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OPTIMAL CONCENTRATION OF PREBIOTIC RAFFINOSE TO INCREASE VIABILITY OF *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus*

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ABSTRACT

Raffinose is an oligosaccharide consisting of three monosaccharide units, namely galactose, glucose, and fructose which are linked by α- (1-6) glycosidic bonds. Raffinose can be obtained mainly from soybeans, green beans, cabbage, broccoli, beets, asparagus, and wheat. Oligosaccharides such as raffinose can be a source of prebiotics because they are not enzymatically hydrolyzed in the stomach and small intestine so they can reach the large intestine. The use of raffinose in Indonesia is still limited due to its relatively high price. This is inversely proportional to Indonesia's abundance of natural sources of raffinose. The purpose of this study was to analyze the optimal concentration of raffinose to increase the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*. The design used was a completely randomized design (CRD) with fixed variables being the concentration of raffinose and the independent variables were the viability of lactic acid bacteria, the pH value, and the total titrated lactic acid levels. *L. acidophilus* was the most sensitive and fast-growing probiotic with high viability in modified MRSB media with the addition of raffinose, followed by *S. thermophilus* and *L. bulgaricus*. The optimal viability of *L. acidophilus* occurred at the addition of 0.3% raffinose with an incubation time of 24 hours. Meanwhile, for *L. bulgaricus* and *S. thermophilus* the optimal viability occurred at the addition of 0.5% raffinose with an incubation time of 24 hours.

1.Introduction

Probiotics are functional food ingredients in the form of live microbes that benefit the health of the host (humans) by increasing the balance of microflora in the gut (Schrezenmeir and de Vrese, 2001, Mohammadi et al., 2013; Saad et al., 2013; Pham and Mohajeri, 2018; Gibson et al., 2017). The microorganisms best known as probiotics are lactic acid bacteria such as Lactobacillus sp., Streptococcus sp., and Bifidobacteria, widely used in yogurt and other dairy products (Schrezenmeir and de Vrese, 2001; Mohammadi et al., 2013; Gibson et al. ., 2017). Probiotics are not permanently in the host's body, so they must be consumed regularly (Saad et al., 2013; Pham and Mohajeri, 2018; Gibson et al., 2017). *L. acidophilus* and *L. bulgaricus* are lactic acid bacteria in the form of rods, gram-positive, non-spore-forming, 0.6-0.9 µm wide and 1.5-6.0 µm long (Gomes and Malcata, 1999; Adamberg et al., 2014). The growth of *L. acidophilus* and *L. bulgaricus* can occur in the temperature range 35°C-45°C, the optimal pH for growth is in the range 5.5-6.0. *L.*

acidophilus and *L. bulgaricus* are homofermentative, producing 0.3-2.0% of a metabolic product in the form of DL-lactic acid in milk (Schrezenmeir and de Vrese, 2001; Mohammadi et al., 2013; Gibson et al., 2017).

The distribution of *L. acidophilus* and *L. bulgaricus* is influenced by several environmental factors including pH, oxygen availability, substrate specifications, and interactions between bacteria (Schrezenmeir and de Vrese, 2001, Mohammadi et al., 2013; Gibson et al., 2017). *L. acidophilus* and *L. bulgaricus* are non-pathogenic and even act as health promoters in the gastrointestinal tract and genital (Gomes and Malcata, 1999; Adamberg et al., 2014). *S. thermophilus* is a lactic acid bacterium in the form of cocci with a diameter of 0.7-0.9 μm that forms chains, including the Gram-positive, non-spore, thermoduric group (Yerlikaya and Ozer, 2014). *S. thermophilus* produces an L (+) lactic acid configuration and does not ferment maltose. *S. thermophilus* and *L. bulgaricus* are lactic acid bacteria used in the fermentation of milk into yogurt (Das et al., 2019; Yerlikaya and Ozer, 2014).

Prebiotics are food ingredients that cannot be digested and can have a positive effect by increasing the growth and activity of a number of probiotic bacterial species in the large intestine, so as to maintain the health of the human digestive tract (Schrezenmeir and de Vrese, 2001; Roberfroid et al., 2010, Pham and Mohajeri, 2018). Food that is not digested by the upper digestive tract is able to reach the colon in an intact state so that it can be used as the main substrate for the growth of probiotic bacteria (Cummings et al., 2001; De Sousa et al., 2011. Ose et al., 2018).

Oligosaccharides such as raffinose can be a prebiotic source because they are not enzymatically hydrolyzed in the stomach and small intestine so they can reach the colon (Cummings et al., 2001; Roberfroid et al., 2010, Palacio et al., 2014, Carlson et al. , 2017). The use of raffinose in Indonesia is still limited because Indonesia still imports raffinose so the price is relatively high. This is inversely proportional to Indonesia's abundance of natural sources of raffinose, so it is necessary to study the effect of the prebiotic raffinose on probiotic viability.

Raffinose ($C_{18}H_{32}0_{16}$) is an oligosaccharide consisting of three monosaccharide units, namely galactose, glucose, and fructose which is linked by α (1-6) glycoside bonds (Amorim et al., 2020b, Teixeira et al., 2012, Martínez-Villaluenga et al., 2005). The chemical structure of raffinose is α-D-galactopyranosil- (1,6) -α-D-glucopyranosil- (1,2) -β-D-fructofuranoside (Adamberg et al., 2018). Raffinose is a non-reducing trisaccharide, dissolves in pyridine, but is difficult to dissolve in alcohol (Teixeira et al., 2012, Martínez-Villaluenga et al., 2005). Raffinose can be obtained mainly from the Leguminaceae (soybeans, green beans) and several other plants such as cabbage, broccoli, beets, asparagus, wheat (Amorim et al., 2020a, Martínez-Villaluenga et al., 2005).

Raffinose can be used as a prebiotic source for digestive tract health (Martínez-Villaluenga et al., 2005). Therefore, it is necessary to research to determine the optimal concentration of raffinose in increasing the viability of *L. acidophilus, L. bulgaricus, S. thermophilus*. In addition, research on the effect of variations in raffinose concentrations on the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* has never been done before. The purpose of this study was to analyze and determine the optimal concentration of raffinose as a carbon source and prebiotic to increase the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*.

2. Materials and methods

2.1. Materials

The research materials were *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus* obtained from IPB Culture Collection (IPBCC), MRSA media (deMan Rogosa and Sharpe Agar, Oxoid), MRSB media (deMan Rogosa and Sharpe Broth, Oxoid), and modified MRSB media which formulated from glucose (Merck), yeast extract (BactoTM), beef extract (Himedia), bacto peptone (Merck), sodium acetate (Sigma), KH_2PO_4 (Merck), $MgSO_4.7H_2O$ (Merck),

$MnSO_4.H_2O$ (Merck), ammonium citrate tribasic (Sigma), Tween® 80 (Merck), agar powder (Merck), raffinose (Sigma).

2.2. Preparation of standard raffinose stock solutions

Standard raffinose stock solution was prepared at a concentration of 5% (w/v). Raffinose is weighed as much as 10 g and dissolved in 200 mL of distilled water, then shaken until homogeneous. Further dilution was carried out to obtain raffinose concentrations of 0.1%, 0.3% and 0.5% (w/v).

2.3. Preparation of *Lactobacillus acidophilus, Lactobacillus bulgaricus, and Streptococcus thermophilus* cultures

Isolates of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* were cultured as much as 2% on MRSB medium (deMan Rogosa and Sharpe Broth, Oxoid) and incubated at 37°C for 24 hours. It was taken as much as 2% to be suspended on modified MRSB media.

2.4. Effect of raffinose concentrations on the viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*

The isolates of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* were each inoculated as much as 2% on MRSB media with the addition of raffinose concentrations of 0%, 0.1%, 0.3%, and 0.5%. Each treatment was inoculated with 3 replications and incubated at 37°C for 0, 6, 12, 18, and 24 hours. The viability of *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* was calculated for the length of incubation time (0, 6, 12, 18, and 24 hours) at 37°C. Furthermore, dilution is made with sterile distilled water according to the incubation time. Incubation for 0 hours was carried out by diluting up to 10^4. Incubation for 6 hours was carried out by diluting up to 10^5. Incubation for 12 hours was carried out by diluting up to 10^6. Incubation for 18 hours was carried out by diluting up to 10^7. Incubation for 24 hours was carried out by diluting up to 10^8. Next, taking 0.1 mL of the sample in two the last dilution was to be inoculated on MRS agar plate with the spread plate method, and then incubated at 37 ° C for 48 hours. The number of colonies of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Streptococcus thermophilus* growing was counted and expressed in Colony Forming Units/ mL.

2.5. Measurement of pH

The value of the degree of acidity (pH) was measured for each treatment with the concentration of raffinose 0%, 0.1%, 0.3% and 0.5% after the incubation period of 0, 6,12, 18, and 24 hours using a pH meter (TOA). The pH meter was calibrated with a phosphate buffer (pH 6.86) and an acetate buffer (pH 4.00).

2.6. Analysis of the total titrated lactic acid levels

Amount of 25 mL of the sample were taken and put into an Erlenmeyer flask containing 100 mL of distilled water. This mixture is added with the PP (phenolphthalein) indicator to test the total lactic acid by 2 to 3 drops. The sample is titrated with 0.1 N NaOH solutions until the color changes to pink, which indicates the end point of the titration has been reached.

2.7. Analysis statistic

The research data were processed statistically using analysis of variance (ANOVA) followed by Duncan's test at the 5% significance level if the results obtained were significantly different between samples using SPSS 17.0 software. The design used was a completely randomized design (CRD) with fixed variables being the concentration of raffinose and the independent variables were the viability of lactic acid bacteria, the pH value and the total titrated lactic acid levels.

3.Results and discussions

3.1. Effect of raffinose concentration on the viability of *Lactobacillus acidophilus*

Table 1 show that *Lactobacillus acidophilus* without the addition of raffinose (control) began to experience a significant increase in viability at 6 and 12 hours incubation. Meanwhile, at the

incubation time of 18 and 24 hours the viability of *Lactobacillus acidophilus* tended to remain. In the 6 and 12 hour incubation period *L. acidophilus* undergoes an exponential phase, where there is significant growth due to rapid cell division, while in the 18 to 24 hour incubation period, the growth has entered a stationary phase, where growth begins to slow down and eventually experiences a number. the same number of bacteria that grow with the number of bacteria that die. This situation is due to the reduced supply of nutrients, the accumulation of toxic metabolites such as bacteriocins and organic acids, and changes in pH that become acidic (Zainuddin et al., 2008). It is undeniable that if the supply of nutrients is reduced, the metabolic activity will also decrease, so that the number of cells will decrease, the accumulation of toxic compounds produced by bacteria which cause death for bacterial cells (Yusriyah & Agustini, 2014).

The addition of 0.1% raffinose showed the same results as the control, except that the growth was more significant, because the number of cells produced was higher than the control. The addition of raffinose by 0.3% and 0.5% had a significant effect on increasing the viability of *L. acidophilus* cells compared to controls and addition of 0.1% raffinose. The growth of L. acidophilus with the addition of rafinosa 0.3% and 0.5% continued to increase even until the incubation time of 24 hours. This happens because raffinose is a prebiotic compound that can be used specifically by *L. acidophilus* as a source of carbon and a source of nutrients in its viability. *L. acidophilus* produces the enzyme glycopyranosidase which is able to hydrolyze the α- (1,6) glycosidic bonds that connect galactose compounds with glucose, and also produces fructofuranosidase enzymes which can hydrolyze β - (1,2) - glycosidic bonds between glucose and fructose (Gänzle & Follador, 2012). This causes *L. acidophilus* to be able to use galactose, glucose and those produced from the hydrolysis of raffinose fructose more effectively as a carbon source for its viability.

Table 1. The results of the viability analysis of *Lactobacillus acidophilus* (CFU/mL) at several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%)

Incubation time (hours)	Viability of *L. acidophilus* (log CFU/mL)			
	Raffinose 0%	Raffinose 0.1%	Raffinose 0.3%	Raffinose 0.5%
0	7.59±0,32^{a}	7.38±0,24^{a}	7.32±0,33^{a}	7.90±0,45^{b}
6	8.28±0,25^{b}	8.01±0,43^{b}	8.32±0,19^{b}	8.27±0,16^{b}
12	8.85±0,18^{c}	9.08±0,15^{c}	8.88±0,12^{c}	8.88±0,23^{c}
18	8.92±0,27^{c}	9.45±0,22^{d}	9.55±0,37^{d}	9.05±0,12^{c}
24	8.90±0,11^{c}	8.77±0,27^{c}	10.10±0,29^{e}	9.61±0,36^{d}

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

The addition of 0.3% raffinose was the most optimal treatment in stimulating the growth of *L. acidophilus* compared to other treatments because it was able to produce the highest increase in viability. Meanwhile, the addition of 0.1% raffinose tended to have a relatively insignificant effect on the increase in the viability of *L. acidophilus* and was comparable to the control treatment. This is due to the addition of raffinose which is still too little in number

Table 2. Total levels of lactic acid titrated *Lactobacillus acidophilus* with several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%) for 24 hours

Incubation time (hours)	Total levels of lactic acid titrated *L. acidophilus* (%)			
	Raffinose 0%	Raffinose 0,10%	Raffinose 0,30%	Raffinose 0,50%
0 jam	0,33±0,03^{a}	0,45±0,01^{c}	0,49±0,03^{c}	0,36±0,02^{b}
6 jam	0,38±0,02^{b}	0,49±0,02^{c}	0,54±0,02^{d}	0,38±0,02^{b}
12 jam	0,45±0,01^{c}	0,52±0,02^{d}	0,68±0,04^{f}	0,46±0,02^{c}
18 jam	0,47±0,03^{c}	0,54±0,01^{d}	0,72±0,02^{f}	0,54±0,01^{d}
24 jam	0,49±0,01^{c}	0,63±0,02^{e}	0,80±0,01^{g}	0,60±0,03^{e}

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

During the 24-hour incubation period for *L. acidophilus* bacteria, there was a significant increase in the total levels of lactic acid ($p < 0.05$), especially in the 0.3% raffinose addition treatment when compared to other treatments (Table 2). The increase in the total levels of lactic acid caused a decrease in the pH value from the range of 7.0 to 4.8 (Figure 1).

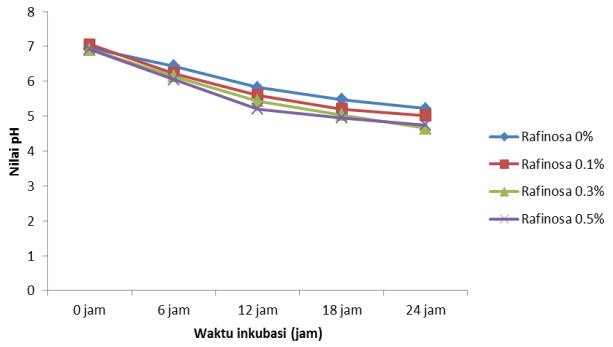

Figure 1. The pH value of *Lactobacillus acidophilus* with several variations in the concentration of raffinose (0%; 0.1%; 0.3%; 0.5%) for 24 hours

Lactic acid compounds as short-chain fatty acids can be used as a carbon source by *Lactobacillus acidophilus* for growth through the β-oxidation metabolic pathway. Apart from lactic acid, several other short-chain fatty acid compounds are also produced including acetic acid, propionate acid, and butyric acid as a result of the metabolism of raffinose by *Lactobacillus acidophilus*. The effects of short-chain fatty acid production and increased viability of probiotics are beneficial, among others, increasing intestinal function, calcium absorption, lipid metabolism, and reducing the risk of colon cancer (Macfarlane & Cummings, 1999).

After entering the stationary phase and death, most of the lactic acid raffinose fermentation will undergo alcohol fermentation by the enzyme lactate dehydrogenase. The higher the total colony count (CFU/ mL) indicates a significant increase in the viability of *Lactobacillus acidophilus*. The increase in lactic acid production during fermentation has a positive correlation with the increase in the viability of *L. acidophilus*. The ability of a carbohydrate source to be fermented is related to the enzymatic hydrolysis system by bacteria (Moreno et al., 2017). Fructofuranosidase is an enzyme that hydrolyzes the fructose group from raffinose at the β- (1,2) end position so that it contributes to fructan metabolism. Some of the factors that affect raffinose fermentability include the structure of the saccharide (level of molecular branching and glycosidic bonds formed), degree of polymerization (length of carbon chains) (Gänzle & Follador, 2012).

Raffinose has a simpler saccharide structure and a low degree of polymerization with a short carbon chain length, making it easier for *L. acidophilus* to ferment it when compared to other prebiotic sources such as inulin, FOS (fructo oligosaccharide), GOS (galacto oligosaccharide), and resistant starch (Gänzle & Follador, 2012). One of the benefits of raffinose as a prebiotic is that it can increase the growth of probiotic bacteria by lowering the intestinal pH to an optimal level, which is influenced by the physicochemical properties of bile acids (Gänzle & Follador, 2012). High concentrations

of raffinose prebiotics can decrease the solubility of bile acids which can reduce their toxicity. The character of raffinose which cannot be digested directly in the human gastrointestinal tract causes raffinose to act as a prebiotic (Date et al., 2014).

3.2. Effect of raffinose concentration on the viability of *Lactobacillus bulgaricus*

Table 3 shows that the higher the concentration of raffinose added, the viability of *Lactobacillus bulgaricus* also increased significantly. The highest increase in the viability of *L. bulgaricus* based on the results of the study was the addition of 0.5% raffinose concentration with an incubation time of 24 hours. Raffinose consists of several types of sugar, namely galactose, glucose, and fructose which can be metabolized by bacteria into lactic acid during the fermentation process. The more raffinose available, the more substrate *L. bulgaricus* can hydrolyze into pyruvic acid which can then be converted into other organic acids such as lactic, propionate, butyric, and acetic acid. The longer the incubation time, the higher the viability of *L. bulgaricus*. The longer incubation time will give *L. bulgaricus* time to ferment raffinose into simple sugars such as glucose, galactose, and fructose to then undergo the process of glycolysis to pyruvic asthma and with the help of the enzyme lactic dehydrogenase, it is converted to lactic acid and growth energy (Yusriyah & Agustini, 2014).

Lactobacillus bulgaricus with control treatment experienced a significant increase in viability at 6 and 12 hours incubation time, but at 18 and 24 hours, incubation time the viability tended to enter the stationary phase. This also happened to the addition of 0.1% raffinose. This shows that *L. bulgaricus* at the incubation period of 6 hours and 12 hours experienced a log phase, namely the bacterial cell phase growing exponentially. During the log phase each cell divides to form two cells, each of which will divide again and so on as long as there are sufficient nutrients in the medium and the environment supports bacterial growth. Whereas at the 18 hours and 24 hour incubation time, the growth has entered a stationary phase towards death, wherein this phase there is no bacterial growth, but most of the bacteria die because the nutrients are depleted. This is due to the addition of the prebiotic raffinose which is too little.

The addition of raffinose by 0.3% and 0.5% had a very significant effect on the viability of *Lactobacillus bulgaricus* cells compared to other treatments. The viability of *L. bulgaricus* on the addition of raffinose 0.3%, and 0.5% continued to increase even at the incubation time of 24 hours. This occurs because the prebiotic raffinose as a carbon source is used optimally by *L. bulgaricus*, so that at the 18 hours and 24 hour incubation time, the viability of *L. bulgaricus* continues to increase and is still in an exponential phase.

During the 24-hour incubation period in the fermentation of raffinose by *L. bulgaricus*, the total lactic acid levels increased significantly ($p < 0.05$) (Table 4). The increase in the total levels of lactic acid caused a significant decrease in the pH value from the range of 7.0 to 5.0 (Figure 2). This significant increase shows that *L. bulgaricus* bacteria are able to produce fructofuranosidase enzymes with high activity so that they can hydrolyze raffinose into glucose, galactose, and fructose monosaccharides which will then be converted into lactic acid products by the enzyme lactic dehydrogenase. The higher the amount of raffinose concentration added to the modified MRS media had an impact on the increase in the total accumulated levels of lactic acid so that a significant decrease in the pH value also occurred with the length of the incubation time.

Table 3. The results of the viability analysis of *Lactobacillus bulgaricus* (CFU/mL) at several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%)

Incubation time (hours)	Viability of *L. bulgaricus* (log CFU/mL)			
	Raffinose 0%	Raffinose 0.1%	Raffinose 0.3%	Raffinose 0.5%
0	6.93±0,14[a]	6.84±0,15[a]	7.38±0,41[b]	7.41±0,25[b]
6	7.49±0,22[b]	7.34±0,22[b]	7.83±0,17[c]	7.76±0,14[c]
12	7.68±0,18[c]	7.75±0,16[c]	8.21±0,26[d]	8.27±0,18[d]
18	7.72±0,10[c]	8.01±0,28[d]	8.69±0,23[e]	8.62±0,28[e]
24	7.89±0,15[c]	8.33±0,13[d]	9.31±0,14[f]	9.61±0,24[f]

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

Table 4. Total levels of lactic acid titrated *Lactobacillus bulgaricus* with several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%) for 24 hours

Incubation time (hours)	Total levels of lactic acid titrated *Lactobacillus bulgaricus* (%)			
	Raffinose 0%	Raffinose 0,10%	Raffinose 0,30%	Raffinose 0,50%
0	0,39±0,02[a]	0,45±0,01[b]	0,54±0,01[c]	0,56±0,02[d]
6	0,43±0,02[b]	0,48±0,01[c]	0,63±0,03[d]	0,63±0,02[d]
12	0,48±0,03[c]	0,50±0,02[c]	0,75±0,02[e]	0,72±0,03[e]
18	0,50±0,01[c]	0,57±0,02[d]	0,82±0,01[f]	0,88±0,03[g]
24	0,54±0,02[c]	0,63±0,01[d]	0,90±0,03[g]	0,99±0,01[h]

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

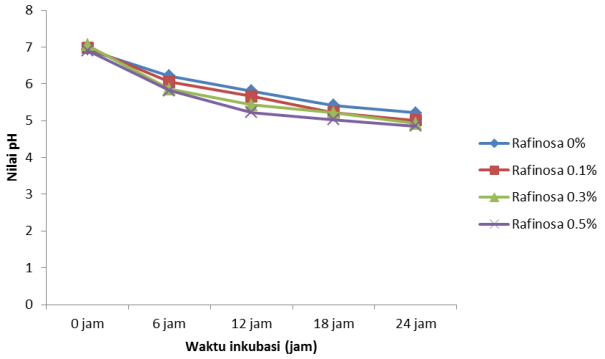

Figure 2. The pH value of *Lactobacillus bulgaricus* with several variations in the concentration of raffinose (0%; 0.1%; 0.3%; 0.5%) for 24 hours

The change in pH value is caused by the formation of organic acids with the main product being lactic acid. The fermentation of carbohydrates by lactic acid bacteria produces organic acids such as lactic and acetate which make the surrounding pH acid so that pathogenic organisms are unable to live (Mohammadi et al., 2013). Thus the change in pH to acid will cause an antimicrobial effect for pathogenic microbes, on the other hand, lactic acid bacteria can still live in an acidic environment with an optimum pH of 3–5. Another mechanism of this antimicrobial property is that lactic acid bacteria also produce antimicrobial peptides such as bacteriocins. Bacteriocins have inhibitory properties because the polypeptides contained can combine with pathogenic bacterial cell membrane proteins so that the cell membrane cannot function properly in terms of selecting molecules in and out of cells (De Sousa et al., 2011).

Lactobacillus acidophilus, Lactobacillus bulgaricus, and *Streptococcus thermophilus* are lactic acid bacteria that are homofermentative, so they can produce lactic acid as the majority product of carbohydrate fermentation and a small portion of acetate via the hexose diphosphate (HDP) pathway or also known as Embden-Meyerhoff, Pathway 2012; Cummings,

et al., 2001). Lactic acid bacteria which are heterofermentative produce lactic acid from carbohydrate fermentation through the hexose monophosphate (HMP) pathway and the pentose phosphate pathway (Gänzle & Follador, 2012). Most of the lactic acid formed during the fermentation process is converted into acetic acid, propionate, and butyric acid through the acetyl-CoA pathway (Gänzle & Follador, 2012; Cummings, et al., 2001).

3.3. Effect of raffinose concentration on the viability of *Streptococcus thermophilus*

Similar to *L. acidophilus* and *L. bulgaricus*, the growth of *Streptococcus thermophilus* was strongly influenced by the concentration of raffinose added to the modified MRSB medium ($p < 0.05$). This is indicated by the increasing growth of the total number of *Streptococcus thermophilus* colonies during the 24-hour incubation period of 1-2 log (CFU / mL) of *Streptococcus thermophilus* colonies in the 0.1%, 0.3%, and 0.5% raffinose treatment (Table 5). Table 5 shows that the higher the raffinose concentration is given, the impact on the increased viability of *Streptococcus thermophilus*. In addition, along with the increasing incubation time, the viability of S. thermophilus also increased for each treatment tested. The highest increase in viability of *S. thermophilus* occurred in the addition of 0.5% raffinose treatment. The increase in total *S. thermophilus* started at the incubation time of 6 hours and then continued to increase even until the incubation time of 24 hours. This shows that at that time it was an exponential phase where the growth of *S. thermophilus* took place optimally due to the availability of sufficient nutrients and environmental conditions that supported its growth.

The decrease in pH during raffinose fermentation by *Streptococcus thermophilus* was influenced by the activity of these bacteria in hydrolyzing raffinose to lactic acid (Figure 3 and Table 6). The production of lactic acid as a result of rhinoceros metabolism caused a significant decrease in the pH value from the range of 7.0 to 4.5. This is related to the increasing number of lactic acid bacteria populations that use raffinose as a carbon source for their growth. The more carbon sources that can be metabolized, the more lactic acid is produced so that the pH will automatically be lower. Streptococcus sp. is responsible for decreasing the initial pH of fermented milk to around 5.0. Then the type of Lactobacillus sp. is responsible for further decreases until the pH reaches 4.5. S. thermophilus also produces the enzyme fructofuranosidase which hydrolyzes raffinose into fructose, glucose, and galactose which are then converted into lactic acid products by the enzyme lactic dehydrogenase (Gänzle & Follador, 2012; Cummings, et al., 2001).

Table 5. The results of the viability analysis of *Streptococcus thermophilus* (CFU/mL) at several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%)

Incubation time (hours)	Viability of *S. thermophilus* (log CFU/mL)			
	Raffinose 0%	Raffinose 0.1%	Raffinose 0.3%	Raffinose 0.5%
0	7.15±0,30^{a}	7.20±0,11^{a}	7.57±0,12^{a}	7.51±0,11^{a}
6	7.64±0,21^{b}	7.70±0,26^{b}	7.80±0,24^{b}	7.66±0,14^{b}
12	7.74±0,18^{b}	7.86±0,14^{b}	8.20±0,18^{c}	8.19±0,20^{c}
18	7.94±0,24^{c}	8.24±0,20^{c}	8.41±0,15^{c}	8.77±0,16^{d}
24	8.37±0,16^{c}	8.32±0,15^{c}	8.84±0,21^{d}	9.39±0,18^{e}

Note: Different letters indicate significantly different values with a real level of 95%, ($\alpha = 5\%$), after a statistical test was carried out with the Duncan test on SPSS 17.0

Table 6. Total levels of lactic acid titrated *Streptococcus thermophilus* with several variations in the concentration of raffinose (0%, 0.1%, 0.3%, 0.5%) for 24 hours

Incubation time (hours)	Total levels of lactic acid titrated *Streptococcus thermophilus* (%)			
	Raffinose 0%	Raffinose 0,10%	Raffinose 0,30%	Raffinose 0,50%
0	0,38±0,02^{a}	0,40±0,02^{a}	0,49±0,02^{d}	0,49±0,01^{d}
6	0,42±0,02^{a}	0,44±0,02^{a}	0,68±0,03^{e}	0,68±0,01^{e}
12	0,56±0,01^{c}	0,48±0,01^{b}	0,72±0,01^{e}	0,87±0,02^{g}
18	0,59±0,01^{c}	0,54±0,01^{b}	0,85±0,02^{f}	0,98±0,02^{h}
24	0,64±0,02^{c}	0,63±0,02^{c}	0,99±0,01^{e}	1,17±0,02^{g}

Note: Different letters indicate significantly different values with a real level of 95%, (α = 5%), after a statistical test was carried out with the Duncan test on SPSS 17.0

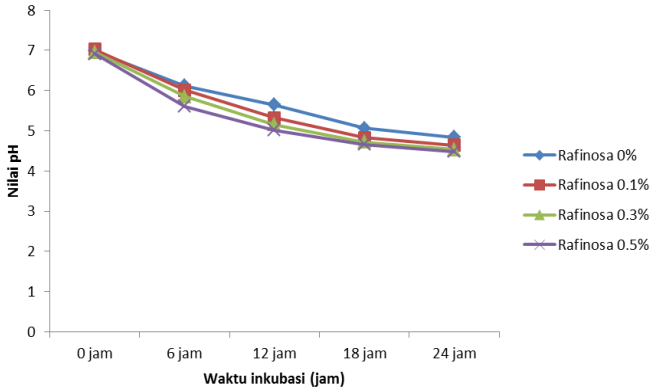

Figure 3. The pH value of *Streptococcus thermophilus* with several variations in the concentration of raffinose (0%; 0.1%; 0.3%; 0.5%) for 24 hours

The benefits of short-chain fatty acids from raffinose metabolism on health, among others, are that these compounds can be absorbed by the intestinal mucosa and play a role in meeting energy needs (Mueller et al., 2016). Lactic acid will make the intestinal conditions acidic so that pathogenic bacteria that cannot stand the acid will die. Acetic acid will be metabolized in muscle, kidney, heart, and brain cells (Rastall, 2013). Propionic acid is a gluconeogenic precursor that suppresses cholesterol synthesis in the liver. *Lactobacillus plantarum* can reduce blood pressure, fibrinogen, and LDL cholesterol and raise HDL cholesterol (De Sousa et al., 2011). Meanwhile, butyric acid is the main energy source for colonocytes, where butyrate is metabolized by the colonic epithelium and functions as a regulator of cell growth and differentiation (Mueller et al., 2016). In addition, butyric acid plays an important role in preventing cancer (Rastall, 2013).

Raffinose fermentation is a source of energy for probiotic bacteria such as Lactobacillus sp. and Bifidobacterium. Along with the energy requirements for growth, the availability of carbohydrates will decrease, so that protein and amino acids will become the dominant metabolic energy sources for probiotic bacteria in the colon (Cummings et al., 2001). This will cause an increase in pathogenic bacteria in the intestine because protein and amino acids are the main nutrient sources for pathogenic bacteria (Adamberg et al., 2014). Therefore, the consumption of raffinose as a carbohydrate compound that is difficult to digest is needed to maintain the balance of the microflora in the intestine. This is what ultimately led to the concept of prebiotics. In the production of short-chain fatty acids, raffinose compounds will be hydrolyzed by the enzyme fructofuranosidase produced by *L. acidophilus, L. plantarum*, and *S. thermophilus* into fructose, glucose, and galactose (Adamberg et al., 2018). Furthermore, fructose, glucose, and galactose undergo a glycolysis process to become pyruvic acid (Teixeira et al., 2012). In the subsequent metabolic process, pyruvic acid will be converted into lactic acid, acetic acid, propionic acid, butyric acid, and CO_2 (Amorim et al., 2020b). *Lactobacillus acidophilus* is the most sensitive lactic acid bacteria and is able to grow quickly and has high viability because it can grow optimally on MRSB media with the addition of 0.3% raffinose. Then followed by *S. thermophilus* and *L. bulgaricus* which were able

to grow optimally and had high viability in the addition of 0.5% raffinose.

4. Conclusions

Raffinose has potential as a prebiotic that provides a carbon source for growth and increases the viability of the probiotics *Lactobacillus acidophilus, Lactobacillus bulgaricus,* and *Streptococcus thermophilus*. *L. acidophilus* was the most sensitive probiotic and was able to grow rapidly and significantly in modified MRSB media with the addition of raffinose, followed by *S. thermophilus* and *L. bulgaricus*. The optimal viability of *L. acidophilus* occurred at the addition of 0.3% raffinose with an incubation time of 24 hours. Meanwhile, for *L. bulgaricus* and *S. thermophilus*, the optimal viability occurred at the addition of 0.5% raffinose with an incubation time of 24 hours. Further research is needed to analyze the content and types of short-chain fatty acids (acetic, propionic, and butyrate) produced during raffinose fermentation by the probiotics *Lactobacillus acidophilus, Lactobacillus bulgaricus*, and *Streptococcus thermophilus*.

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