Analysis of the growth and characteristics of microorganisms in an unchilled yoghurt

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3. Summary

In this work, two culture media were tested based on the composition of the nutrient Agar and MacConkey Agar, with different lactose concentration. The main objective was to produce a solid culture medium for didactic use, easy to produce and inexpensive that would give some indications regarding the characteristics of the microorganisms cultured. Medium I consisting of: agar, yeast extract, peptone, NaCl, neutral red at 2% ethanol and 10 g of lactose per liter seems to present less beneficial conditions for the growth of microorganisms than medium II, with the same composition, but with 5 g of lactose per liter. Thus, it was possible to arrive at a medium with good conditions for bacterial growth and less expensive, as it uses less lactose per assay performed.

The food used in the sample was yogurt rich in probiotics, as it is: very important in the human diet, due to its health benefits; sensitive during exposure time on supermarket shelves and at home.

The microscopic study of the morphological characteristics of the microorganisms present in the yogurt allowed the observation of microorganisms in the form of: bacilli, Gram negative and catalase positive, these are compatible with coliform bacteria; cocci, Gram positive, catalase positive, compatible with *Staphilococus aureus*; cocci, Gram positive, catalase negative compatible with *Streptococcus thermophilus*, *Lactococcus lactis* or *Enterococcus*.

Coliform bacteria and *Staphilococus aureus* are not part of the microorganisms present in a yogurt that follows food safety standards. Thus, we confirmed that contamination occurred during the time that the sample was not refrigerated.

4. Introduction

Some microorganisms are used in the production of dairy products (cheese, fermented milk or yoghurts) and are essential for obtaining them and thus have a beneficial effect on human nutrition. Others have the ability to spoil food by altering the organoleptic characteristics. Only a small number of microorganisms, pathogenic microorganisms, have the ability to cause disease. These can cause infections or food poisoning (Trickket, 2001). Foodborne pathogenic microorganisms and their toxins are the cause of numerous cases of foodborne illness worldwide (Lacasse, 1995).

In the industry, food can be contaminated during the processing stage due to malfunction or inadequate cleaning of the equipment, use of cleaning material not suitable for the purpose, insect and rodent infestations, or even due to inadequate storage (Cunha, 2017).

In this work, we tried to produce a culture medium for didactic use, easy to produce and inexpensive, which would give some indications regarding the characteristics of the microorganisms cultivated. Thus, two media were produced based on almost all the constituents of the nutrient agar medium, which is a crop maintenance medium, and some constituents of the MacConkey agar medium, which is a selective and differential medium. The only difference between the two media is the concentration of lactose used, allowing you to select the least expensive one that produces good results.

The sample used was yogurt produced with cow's milk and enriched with bifidobacteria. The choice for this food was made based on: its wide use in human food, due to its health benefits and the sensitivity it has during the time of exposure on supermarket shelves and at home.

The morphological characteristics of the microorganisms present in the yogurt were studied under an optical microscope, performing the Gram stain and the catalase test to the different CFU present in the samples.

Finally, by identifying some colony-forming units (CFUs) present, it was possible to verify if contamination occurred during the time (24 hours) in which the sample was not refrigerated, allowing the students' awareness of this method of preserving food quality.

5. Research Objectives

To produce a culture medium for didactic use, easy to produce and inexpensive, which provides some indications regarding the characteristics of microorganisms cultured using solid yogurt rich in bifidobacteria.

To study under the Optical Microscope the morphological characteristics of the CFU of the microorganisms present in yogurt.

Apply the Gram stain and the catalase test to the different CFU present in the samples.

Show the importance of refrigeration in the preservation of yogurt.

6. Theoretical framework

In the realization of this work, several concepts associated with the preservation of dairy foods, bacteria associated with yogurt production and culture media were addressed.

Food safety, according to the *Codex Alimentarius*, is defined as the guarantee that food will not cause harm to the consumer, as long as it is prepared or ingested in accordance with its intended use, and is linked to food hygiene. According to Regulation (EC) No 852/2004, it is defined as the set of measures and conditions necessary to control hazards and ensure that foodstuffs are fit for human consumption. Failures in food security lead to yield losses (Trickett, 2001).

There are three types of pre-established microbiological criteria: laws and regulations; microbiological specifications and guide values. The first concerns microbiological criteria existing in Community legislation and regulations. The second is related to microbiological criteria defined as a contractual agreement in commercial exchanges, which vary according to operator, contract, country, etc. Finally, the third addresses the microbiological criteria that help industry and other operators to define the acceptable and unacceptable limits of their products. These microbiological criteria serve as a guideline for the identification of situations that require greater attention and monitoring, in order to ensure compliance with Good Manufacturing Practices, or even to highlight the need for corrective measures (Santos *et al,* 2005).

In fact, it is not only food industry professionals who need to be careful when handling food, consumers should always keep the temperature of refrigerators in their homes low. Thus, they limit the growth of bacteria, if they are present in ready-to-eat foods (the World Health Organization – WHO, advises to refrigerate food below 5ºC) and must respect all hygiene and food handling precautions, even the food that is going to be cooked, as well as the spaces and equipment that come into contact with food, such as slaughterhouses (Guerra, 2014).

The destitution of food can be associated with non-compliance with storage rules, such as: grouping foodstuffs by household; food products must be separated from non-food

products; the products should not be placed directly on the floor and should preferably be away from the wall; check the expiry dates of the products to be stored, in order to ensure that products with shorter shelf lives are first in – first out; Immediately discard products that are in broken, dented or broken packaging. These rules must also be followed in the storage of refrigerated and frozen products (Mendonça and Ramalhosa, 2014).

Refrigerated products should be stored in refrigerators with a temperature between 1 °C and 4 °C (Baptista and Linhares, 2005). Refrigerators must be periodically sanitized, because there are microorganisms that multiply at the refrigeration temperature, and gradually colonize it. In addition, it is important that the capacity of the equipment is not exceeded, so that the air circulates inside. The refrigerator door should be opened as few times as possible and for the shortest period of time (Mendonça and Ramalhosa, 2014).

The binomial time/temperature used during food processing and storage is essential to inhibit the multiplication of pathogenic microorganisms (Lacasse, 1995).

The natural beneficial microorganisms in milk include Lactobacilli, Streptococci and Lactococci, which compete with pathogenic bacteria. These are Gram-positive, non-sporulated, catalase-negative, cytochrome-free, anaerobic, but aerotolerant, acid-tolerant and strictly fermentative microorganisms (Holzapfel *et al.,* 2001).

Yogurt is an elaborate food that is produced from full-fat, semi-skimmed or skimmed milk, and results from the action of lactic acid bacteria on milk. These microbial agents are *Streptococcus termophilus*, *Lactobacillus bulgaricus*, or *Lactobacillus acidophilus,* which use lactose to produce lactic acid, responsible for acidity, and other substances that give characteristic aromas and flavors, while the milk coagulates and the consistency of yogurt emerges (Mendonça and Ferreira, 2014). Its activity is higher if the temperature inside the greenhouse is between 30ºC and 45ºC, with 37ºC being its optimal temperature. Above this interval the bacteria do not survive, and below it the speed of the process is greatly reduced (Pacheco, 2022).

The beneficial effects of probiotics began to be studied at the beginning of the twentieth century, with the Russian microbiologist Eli Metchnikoff, who proposed a theory about the prolongation of life based on the daily consumption of fermented milks by the peoples of the Balkans. Metchnikoff's experiments led him to believe that lactic acid bacteria could establish themselves in the intestinal tract and prevent the multiplication and even decrease the number of putrefactive bacteria (Vasiljevic & Shah, 2008).

Research conducted by Tabbers (2011) showed that the administration of *Bifidobacterium breve* in children with constipation increased the frequency of bowel movements, improved stool consistency and reduced abdominal pain. In other studies, it was possible to verify that the administration of probiotics contributed to a better colonization of the intestinal microbiota, reducing constipation, diarrhea, flatulence, abdominal distension and abdominal pain. In addition, probiotics have also been shown to be efficient and safe in maintaining and remitting ulcerative colitis and reducing the incidence and severity of necrotizing enterocolitis (Monteiro and Torres, 2012).

Probiotics are live microorganisms, added in adequate amounts, that confer health benefits on the host. The main microorganisms used as probiotics come from mono- or multicultures represented by bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* and, to a lesser extent, *Enterococcus* and *Streptococcus* (Saad, 2006; Stefe *et al*., 2008; Santos, 2010). The genus *Bifidobacterium* is composed of strict or anaerobic, gram-positive aerobic bacteria that predominate in the large intestine (Paschoal *et al*., 2008). This genus includes thirty species and the most frequently used are*: B. adolescents, B. animalis, B. bifidum, B. breve, B. infantis, B. lactis, B. longum, B. thermophilum* (Paschoal *et al*., 2008). *Lactococcus lactis* is a gram-positive lactic acid-producing bacterium extensively used in the manufacture of dairy and other fermented products. As a probiotic,

it improves immune function, decreasing the occurrence of allergic episodes. It is also related to the reduction of symptoms associated with inflammatory bowel diseases and improves the frequency of bowel movements and the consistency of stool (Song *et al* 2017).

Bacteria of the genus *Lactobacillus* are lactic acid, rod-like, Gram positive, non-sporulated and catalase negative (Zanini, 2012). Studies carried out with *Streptococcus thermophilus* have been found to form large, unraised CFUs with wavy borders, cocci-shaped, Gram-positive and catalase-negative (Zirnstein, 1999). By analyzing the bibliography, we have made sure that *Staphilococus aureus* forms CFU with relief, is Gram positive cocci and catalase positive. Bifidobacteria form bumpy CFUs, are Gram-positive bacilli and catalase-negative. Total coliforms are Gram-negative and catalase-positive bacilli (Silva, 2020).

Staphylococci are saprophytic bacteria of the skin, they can inhabit the digestive system, the urinary tract, the nasal and oral mucosa, and the conjunctiva of the eyes (Ferreira and Sousa, 2000). Food poisoning by *S. aureus* can be caused by various foods with a high moisture content and a high percentage of protein, including: meat and meat products; poultry, eggs, milk and dairy products. In fact, any food subject to manipulation after processing and kept at storage temperatures between 10 and 45ºC before consumption can be involved in food poisoning caused by this bacterium. We must: keep food out of the dangerous temperature zone (between 10 and 65ºC) and cover refrigerated food to avoid cross-contamination (Estevinho, 2014).

The family Enterobacteriaceae consists of rod-shaped, aerobic, or facultative anaerobic Gram-negative bacteria. They are glucose fermenters, producers of catalase and cytochrome oxidase negative (Ferreira and Sousa, 2000). This includes several species that are very important to humans, including many microorganisms from the human and animal intestinal tract, such as *Escherichia coli*, and other soil and water inhabitants that may be involved in pathogenic processes, such as *Salmonella* and *Yersínia* (Cunha, 2017). Enterobacteriaceae include coliforms, whose morphological and physiological characteristics can be evaluated by cultivation in selective culture media. They are negative, non-spore-forming oxidases that ferment lactose in addition to glucose, producing gas and acid when incubated at 36°C for 48 hours in a culture medium with bile salts and detergents, as they contain the enzyme β-D-galactosidase, which breaks the β-1,4 bonds of lactose (Pelczar, *et al*., 1997). Some coliform bacteria such as *Escherichia coli* are part of the normal gut flora of humans and warm-blooded animals, for this reason these bacteria are considered indicators of fecal contamination (Cunha, 2017). Salmonella is another bacterium belonging to the coliform group and can be present in drinking water contaminated with effluents containing fecal matter. This same water can also contaminate foodstuffs if it is used to wash equipment, premises, packaging or is inadvertently used as an ingredient. Contamination of the food chain with salmonella usually occurs cross- (Bernardo, 2014). Finally, *Pseudomonas aeruginosa* is an aerobic bacterium belonging to the group of coliforms responsible for food poisoning, which is characterized by being: Gram negative, rod-shaped and motile. Its mobility is ensured by one or more polar flagella, and it is catalase and oxidase positive. It has been considered as a new indicator of fecal contamination, although this bacterium is not a usual inhabitant of the gastrointestinal tract, it is excreted by about 12% of the adult population (Ferreira and Sousa, 1998).

Samples should preferably be analysed within 24 hours of receipt (Cunha, 2017).

In the spreading technique, the sample (0.1 ml) is distributed over the surface of a solidified culture medium with the help of a spreader and, after incubation, the colonies developed are counted (Cunha, 2017).

The Nutrient agar is a general medium, characterized by its basic nutrient composition, being suitable for a multitude of microbiological applications. The simple constitution of the medium, composed of peptone, beef extract, sodium chloride and agar,

ensures the supply of the nutrients necessary for the replication of microorganisms. Beef extract, rich in water-soluble substances such as carbohydrates and vitamins, along with organic nitrogen compounds and salts, complements peptones, which are the main sources of organic nitrogen. In addition, sodium chloride is incorporated into the medium, with the main function being to maintain osmotic balance, ensuring that the pH of the medium remains stable during bacterial growth. Lastly, agar functions as a solidifying agent. Its inclusion ensures a stable surface for bacterial growth, facilitating the observation of colony morphology and allowing accurate enumeration of organisms. One of its notable advantages is the prolonged survival of crops at room temperature, eliminating the risk of overgrowth, a common problem with more nutrient-rich media. So, due to its simplicity and effectiveness, this formula has been maintained over the years and is still widely recommended for the microbiological examination of various samples, according to standard methods. In addition, it is used for counting organisms in various materials, including water, sewage, dairy, and feces. Delving deeper into its composition, Nutrient agar is devoid of any indicators, selective agents, differential ingredients and enriching substances. The beginning of the production of Nutrient agar dates back to 1917, when it was first published by the American Public Health Association (Tankeshwarin, 2024)

MacConkey Agar medium is a selective and differential culture medium developed by Alfred Theodore MacConkey in 1900. It is widely used in microbiology for the isolation and differentiation of enterobacteria, especially *Escherichia coli* and *Salmonella spp.* This medium is prepared with peptone, lactose, bile salts, crystal violet and phenol red. The bile salts and violet crystal inhibit the growth of gram-positive bacteria, allowing only the growth of gram-negative bacteria. In addition, the presence of lactose as a carbon source allows the identification of bacteria capable of fermenting this sugar. The lactose-fermenting bacteria will produce lactic acid during the fermentation process, which results in a decrease in the pH of the medium. This leads to the change of color of the pH indicator present in the MacConkey medium, from red to yellow, indicating lactose fermentation (Mazura-Reetz, 1979).

The method of counting microorganisms in plates is a method that can be used to count large microbial groups, such as mesophilic aerobes, psychotropics, thermophiles, molds and yeasts, varying the type of medium, temperature and incubation time (Silva *et al.*, 2007). In this method, samples are homogenized, serially diluted in appropriate diluent and are incorporated into a medium or spread over a medium. After incubation, all visible colonies are counted (Jay, 1998). The goal of dilutions is to have a countable number of colonies. The microorganism count at 30ºC (ISO 4833:2003) is a parameter that allows estimating the total microbial population of the food, without identifying the types of microorganisms present. The assessment of the number of microorganisms at 30ºC is used as an indicator of the increase in the level of microbial contamination in food (Cunha, 2017).

Developed by Christian Gram in 1884, Gram staining is a technique for distinguishing bacterial cells, based on the different chemical and structural constitution of the cell wall. This technique allows the morphological characterization of the bacterium and its classification into two groups according to its ability to retain or not the violet color of the violet crystal solution (Madigan *et al.*, 1997). After the smear is fixed by heat, the bacteria are stained by the violet crystal. Then Lugol (a solution with iodine in equilibrium with potassium iodide) is added, which acts as a mordant, and in which the iodine ions (I^-) will precipitate the violet crystal forming a precipitated violet-iodine crystal. Then, with the application of the acetone:alcohol solution, which causes the solubilization of the membranes, the violet-lugol crystal complex is retained by the thick layer of peptidoglycans in the cell wall of Gram-positive ($Gram^+$) bacteria, which turn an intense purple/violet color. In Gram-negative ($Gram^-$) cells, the violet-lugol crystal complex is removed, so after this process these cells become discolored. To visualize Gram-negative bacteria, a secondary basic dye (safranin) is added that acts as a contrastant (Cunha, 2017).

In the Catalase test, hydrogen peroxide is an end product of the oxidative metabolism of carbohydrates, being an extremely toxic compound for microorganisms, which through catalase break it down into water and oxygen. Catalase is an enzyme that is present in most facultative aerobic or anaerobic bacteria and absent in strict anaerobes. The reaction is visualized by the formation of bubbles when emulsifying a culture with hydrogen peroxide (Madigan *et al*., 1997). This test helps to differentiate the genus *Staphylococcus* – catalase-positive, from the *genus Streptococcus* – catalase-negative (Cunha, 2017).

7. Materials and methods

7.1 Biological material

To carry out this work, a yogurt rich in bifidobacteria was used for twenty-four hours at temperatures ranging from 15-22 degrees. The yogurt used in the study sample was natural and within the expiration date, that is, it had no added flavorings or dyes and did not present a risk of being consumed.

After the stock sample (10^{-1}), using 25g of aseptically removed yogurt and 225ml of 0.1% peptone water, samples 10^{-2}, 10^{-3} and 10^{-4} were produced. To do this, we collected 1 ml of the stock sample and added 9 ml of peptone water to obtain the 10^{-2} sample, for the remaining dilutions 1 ml of the previous sample was always used and 9 ml of peptone water was added.

The samples were homogenized for three minutes and spread on the surface with a spreader over the solid medium.

7.2 Methods

7.2.1 Culture Media

The work bench was disinfected and tidied up.

For the analysis of the growth of the microorganisms, two culture media (medium I and medium II) were used, which result from the selection of some constituents of the official formulas of the culture media: Nutrient Agar and MacConkey Agar. Thus, medium I consists of: 1.5 g of yeast extract, 5 g of peptone, 5 g of NaCl, 1.5 ml of neutral red at 2% ethanol, 15 g of agar and 10 g of lactose per liter. Medium II, on the other hand, has the same composition, but the amount of lactose has been reduced by half, i.e. 5 g of lactose per litre.

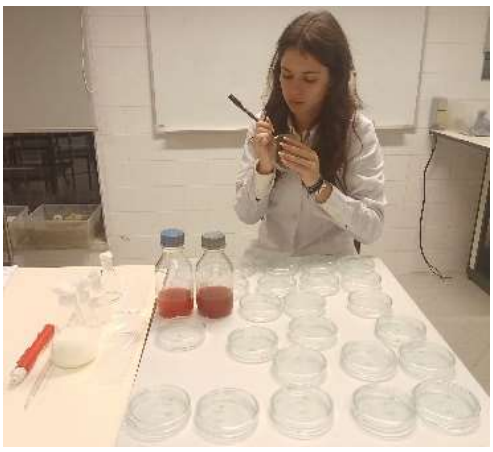

Figure 1 - Identification of Petri dishes.

In the production of the culture media, we started by adding distilled water to two Erlenmeyr flasks and only then weighing/measuring and adding the substances to the distilled water. The dissolution was promoted by agitating the entire contents using a glass rod and heating the solution with the help of a heating plate. Next, the pH was set to 7.0. Finally, the Petri dishes to be used and the two culture media were sterilized using a vertical autoclave for 20 minutes at 121ºC. This was followed by the identification

of the Petri dishes with the name of the material, culture medium, dilution and date of the assay (Figure 1).

In the activity, twenty-four Petri dishes were prepared, twelve filled with medium I and twelve with medium II. The petri dishes were open for as little time as possible and, while open, all the work was done with gloves, without talking and next to the flame of the Bunsen burner. After cooling and solidification of the media in the Petri dishes, 0.1 ml of the sample was inoculated using a spreader in three replicates by sample dilution. Thus, 0.1 ml was seeded with concentration 10^{-4} in plates 10 to 12 for media I and II, then sample 10^{-3} was sown in plates 7 to 9 for the same media, then sample 10^{-2} was spread on plates 4 to 6 in both media and finally 0.1 ml of sample 10^{-1} was added to plates 1 to 3 in medium I and II. 15 minutes and the plates were incubated at 30ºC for 40 hours.

CFU counts were performed at the end of 20h and 40h in all petri dishes. Plates with more than 15 colonies and less than 150 (10^{-2} dilution) were selected.

7.2.2 Microbiological Analyses

In this work, the catalase test was also performed after 20 hours of incubation and the Gram stain and observation under an optical microscope with 1000x magnification at the end of the 40 hours of study.

The catalase reaction is visualized by the formation of bubbles when mixing a culture with hydrogen peroxide (Madigan et al., 1997).

A drop of the reagent (hydrogen peroxide) and a drop of distilled water were placed on a slide. Then, using a loop, the colony under study was inoculated first in the drop of water and second in the drop of hydrogen peroxide. The catalase reaction is positive when oxygen bubbles are produced in the hydrogen peroxide droplet and has been well performed if bubbles are not produced in the distilled water droplet (Cunha, 2017).

Regarding Gram's coloring, he began by placing a small drop of water on a glass slide. With the help of a sterile loop, a small sample of the bacterial culture is taken and the cells are spread in the drop of distilled and sterile water, placed on the slide. After the slide is dry with the help of the lamp, we should cover it with a few drops of the Violet crystal solution, letting it react for 1 minute. With the blade tilted, rinse gently for a few seconds with distilled water. Then cover the blade with Lugol and leave it on for 1 minute. With the blade tilted, rinse gently for a few seconds with distilled water, and then pour a solution of acetone and alcohol in equal parts over the smear, never for a period longer than 30 seconds and until the violet color is not visible. With the blade tilted, rinse for a few seconds with distilled water. Finally, cover the blade with Safranin for 10 seconds and rinse with distilled water. Allow the blade to air dry. At the end, the slide is observed under the microscope in the immersion objective (100x). Bacterial cells that are blue or violet are called Gram positive, while dark pink to red are called Gram negative. (Cunha, 2017).

8. Results

8.1 Culture Media

The number of CFU in the Petri dishes with medium I and II was obtained at the end of 20 and 40 hours of incubation (Figure 2).

Plate	Dilution	Medium I				Middle II			
		20H	20H. - Average	40H	40H - Average	20H	20H - Average	40H	40H - Average
1	10^{-1}	387	336,7	511	500,3	826	859,7	NC	NC
2		421		573		842		NC	
3		202		417		911		NC	
4	**10^{-2}**	**30**	**36,3**	**38**	**48,3**	**73**	**78,7**	**82**	**93,0**
5		**28**		**29**		**106**		**128**	
6		**51**		**78**		**57**		**69**	
7	10^{-3}	12	23,3	28	42,3	63	57	74	65
8		20		44		51		52	
9		38		55		57		69	
10	10^{-4}	0	10,3	6	15	29	20,3	55	32
11		21		22		20		25	
12		10		17		12		16	

Figure 2 - Table with the counts of the number of CFU in all Petri dishes and averages at each dilution and a half at the end of 20H and 40H of growth.

We found that the number of colony-forming units (CFU) increased with the incubation time for all dilutions (Figures 3 and 4) and with the decrease in the amount of lactose in the medium (Figures 4 and 5) and decreased with the increase of dilutions (Figures 5 and 6).

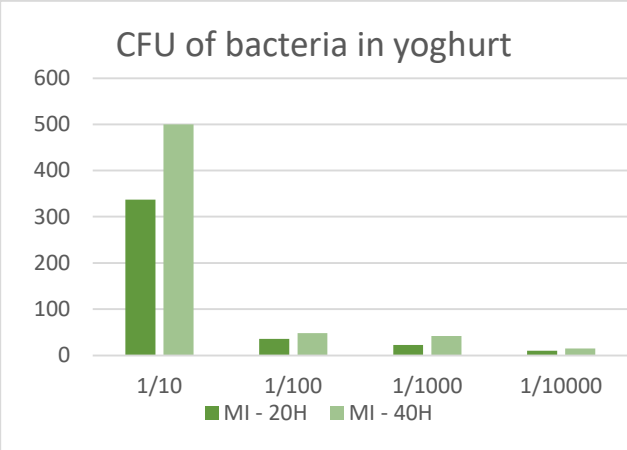

Figure 3 - Comparative graph of the number of CFU between the same medium with different sample dilutions and two different counting times (20H, 40H).

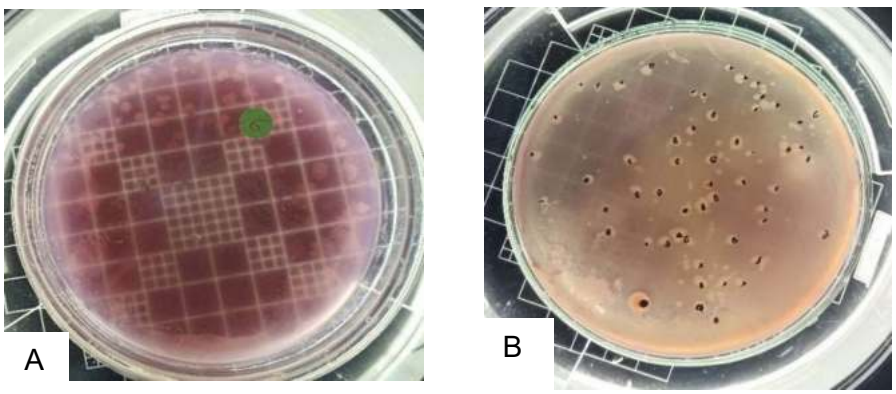

Figure 4 – CFU in the I6-20H Petri dish (A) and UFC in the II4-40H Petri dish (B).

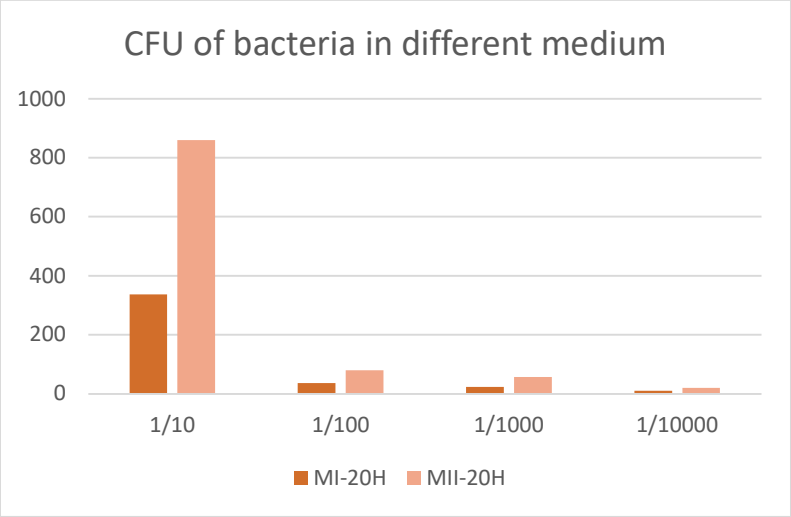

Figure 5 – Comparative graph of the CFU number between different lactose concentrations (medium I and medium II) in the different dilutions of the yogurt sample.

The culture medium transitioned from reddish-pink to yellowish over time (Figure 4).

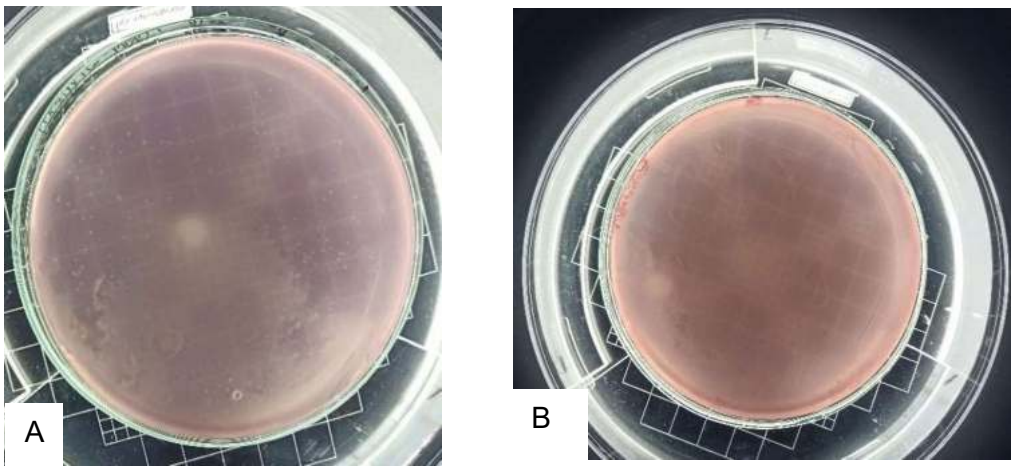

Figure 6 - CFU in the I3-20H Petri Dish (A) and CFU in the I10-20H Petri Dish (B).

The 10^{-2} dilution is the smallest dilution that allows between 15 and 150 CFU to be counted after 20 hours of growth. Thus, we will calculate the number of CFU/ml for medium I and medium II using the average of the number of CFU obtained for this dilution in the two culture media. Using the formula: $n = c/(v \times d)$, where

c = average of the number of colonies in the various replicates

v = inoculum volume

d = dilution factor corresponding to plates with counts

In medium I we have 3.6×10^4 CFU/ml and in medium II we have 7.9×10^4 CFU/ml, that is, we have more CFU/ml in medium II than in medium I for a 10^{-2} dilution.

8.2 Microbiological Analysis

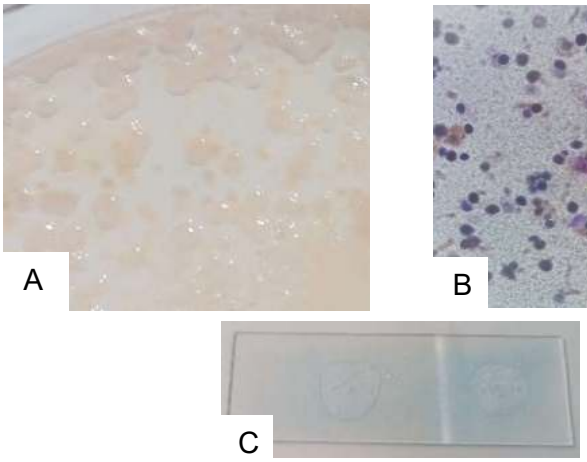

Figure 7 - Typical appearance of CFU (A) in the form of cocci, Gram positive, magnified 1000x (B) and catalase negative (C).

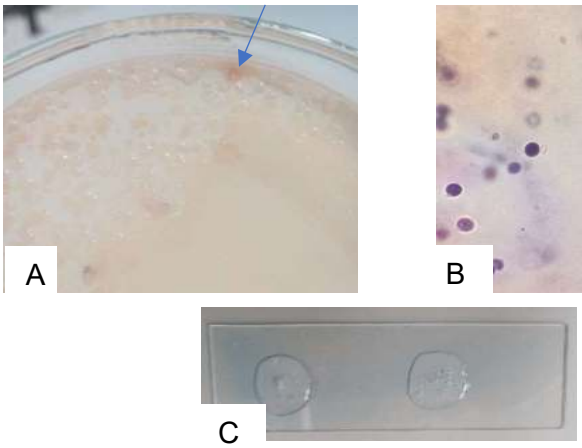

Figure 8 - Typical appearance of CFU (A) in the form of cocci, Gram positive, magnified 1000x (B) and catalase positive (C).

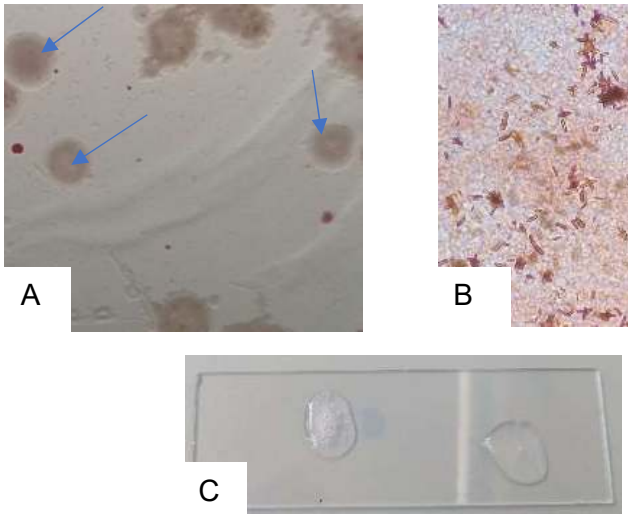

Figure 9 - Typical appearance of CFU (A) in the form of bacilli, Gram negative, magnified 1000x (B) and catalase positive (C).

9. Discussion

Figure 2 shows that the number of colony-forming units (CFU) increased with the incubation time for all dilutions (Figures 3 and 4) and with the decrease in the amount of lactose in the medium (Figures 4 and 5) and decreased with increasing dilutions (Figures 5 and 6). These results are in line with expectations, as the peptone and yeast extract available in the media provide the bacteria with nutrients to grow and multiply and the lactose allows the production of energy for bacterial metabolism (TankeshwarIN, 2024; Mazura-Reetz, 1979). Interestingly, in medium I consisting of: 1.5 g of yeast extract, 5 g of peptone, 5 g of NaCl, 1.5 ml of neutral red at 2% in ethanol, 15 g of agar and 10 g of lactose per liter we obtained lower growth than in medium II, whose composition is: 1.5 g of yeast extract, 5 g of peptone, 5 g of NaCl, 1.5 ml of 2% neutral red in ethanol, 15 g of agar and 5 g of lactose per liter of solution. Thus, the medium with less lactose presents greater growth, probably this nutrient becomes limiting when present in higher values, as happens in medium I. In figure 4, in addition to counting the number of CFU, it was possible to know that the production of lactic acid after 40 hours is higher than after 20 hours. as the middle turned from red to yellowish, indicating that it has become more acidic. Regarding the results obtained in the different dilutions, it is natural that the higher the dilution, the lower the number of CFU possible to develop, given that we have a smaller sample quantity (Cunha, 2017).

In the microbiological analysis, it was possible to distinguish three groups of bacteria (figures 7, 8 and 9). The group depicted in figure 7 has characteristics compatible with *Streptococcus thermophilus, Lactococcus lactis and Enterococcus*, as it was found that they form large, reliefless CFUs with wavy borders, have the shape of coconuts, are Gram positive and catalase negative (Zirnstein, 1999) and are part of the bacteria used in the production of probiotic-rich yogurt (Song *et al* 2017; Saad, 2006; Stefe *et al*., 2008; Santos, 2010). The group depicted in figure 8 has typical characteristics of *Staphilococus aureus,* as it forms a fuse with relief, are Gram positive cocci and catalase positive (Silva, 2020). Finally, group 9 bacteria are compatible with coliforms, as they are Gram-negative and catalase-positive bacilli (Silva, 2020). Since *Staphilococus aureus* and coliform bacteria are not part of the microorganisms present in a yogurt that follows food safety standards (Estevinho, 2014; Cunha, 2017), confirming that contamination occurred during the time the sample was not refrigerated.

10. Conclusions

It can be concluded that the number of colony-forming units (CFU) increases with the incubation time for all dilutions and with the decrease of lactose present in the medium and decreased with the increase of dilutions. The media obtained (medium I and medium II) provide information on lactic acid production, as the medium changes from red to yellowish over the time of growth of the CFU, indicating that it has become more acidic. These results allowed us to achieve one of the objectives of the work, the production of a medium for didactic use, easy to produce, with good growth, which provides some indications regarding the characteristics of the microorganisms cultured and less expensive, as it uses less amount of lactose per experimental assay.

A new assay can be tested with only two dilutions, since the 10^{-2} dilution is the smallest that allows between 15 and 150 CFU to be counted after 20 hours of growth and to add a third medium that has the same composition as medium I and II, but that has 2.5g of lactose per liter of solution. Thus, it would be possible to analyze the behavior of bacterial growth in a medium with less lactose than those already analyzed (medium I and medium II).

It was possible to distinguish three groups of bacteria (cocci, gram positive and catalase negative; cocci, gram positive and catalase positive and bacilli, gram negative and catalase positive). Bacteria that present as cocci, gram-positive, and catalase-negative are compatible with *Streptococcus thermophilus, Lactococcus lactis, and Enterococcus*. The group consisting of cocci-shaped, gram-positive and catalase-positive bacteria corresponds to *Staphilococus aureus.* Finally, bacteria in the form of bacilli, Gram-negative and catalase-positive correspond to coliforms. Thus, it was possible to accomplish two more objectives of the work, since it was possible to study the morphological characteristics of the CFU of the microorganisms present in the yogurt and to apply the Gram stain and the catalase test to the different CFU present in the samples, contributing to their classification. *Staphilococus aureus* and coliform bacteria are only part of contaminated yogurts, demonstrating the importance of keeping yogurts at temperatures below 5ºC. Thus, the last objective of the work was achieved: to show the importance of refrigeration in the preservation of yogurt.

Chromogenic media are the simplest, fastest and most efficient way for incubation, differentiation and selection of microorganisms **(**Engelkirk, P. *et al,* 2011). Thus, in a future trial, these means can be applied in the study of coliforms to identify the species that contaminate the food. *E. coli* would be identified using the TBX culture medium, as it has colors in shades of blue/green (Cunha, 2017). *Salmonella*, on the other hand, would be identified by the IBISA medium, as its CFUs stain green on yellow agar (Biomerieu).

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