

Bioprospecting of *Lactobacillus sp.* Starter Culture from Colostrum Breast Milk for Probiotic Milk Production from Soybeans

Mufti Hatur Rahmah(*)¹, Masriany Masriany², Ramlah Ramlah¹,
Isdaryanti Isdaryanti¹

¹ Biology Department, Fakultas Teacher Training and Education,
Universitas Sulawesi Barat;

² Biology Department, Fakultas of Science and Technology, UIN Alauddin Makassar

*Corresponding author: muftihaturrahmah@unsulbar.ac.id

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Abstract

Lactobacillus sp. is a bacterium that naturally exists in various environments, including in breast milk colostrum. This bacterium belongs to a group of lactic acid bacteria that produce lactic acid as the main product of sugar fermentation so that it has probiotic properties that also play a role in the fermentation process. This study aims to produce probiotic milk using *Lactobacillus sp.* isolates from breast milk colostrum with soybean (*Glycine max* (L.) Merril.) as the base material and then test the total lactic acid content, pH value, reducing sugar and the number of lactic acid bacteria. The production of soybean fermented milk is based on the analysis of total lactic acid content by acid-base titration method with 0.1 N NaOH standard solution, pH value by using universal pH meter, reducing sugar test by spectrophotometric method of Nelson-Somogyi and the number of lactic acid bacteria by using Glucose Yeast Pepton Agar (GYPA) medium added with CaCO₃ then calculated by using Standard Plate Count (SPC) method with TPC value (Total Plate Number) is 2.3. 10¹² colonies/ml, 2.5. 10¹² colonies/ml, 4.5. 10¹⁴ colonies/ml, 4.1. 10¹⁴ colonies/ml. From the results of the study it is known that *Lactobacillus sp.* isolates from breast milk colostrum fluid can ferment soybean milk (*Glycine max* (L.) Merril.).

Keywords : Colostrum Breast Milk, *Glycine max*, *Lactobacillus sp.*, Probiotic Milk, Soybean



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INTRODUCTION

Food and nutrition development is a cross-sectoral development effort that is interrelated, aiming to meet the community's food evenly both in quantity and nutritional quality so that one of the basic needs is fulfilled in order to improve the welfare of the community. Food and nutrition issues are closely related to calories and protein. Calorie consumption every day for all levels of society is not as difficult as protein (Nirmagustina & Rani, 2013). One commodity that can be a source of high protein is soybeans (*Glycine max* (L.) Merril.), which has a very good chemical composition for the fulfillment of nutrients, especially protein for the body (Eva & Utami, 2014).

The utilization of agricultural products in the form of soybean commodities has been widely carried out, for example, its use for side dishes, crackers, soy sauce, and soy milk. However, efforts need to be made to diversify the use of soybean commodities with the aim of increasing nutritional value, obtaining more diverse food preparations, obtaining added value economically, improving product quality, or optimizing the quality of biotechnology-based products. Efforts to diversify the processing of soybeans are by making acidic soybean probiotic milk fermented with lactic acid bacteria. (Nirmagustina & Rani, 2013)

Lactic acid bacteria are present in breast milk from the time milk is released and will grow when the growth requirements are appropriate, such as temperature, oxygen, acidity, and the content of nutrients available in the breast milk (Ladokun & Oni, 2014). Most of the content of breast milk is carbohydrates (lactose and oligosaccharides) which are fermentation substrates, where lactic acid bacteria break down lactose into lactic acid (Laksito et al., 2020). Some studies suggest that breast milk colostrum contains lactic acid bacteria, namely the genus *Lactobacillus* such as *Lactobacillus gasseri* and *Lactobacillus fermentum* (Rossi et al., 2018). This relate to the research (Liu et al., 2020) by isolating lactic acid bacteria from breast milk colostrum and giving positive results with the presence of lactic acid bacteria, namely *Lactobacillus sp.* (Liu et al., 2020). Lactic acid bacteria isolated from breast milk colostrum will be fermented into soy bean milk containing carbohydrates to determine its bioprospection in producing probiotic soy bean milk.

The fermentation process carried out by *Lactobacillus sp* will increase the nutritional value of soybeans (Ramadhany, 2015). Increasing the quality of fermented food products has an impact on increasing the acceptance value of these food products by consumers. Thus, this study was conducted for the purpose of producing probiotic soybean milk using lactic acid bacteria isolates that have been isolated by *Lactobacillus sp* derived from breast milk colostrum.

METHOD

This research used an experimental method conducted at the Microbiology Laboratory, Universitas Sulawesi Barat. The tools used in the process of conducting this research include blender, basin, stirring rod, test tube rack, funnel, baker's glass, measuring cup, anaerobic incubator, filter cloth, stove, erlenmeyer flask, petri dish, pipette, bunsen, oven, autoclave, bulbous ose, test tube, spectrophotometry, biuret, analytical balance, and water bath. While the materials used include distilled water, aluminum foil, 70% alcohol, *Lactobacillus sp isolate from breast milk colostrum fluid*, glucose, phenothalein indicator, soybean (*Glycine max* (L.) Merrill.), starter medium, skim milk powder, GYP medium (Glucose Yeast Pepton Agar) + CaCO₃, pH meter, 0.1 N Sodium Hydroxide standard solution, Nelson reagent, Arsenomolybdat reagent. The stages of this research were carried out through several steps as follows:

Preparation

Soybean (*Glycine max* (L.) Merrill.) samples were sorted to obtain good beans. Soy beans were soaked in 500 g of water for 8-10 hours, then immersed in boiling water for 10 minutes. The skin of the soybean was peeled off.

Soy Bean Milk Production

Cleaned soybeans are pureed using a blender by adding water with a ratio of water : soybeans is 8: 1. Then the milk is filtered using a sterile filter. The filtrate was added 5% sugar and brought to a boil while stirring. 5% skim milk was added and stirred for 5 minutes over low heat. The milk was put into a sterile erlenmeyer and cooled at 37°C.

Starter Medium Preparation

Yeast extract 5 g, Lactose 5 g, Glucose 5 g, CaCO₃ 0.2 g were weighed then added to distilled water to a volume of 1000 ml, then dissolved and measured pH 4-5 (Rahmah, 2021). The medium was sterilized using an autoclave at 121°C for 15 minutes.

Fermentation of Soya

Soy bean milk was pasteurized at 80°C for 15 minutes, then cooled to 37°C. After that, soy bean milk was inoculated with 3% starter. Fermentation for 24 hours at 37°C. During the fermentation process, various parameters were analyzed, including: total titratable acid, acidity (pH), cell density of *Lactobacillus sp*, and sugar reduction.

Data Analysis

Analysis of Total Lactic Acid

Lactic acid content in the fermentation media was analyzed by the total titrated acid method using 0.1 N NaOH standard solution. Soybean fermented milk was pipetted as much as 10 ml, then added with 1 - 2 drops of phenothalein indicator and then titrated with 0.1 N NaOH standard solution until a pink color change occurred. Furthermore, the total lactic acid content was calculated with the following formula :

$$\% \text{ Laktat Acid} = \frac{\text{ml NaOH} \times \text{N NaOH} \times 0,09}{\text{ml sample}} \times 100\%$$

pH measurement

pH measurement using a digital pH meter (AMT20 Benchtop -1.00 ~15.00 pH/mV ATC)

Analysis of Total Density of Lactobacillus sp by Standard Plate Count method

Total lactic acid bacteria analysis using GYP4 + CaCO₃ medium.

Composition :

Glucose 1%, Yeast Extract 1%, peptone 1%, mineral solution 1 ml per 200 ml media, agar 1.5%, CaCO₃ 1% were added to distilled water to a volume of 1000 ml.

Composition of mineral solution :

MnSO₄ 200 mg, FeSO₄ 200 mg, MgSO₄ 7H₂O 400 mg, HCl 1 drop, added distilled water to a volume of 100 ml.

Stage :

Soy bean fermented milk as much as 1 ml was diluted into 9 ml of sterile distilled water, then shaken until homogeneous. From this mixture, a 10^{-1} dilution was obtained. Further dilutions were carried out up to the desired dilution level (10^{-13}). 1 ml of each of the last 7 dilutions was put into a petri dish and poured with 10 ml of GYPA + CaCO_3 medium, homogenized by rotating several times. Then allowed to solidify and incubated at 37°C for 1 x 24 hours. Bacterial colonies that are round in shape are surrounded by a clear zone. Calculated by the Standard Plate Count method.

Analysis of Sugar Reduction Content

The reducing sugar content of the fermentation liquid was analyzed by means of Spectrophotometry, *Nelson-Somogyi* method. The determination of reducing sugar includes the following steps :

Standard Curve Determination

Standard glucose solution (0.1g glucose anhydant/100 ml) made from the glucose solution in 6 dilutions so as to obtain a standard glucose solution with concentrations: 0, 10, 20, 30, 40, and 50 ppm/10 ml, prepared 6 clean test tubes, each filled with 2 ml of the standard glucose solution above. One tube was filled with 2 ml of distilled water as a blank, added to each of the above tubes 2 ml of Nelson's reagent, heated all the tubes on a boiling water bath for 10 minutes, took out all the tubes and immediately cooled them together in a glass cup containing cold water so that the temperature of the tubes reached 25°C , after cooling, add 2 ml of Arsenomolybdat reagent, shaken until all Cu_2O precipitates are dissolved again, after all Cu_2O precipitates are completely dissolved, add 2 ml of distilled water, shaken until homogeneous, make a standard curve showing the relationship between glucose concentration and OD optical density.

Determination of Sugar Reduction

Each test tube is filled with a sample solution that has a reducing sugar content of about 10-50 ppm / 50 ml. Taking 2 ml of the clear sample solution pipette into a clean test tube, adding 2 ml of Nelson reagent, and then treated as in the preparation of the standard curve above, the amount of reducing sugar can be determined based on the OD of the sample solution and the standard curve of glucose solution.

Data Collection

Data were obtained from the results of total acid analysis, pH value measurement, lactic acid bacteria count, and determination of sugar reduction.

Data Analysis

The data obtained were analyzed by linear regression using a standard curve.

RESULTS AND DISCUSSION

Results of Total Lactic Acid Level Analysis using acid-base titration method with 0.1 N Sodium Hydroxide solution

The calculation of acid content was carried out on two samples of soybean milk, namely sample a and sample b both at the time before and after fermentation presented in table 1.

Table 1. Total Acid Content of Soybean Milk

Sample	Sample Weight (ml)	Titrant Volume (ml)	Total Acid Content (%)
(a before fermentation)	10	1,2	0,108
(b before fermentation)	10	1,4	0,126
(a' after fermentation)	10	4,2	0,378
(b' after fermentation)	10	4,6	0,414

Results of pH testing of soybean milk samples

The pH test was conducted on soybean milk samples both before fermentation and after fermentation as shown in Table 2.

Table 2. pH of Soybean Milk

Parameters	Soy Milk Sample			
	Before Fermentation		After Fermentation	
	a	b	a'	b'
pH	6,0	5,5	4,5	5,0

Results of Total Density Analysis of Lactobacillus sp using GYP + CaCO₃ Medium with SPC Method

Calculation of Lactic Acid Bacteria was carried out on two soybean milk samples both at the time before fermentation and at the time after fermentation. In this study, it is designed to use soy milk samples twice so that there are two soy milk samples, namely sample a and sample b, each of which will be analyzed with several test parameters. The results of the calculation of Lactic Acid Bacteria at the time before fermentation are listed in Table 3 and Table 4. While the results of the calculation of Lactic Acid Bacteria at the time after fermentation are listed in table 5 and in table 6.

Calculation results of Lactic Acid Bacteria in soy milk at the time before fermentation

Table 3. Soy bean milk a (before fermentation)

Description	Dilution		
	10 ⁻¹¹	10 ⁻¹²	10 ⁻¹³
Colony Count of Lactic Acid Bacteria	23	7	5

Total Plate Count (TPC) of bacteria, colony requirement 30 - 300 colonies

TPC value = $23 \times 1/10^{-11} = 2.3 \cdot 10^{12}$ colonies/ml (does not meet TPC calculation requirements) lactate

Table 4. Soy bean milk b (before fermentation)

Description	Dilution		
	10 ⁻¹¹	10 ⁻¹²	10 ⁻¹³
Colony Count of Lactic Acid Bacteria	25	15	10

Total Plate Count (TPC) of bacteria, colony requirement 30 - 300 colonies
 TPC value = $25 \times 1 / 10^{-11} = 2.5 \cdot 10^{12}$ colonies/ml (does not meet TPC calculation requirements) lactate

Calculation results of Lactic Acid Bacteria in soy milk at the time after fermentation

Table 5. Soy bean milk a' (after fermentation)

Description	Dilution		
	10 ⁻¹¹	10 ⁻¹²	10 ⁻¹³
Colony Count of Lactic Acid Bacteria	120	95	45

Total Plate Count (TPC) of bacteria, colony requirement 30 - 300 colonies
 TPC value = $45 \times 1 / 10^{-11} = 4.5 \cdot 10^{12}$ colonies/ml (eligible for TPC calculation)

Table 6. Soy bean milk b' (after fermentation)

Description	Dilution		
	10 ⁻¹¹	10 ⁻¹²	10 ⁻¹³
Colony Count of Lactic Acid Bacteria	110	70	41

Total Plate Count (TPC) of bacteria, colony requirement 30 - 300 colonies
 TPC value = $41 \times 1 / 10^{-11} = 4.1 \cdot 10^{12}$ colonies/ml (eligible for TPC calculation)

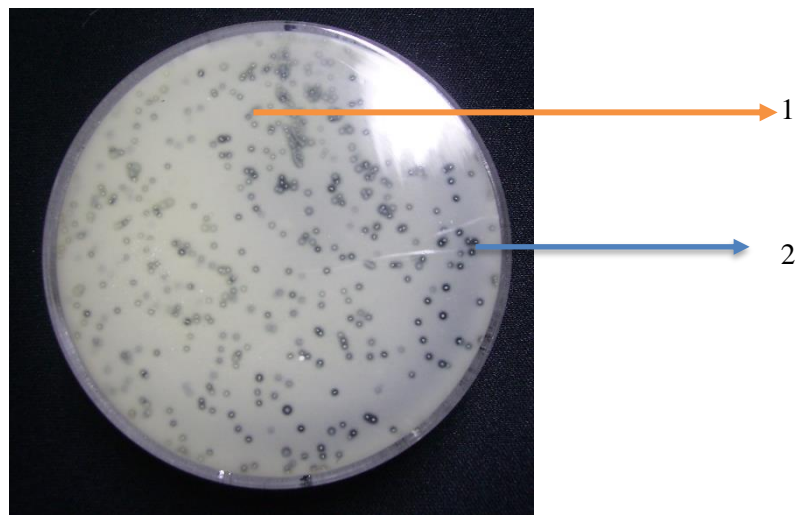


Figure 1. Colonies of *Lactobacillus* sp in fermented soybean milk using GYP A + CaCO₃ medium
 (Note: point 1 = GYP A + CaCO₃ Medium, point 2 = Colonies of Lactic Acid Bacteria (*Lactobacillus* sp))

Results of Sugar Reduction Content Analysis

The results of the analysis of reducing sugar content obtained by determining the Standard Curve of the standard glucose solution are shown in Table 7.

Table 7. Curve determination results of standard sugar solution

Absorbance (Y)	Concentration (level/ppm)
0,155	10
0,285	20
0,412	30
0,589	40
0,710	50

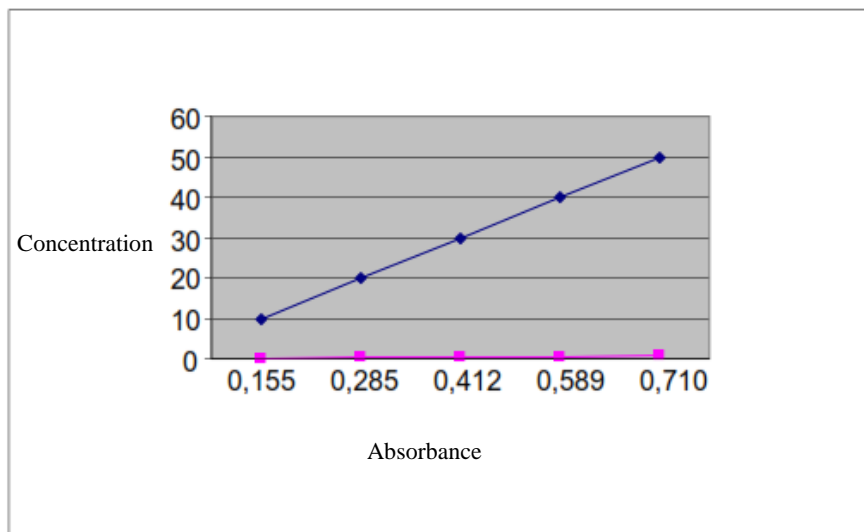


Figure 2. Standard Glucose Solution Curve

$$Y = a + bx$$

$$Y = 0,0060 + 0,0141x$$

The results of the sugar reduction test of the soybean milk sample solution are listed in Table 8.

Table 8. Sugar Reduction Test Result of Soybean Milk

Soy Milk sample name	Cocentration (level/ppm) (Y)	Absorbance (X)
a	30	0,429
b	27,3	0,391
a'	16,3	0,237
b'	13,5	0,197

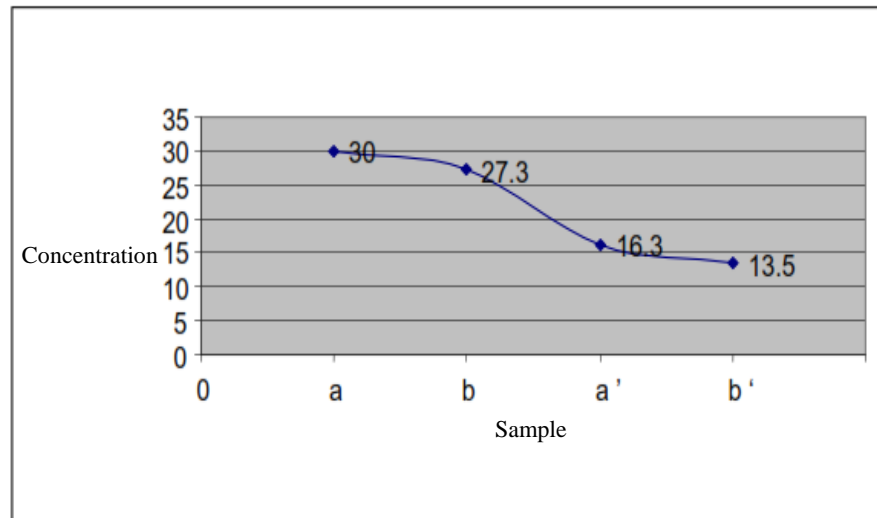


Figure 3. Sugar Reduction Test Solution Curve of Soy bean Milk

Discussion

Soy Bean Milk Production

Soy bean milk was made as much as 700 ml with a ratio of 1: 8 between soybeans and water. From this ratio, soy bean milk is diluted and white in color. Soy bean milk before pasteurization is added with 5% glucose, then 5% skim milk is added with the aim of adding aroma, acidity and covering the smell of langau that can be caused. Then soybean milk was divided into 4, namely milk a and b for the time before fermentation, milk a' and b' for the time after fermentation for 1 x 24 hours. The results of the milk that has been made show that milk made with soy bean base and then added with 5% skim milk and 5% glucose has a smoother, more homogeneous texture and gives a little vegetable aroma this may be due to the basic ingredients of soy bean milk derived from vegetables.

During the fermentation process, lactic acid bacteria perform a metabolic process where lactic acid bacteria can metabolize various monosaccharides into glucose-6-phosphate and then metabolize through the EMP (Embden Meyerhoff Parnas) pathway (Niyibituronsa et al., 2019). Lactic acid bacteria are able to break down the components contained in soybean fermented milk to produce various kinds of metabolites in the form of secondary metabolites and primary metabolites, causing changes in soybean milk, both in terms of appearance, texture, aroma, taste, and chemical composition (Rossi et al., 2018).

Lactic Acid Bacteria Count

The Total Plate Count (TPC) values of lactic acid bacteria in soy bean milk before fermentation were $2.3, 10^{12}$ colonies/ml and $2.5, 10^{12}$ colonies/ml, and the TPC values after fermentation were $4.5, 10^{14}$ colonies/ml and $4.1, 10^{14}$ colonies/ml. From the observation before fermentation, the number of colonies obtained was less than the number of colonies after fermentation, this proves that lactic acid bacteria after

fermentation have a very fast growth and lactic acid bacteria from breast milk colostrum liquid isolates provide quite good results and are able to utilize soybean components as an energy source so that bacterial growth is very fast which can be seen in tables 3, 4, 5, and 6.

Based on the results of research from various parameters before and after fermentation time that soybean milk for the number of lactic acid bacteria has increased the number of colonies so that the total acid content from before fermentation has increased compared to milk at the time after fermentation, which is indicated by the pH value of soybean milk after fermentation has a more acidic pH value than before fermentation time so that the reduced sugar content in soybean milk has a decreasing absorption, this is due to the content of glucose solution in soybean milk has been decomposed into lactic acid (Adriani et al., 2019). The production of soybean fermented milk is analyzed, which includes various parameters, as follows :

Total Acid Content

Based on the calculation of the total amount of lactic acid produced by lactic acid bacteria in soy bean milk a obtained 0.108% and soy bean milk b obtained 0.126%, this indicates that the bacteria in soy bean milk lack metabolic activity so that they cannot decompose chemical compounds into lactic acid. While the calculation of the total amount of lactic acid in soy bean milk a' obtained 0.378% and soy bean milk b' obtained 0.414%, this indicates that the total acid content after fermentation is much greater because lactic acid bacteria in soy bean milk a' and b' perform metabolic activities. Based on the existing requirements that good soybean milk should have a total lactic acid content of not less than 0.5% (Bangun, 2019). The formation of lactic acid by fermentation process is the result of the breakdown of glucose into fructose 1,6 diphosphate and then into lactic acid with the help of enzymes β -galactosidase, glycolase, and lactate dehydrogenase produced by lactic acid bacteria will be converted into lactic acid. (Niyibituronsa et al., 2019).

pH Value

Measurement of the pH value of soybean milk shows that the pH of milk a is 6.0, the pH of milk b is 5.5, the pH of milk a' is 4.5 and the pH of milk b' is 5.0 which indicates that soybean milk has a fairly good acidity level and the fermentation process has occurred. The increase in the number of pH values is influenced by the increasing total acid content which is influenced by the large number of lactic acid bacteria in soybean fermented milk. From these results, the acidity of fermented milk is obtained which is very good because according to (Nakagawa et al., 2015), the optimal growth conditions for lactic acid bacteria are at 37°C, pH 3.0 - 8.0. Lactic acid bacteria have a level of substrate utilization efficiency depending on the type of fermentation. While the type of bacteria produced from the fermentation is homofermentative bacteria that are able to convert 95% of the substrate glucose into lactic acid. (Tiska et al., 2015). Heterofermentative lactic acid bacteria can utilize 90% of the sugar present in the substrate. According to (Jonathan et al., 2022), carbohydrate fermentation by lactic acid

bacteria is carried out through the conversion of carbohydrates to glucose and then glucose is fermented through the hexose diphosphate pathway to produce lactic acid as the main product. These organic acids will cause the pH value of soybean fermented milk to be low.

Sugar Reduction

In the determination of reducing sugar in soybean milk before fermentation and after fermentation, previously the addition of arsenomolybdat reagent was carried out as treatment of standard glucose solution then the sample solution was put into spectrophotometry and obtained absorption for milk a of 0.429 ppm, milk b of 0.391 ppm, milk a' of 0.237 ppm and milk b' of 0.197 ppm. Reduced sugar content before fermentation is greater than milk after fermentation. The occurrence of a decrease in reducing sugar content is due to the fact that in soybean milk after fermentation time there is an increase in the number of lactic acid bacteria which then increases the total acid content so that the glucose solution in fermented soybean milk has decreased absorption because it is reduced to lactic acid. According to [Pujiati & Primiani \(2016\)](#), factors affecting the growth and survival of lactic acid bacteria are very diverse, the chemical composition and nutrient content of the media are very influential. Only lactic acid bacteria are able to break down glucose in milk, also able to ferment carbohydrates, the type of carbohydrates available determines the type of lactic acid bacteria to grow. in milk, the bacteria that grow are those that are able to ferment glucose and lactose.

CONCLUSION

Lactobacillus sp. isolates from breast milk colostrum fluid have prospects in fermenting soy bean milk into probiotic milk. This can be seen from the results of fermentation for 24 hours showing good results because it has met the requirements for the number of colonies of lactic acid bacteria, pH value, total acid content, and reducing sugar test with Total Plate Count (TPC) values of $2.3 \cdot 10^{12}$ colonies/ml, $2.5 \cdot 10^{12}$ colonies/ml, $4.5 \cdot 10^{14}$ colonies/ml, $4.1 \cdot 10^{14}$ colonies/ml.

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