al., the *B. bifidum* colony looks smooth round, white, and each measures about

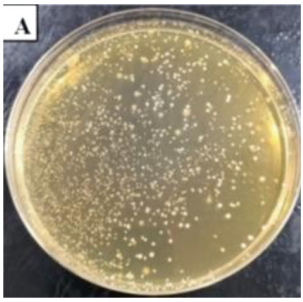

Fig. 1. Colony *L. acidophilus* ATCC 4356 on MRSA media.

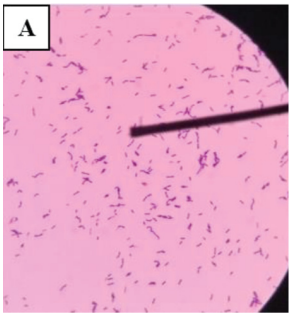

Fig. 2. *L. acidophilus* ATCC 4356 on Gram staining.

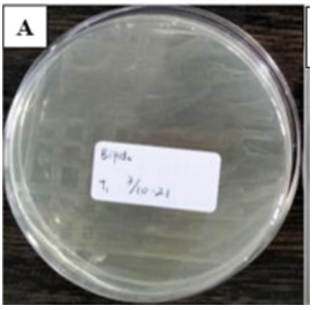

Fig. 3. Colony *B. bifidum* ATCC 29521 on MRSA media.

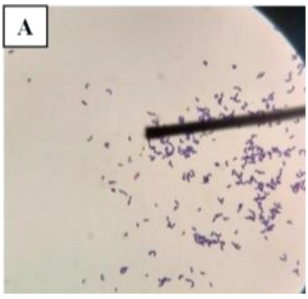

Fig. 4. *B.bifidum* ATCC 29521 on Gram staining.

1–1.5 mm. The difference may be due to differences in culture techniques, where *B. bifidum* in Li et al. was planted with *pour plate* method, while *B. bifidum* in this study was planted with *streak* method [10]. In Fig. 4 can be seen the presence of a picture of dark purple bacteria and a short rod shape with several bacterial formations forming the letter Y, according to the image *of B. bifidum* in the existing literature [11].

Table 1. Comparison of the number of BAL colonies on soyghurt before and after dry freeze

Group trial	TPC results		Difference decrease (CFU/ml)
	Before freeze- drying (CFU/ml)	*After freeze- drying (CFU/ml)*	
KK (−)	0	0	0
K1	$2{,}53 \times 10^{9}$	$3{,}45 \times 10^{4}$	$7{,}30 \times 10^{4}$
K2	$3{,}64 \times 10^{13}$	$2{,}39 \times 10^{11}$	$1{,}52 \times 10^{2}$
K3	$6{,}35 \times 10^{26}$	$1{,}55 \times 10^{23}$	$4{,}09 \times 10^{3}$
K4	$8{,}29 \times 10^{32}$	$2{,}34 \times 10^{29}$	$3{,}54 \times 10^{3}$
K5	$3{,}80 \times 10^{19}$	$6{,}35 \times 10^{14}$	$6{,}00 \times 10^{4}$

Description: KK (−) (soy milk), K1 (soyghurt), K2 (soyghurt + inulin 0.5% + sucrose 10%), K3 (soyghurt + inulin 1% + sucrose 10%), K4 (soyghurt + inulin 3% + sucrose 10%), K5 (soyghurt + inulin 5% + sucrose 10%).

3.3 Number of Bacterial Colonies in Soyghurt Before and After the Dry Frozen Process

Based on the results of the calculation of TPC values before freezing dry in Table 1 below, the number of colonies *L. acidophilus* and *B. bifidum* is lowest in the group that contains only soyghurt without any addition (K1), which is 2.53×10^{9} CFU/ml. The value meets the minimum standard recommended by the SNI for the number of bacterial colonies in yogurt products, which is 10^{6} to 10^{8} CFU/ml. Research conducted by Al-Ansari et al. [8] showed that the number of colonies of *L. acidophilus* in imported soy milk without added sugar was 1.8×10^{7} CFU/ml. This shows that the carbohydrate components contained in US soy milk are actually enough to produce a minimum amount of TPC probiotic drinks, especially if the probiotic drink contains two types of bacteria as in this study whose colony number has a difference of 10^{2} CFU/ml higher than the Al-Ansari et al. [8] which only contains one type of bacteria. However, the number of colonies in K1 decreased to 3.45×10^{4} CFU/ml, indicating that K1 in dry frozen form is not eligible for consumption.

The p value of 0.000 ($p < 0.05$) was obtained for *the Saphiro Wilk* test on TPC results data before the dry freeze process indicating that the distribution of the data is not normal. Then the data analysis continued with the *Kruskal- Wallis* test and obtained a p value of 0.007 ($p < 0.05$) which means there is a meaningful relationship in the addition of sucrose and inulin to the increase in the number of colonies in soyghurt before dry freeze. Mann Whitney's *Post Hoc* test was conducted and there was a significant difference in each group with a value of p 0.025 ($p < 0.05$) (See Table 2).

The highest bacterial growth rate is found in K4 when viewed from Table 1. The number of K4 colonies before the dry freeze process is 8.29×10^{32} CFU/ml, while after the dry freeze process it becomes $2{,}34 \times 10^{29}$ CFU/ml. These results do not fit the hypothesis that the highest number of bacterial colonies should belong to the group with the highest concentrations of sucrose and inulin, K5. The number of colonies in K5, both before and after dry freeze, is lower than K4. According to research conducted by

Table 2. *Mann Whitney's Post Hoc* test results before freezing dry and after freezing dry

Group 1	Group 2	Value P	Interpretation
KK (−)	K1	0,025	Significant
	K2	0,025	Significant
	K3	0,025	Significant
	K4	0,025	Significant
	K5	0,025	Significant
K1	K2	0,025	Significant
	K3	0,025	Significant
	K4	0,025	Significant
	K5	0,025	Significant
K2	K3	0,025	Significant
	K4	0,025	Significant
	K5	0,025	Significant
K3	K4	0,025	Significant
	K5	0,025	Significant
K4	K5	0,025	Significant

Description: KK (−) (soy milk), K1 (soyghurt), K2 (soyghurt + inulin 0.5% + sucrose 10%), K3 (soyghurt + inulin 1% + sucrose 10%), K4 (soyghurt + inulin 3% + sucrose 10%), K5 (soyghurt + inulin 5% + sucrose 10%).

Setiarto et al. (2016), of the three concentrations of inulin tested, namely 0.1%, 0.3%, and 0.5%, the concentration of 0.5% as the highest concentration is the most effective concentration in increasing the growth rate of *L. acidophilus* [7]. Danirmala in his study consisting of four test groups with sucrose concentrations of 0%, 5%, 10%, and 15% respectively stated that the group with a 15% sucrose concentration was the group that had the most number of colonies [8]. This indicates that the higher the concentration of inulin and sucrose given, the higher the number of colonies obtained.

The two studies above only used one additional type of prebiotic, while this study used two additional types of prebiotics, such as research conducted by Tari et al. [3]. Tari et al. [3] in his research on the viability of *S. thermophillus*, *L. bulgaricus*, and *L. plantarum* in dried frozen purple yam yogurt made four test groups that each contained 10% skim milk with different concentrations of sucrose, namely 0%, 2.5%, 5%, and 7.5%. Tari et al. [3] stated that the largest number of bacterial colonies were found in groups containing 5% sucrose and skim milk 10%. In addition, there is a study on the viability of probiotic bacteria *L. casei*, *L. acidophilus*, and *L. plantarum* in soy milk conducted by Nisa et al. [4] using sucrose (5% and 10%) and 10% detrines. It was noted that the number of BAL colonies in the group containing a mixture of 10% sucrose and 10% dextrin was even lower. The equation of the two studies is that the largest number of colonies was not owned by the test group in which sucrose concentrations contained

Table 3. *Mann Whitney's Post Hoc* test results difference in TPC results

Group 1	Group 2	Value P	Interpretation
KK (−)	K1	0,025	Significant
	K2	0,025	Significant
	K3	0,025	Significant
	K4	0,025	Significant
	K5	0,025	Significant
K1	K2	0,025	Significant
	K3	0,025	Significant
	K4	0,025	Significant
	K5	0,025	Significant
K2	K3	0,025	Significant
	K4	0,025	Significant
	K5	0,025	Significant
K3	K4	0,025	Significant
	K5	0,025	Significant
K4	K5	0,025	Significant

Description: KK (−) (soy milk), K1 (soyghurt), K2 (soyghurt + inulin 0.5% + sucrose 10%), K3 (soyghurt + inulin 1% + sucrose 10%), K4 (soyghurt + inulin 3% + sucrose 10%), K5 (soyghurt + inulin 5% + sucrose 10%).

the highest concentrations of sucrose. Both argue that the concentration of sucrose is too high when there has been another prebiotic mixture, it will result in osmotic imbalance inside and outside the bacterial cells so that the condition will cause bacterial cells to lyze and eventually die [3, 4].

The p value in *the Saphiro Wilk* test data difference in TPC results is 0.000 ($p < 0.05$), therefore the data analysis continued with the *Kruskal-Wallis* test and obtained a value of p 0.007 ($p < 0.05$) which means there is a meaningful relationship in the addition of sucrose and inulin to the rate of decrease in the number of colonies *L. acidophilus* and *B. bifidum* after the dry freeze process is carried out. Then continued with *the Post Hoc Mann Whitney* test which turned out to be a significant difference in each group with a value of p 0.025 ($p < 0.05$) (See Table 3).

The number of colonies in the entire test group decreased when viewed from the results of TPC in Table 1. The most decreases were owned by groups containing only soyghurt (K1) and groups containing soyghurt with 10% sucrose and the highest concentration of inulin addition (K6), which was 10^4 CFU/ml. The lowest decrease was in the group containing soyghurt with 10% sucrose and the lowest concentration of inulin addition (K2), which was 10^2 CFU/ml. While the group containing soyghurt with sucrose 10% and inulin 1% (K3) and the group containing soyghurt with sucrose 10% and inulin 3% (K4) experienced a decrease in the number of colonies by 10^3 CFU/ml.

That is, the number of bacterial colonies in this study decreased by 2 to 4 log cycles after the dry freeze process was carried out. The decrease is higher than the decrease in the number of colonies in the Tari et al. study which is only about 1 log cycle [3].

Decrease in the number of colonies in soyghurt after dry freezing occurs because during the freezing of products in the dry freezing process or lyophilization there is damage to the structure and function of bacterial cells due to loss of cell stability. Osmotic shock experienced by bacterial cells due to the loss of water in large quantities during the drying process is suspected as a major factor in bacterial cell damage [9]. Therefore, to minimize the occurrence of these events, it is necessary to add a protective material of bacterial cells called cryoprotectants, one of which is sucrose [3, 4]. Sucrose is one of the cryoprotectants that meets these criteria. As cryoprotectants, sucrose is thought to balance turgor pressure by decreasing water activity, preventing oxidative damage, and making lipid membrane structures and proteins more stable at low water activity as during the drying process [4].

In addition to acting as cryoprotectants, sucrose can also act as a prebiotic. Sucrose is a natural disaccharide that will be broken down by bacterial cells into glucose and fructose as monosaccharides [12]. Additional prebiotics in the form of inulin are needed so that the rate of growth and development of bacteria increases and the number of bacterial colonies will remain high in excess of the standard SNI of probiotic drinks despite the decrease due to the lyophilization process. Inulin is a group of natural polysaccharides of carbohydrates consisting of fructose monosaccharides at each end of the strand contain glucose [7, 13]. Glucose is metabolized by bacteria through the *Embden Meyerhoff Parnas* (EMP) pathway [14].

During anaerobic conditions, glucose is broken down in the glycolysis process into glucose 6-phosphate which is then converted into fructose 6-phosphate. The molecule is broken down into two glyceraldehyde 3-phosphate molecules that will undergo oxidation and phosphorylation into 3-phosphoglycerate. Furthermore 3-phosphoglycerate is processed into phospholiolpiruvat producing two pyruvate molecules. Pyruvate is then reduced to lactate [15]. Lactic acid will be used by bacteria as a source of carbon through metabolic pathways β-oxidase into energy for bacterial growth, reproduction, and activity [13]. In the presence of additional sucrose and inulin, the energy needs of bacteria during the *lag* phase will certainly be met properly so that the stationary phase can last longer. In addition, inulin has a prebiotic or bifidogenic effect that works by increasing growth rate, stimulating development and starting the metabolic action of bacteria in the colon, particularly *Lactobacillus sp. Bifidobacterium sp* [16].

In Table 1 it can also be seen that between the number of colonies before and after the freeze is dry, the difference in the number of colonies in K2, K3, and K4 as the test groups added inulin and sucrose is lower than the K1 that is not added inulin and sucrose. That is, *L. acidophilus* and *B. bifidum* in K1 rely only on the carbohydrate components of soy milk to survive and replicate. Both BAL do not get inulin and sucrose that act as prebiotics and can increase their growth rate [3, 13]. In addition, because it does not get sucrose as a cryoprotector, the possibility of damage to the structure and function of BAL cells during the freezing process of the product will certainly be greater [3]. This is what resulted in a high decrease in the number of colonies in K1.

In addition, it can be seen that the decrease in the number of colonies in K5 is as large as K1 whereas K5 is a test group with the highest concentrations of inulin and sucrose. Most likely the event was caused by three main causes. First, osmotic shock experienced by bacterial cells during the freezing process of the product will result in damage to the structure and function of these cells. Second, too high a concentration of prebiotic mixtures will result in osmotic imbalances inside and outside bacterial cells so that the condition can cause bacterial cells to lyze and eventually die [3, 4]. This is one of the reasons why the 10% sucrose concentration is prefera over the 15% concentration. Third, due to the high concentration of inulin and sucrose, the bacterial population has indeed increased greatly but this is also followed by an increase in toxic waste from the metabolism of these bacteria so that the bacterial mortality rate will be higher than the growth rate [15].

Exposure to oxygen to both bacteria can cause some partially reduced compounds to be produced, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and highly reactive radical hydroxyl (HO). Each of these three compounds can cause oxidative stress so that damage to cellular proteins, lipids, and DNA can occur. In addition, bacterial metabolic processes can produce ho byproducts that are highly toxic through fenton reactions. H_2O_2 reacts with Fe^{2+} to generate HO. HO can damage cell proteins that cause ATP production to decrease so that the energy possessed by bacterial cells to carry out growth becomes greatly reduced. Then HO can cause damage to phosphodiester bonds in the structure of DNA and damage to lipid structures in the plasma membrane so that bacterial cells become lysis [16].

The high population of bacteria makes the decline in nutrient intake quickly occur because of competition between cultures that are high enough that the mortality rate of bacterial cells will be much higher than the growth rate. Sucrose can indeed balance the turgor pressure of the cell but if the concentration is too high, then osmotic imbalance inside and outside the bacterial cell can occur so that bacterial cells lyze and eventually die [17]. Bacteria that have died do not have the ability to form colonies so at the time calculated using *a colony counter*, it appears that the number of bacterial colonies is lower [4].

4 Conclusion

The conclusion of the study was that the number of colonies in the entire trial group decreased after the dry freeze process was carried out. Hanya group of dry frozen soyghurt without any additions that do not meet SNI standards. Statistical tests showed that the addition of inulin and sucrose to dried frozen soyghurt had a significant rate of colony decline after the dry freeze process by providing significantly improved growth of *L. acidophilus* and *B. bifidum* (H1). accepted). The combination of 3% inulin and 10% sucrose provides the highest TPC result, which is 2.34×10^{29} CFU/ml. This suggests that the higher the concentration of inulin and sucrose additions to soyghurt does not guarantee that the number of BAL colonies in them will also become increasingly high (H2 is rejected).

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