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Tolerance of probiotic microorganisms to sodium chloride

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ABSTRACT: Since the increasing consumption of packaged and ready-to-eat foods leads to excessive sodium intake, this study aims to investigate the effects of various sodium concentrations on probiotic microorganisms, which are key members of the human microbiota. A total of 5 probiotic microorganisms (3 different *Lactobacillus plantarum* (*L. plantarum*) strains, *Lactobacillus kefiri* (*L. kefiri*), *Lactobacillus acidophilus* (*L. acidophilus*)) were used in the present study. In order to find out the impact of varying concentrations of sodium chloride (1%, 2%, 6%, and 10%) on probiotic microorganisms viability, the formed colonies on de Man Ragosa Sharp (MRS) agar were assessed by plate count. Bacterial growth curves were prepared according to the visible colonies on the plates. It was found that, *Lactobacillus acidophilus* ATCC 4356 demonstrated significantly higher tolerance to sodium, particularly at the concentrations of 6% and 10%. In contrast, all of the *L. plantarum* strains exhibited limited tolerance at higher concentrations. The survival rate of *L. acidophilus* and *L. kefiri* at high concentrations of sodium for extend time is promising in order to develop new probiotic preparations in the pharmaceutical industry.

KEYWORDS: Probiotics; Sodium chloride; Lactobacillus; Packaged products

1. INTRODUCTION

The term "probiotic" means "for life" (Latin, "*pro*" and "*bios*") and is used to describe bacteria providing beneficial effects not just for humans but also for animals. The importance of probiotics was first highlighted in 2001 when their definition was formally established as "live microorganisms that, when administered in sufficient amounts, confer numerous health benefits to the host" [1]. This definition was further refined in 2014 by a panel of scientists from the International Scientific Association for Probiotics and Prebiotics (ISAPP), emphasizing that it "encompasses a wide range of microbes and applications while capturing the essence of probiotics" [2]. The Russian scientist Elie Metchnikoff was the first scientist to hypothesize that certain bacteria could have beneficial effects on living organisms, thus initiating scientific research on this subject. In the early 19th century, Metchnikoff attributed the longevity of rural Bulgarians to their frequent consumption of fermented dairy products and suggested that specific bacterial species could exert positive effects on digestive tract [3]. Moreover, probiotic microorganisms show beneficial effects by improving the natural microflora of the host. Studies have shown that probiotic microorganisms provide improvement in metabolic parameters because of their direct enzymatic and metabolic effects such as regulation of intestinal microbiota and modulation of immune system [4], anti-toxigenic [5], antimicrobial [6], anti-inflammatory [7] and antioxidant [8] effects.

Lactic acid bacteria (LAB) are gram-positive, non-spore-forming, non-pathogenic microorganisms that have been important with their producing metabolites, enhancing food safety, flavour, and preservation. Also, LAB have been identified as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration (USFDA), which makes them great choices for use as probiotics in food manufacturing. They are widely used in traditional food fermentation progress such as yogurt, boza, kefir and cheese [9]. Probiotic microorganisms have a wide range of uses from fermented food products to pharmaceutical preparations. Probiotic products may contain one or more selected microbial strains including *Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus, Enterococcus, Bacillus,* and some yeast strains of the genus *Saccharomyces*. Among probiotic bacteria, especially *Lactobacillus* and *Bifidobacterium* species are the most preferred strains in the development of probiotic preparations with their beneficial effects in the microbiota of healthy people or in the content of dairy products [10].

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Salt, also known as sodium chloride (NaCl), is an essential component of the human diet. As a seasoning used in daily life, it plays a crucial role in enhancing the flavour of food, regulating food quality, and supporting vital bodily functions [11]. However, in recent years, people have been consuming excessive amounts of salt, averaging 10.8 g/day, which is more than the double of the maximum intake (5 g/day) recommended by the World Health Organization (WHO) [12].

Due to the important role of salt in the human diet, the importance of investigating the effects of sodium consumption on human health has increased. The global increase in the consumption of packaged foods has led to excessive sodium intake. Consequently, the prevalence of various diseases has also increased. High-salt diets have been shown to alter the intestinal microbiome by reducing beneficial bacteria and causing dysbiosis. These changes in the intestinal microbiome are thought to play a role in the pathogenesis of Irritable Bowel Disease (IBD), with high salt consumption exacerbating the symptoms of this condition. Furthermore, excessive salt intake has been reported to increase the risk of various cardiovascular diseases, such as hypertension [13,14].

The aim of the present study was to evaluate the tolerance of selected probiotic microorganisms to different concentrations of sodium chloride (1%, 2%, 6% and 10%) and to investigate how sodium influences the growth of these probiotic microorganisms.

2. RESULTS

2.1 Identification of Isolated Strains

The probiotic strains isolated from different sources were subjected to a series of tests to identificate their characteristics (Table 1).

Table 1. Identification tests of the isolated probiotic strains.

Strains	Gram Staining	Catalase Test	Arginine Hydrolysis Test	Voges-Proskauer Test	Gas Production from Glucose	Temperature	Source
***L. plantarum* 1c-2.2.A**	Gram (+)	-	+	-	-	37°C	Homemade Whey
***L. plantarum* 2a-2A**	Gram (+)	-	+	-	-	37°C	Pharmaceutical Product
***L. kefiri* 1a-A1**	Gram (+)	-	-	-	-	37°C	Homemade Kefir
***L. plantarum* ATCC 14917**	Gram (+)	-	+	-	-	37°C	ATCC®
***L. acidophilus* ATCC 4356**	Gram (+)	-	+	-	-	37°C	ATCC®

2.2 Determination of the Impact of Sodium Chloride on Probiotic Microorganisms Viability

L. acidophilus ATCC 4356 showed tolerance to sodium chloride, especially at the concentrations of 6% and 10% when compared to other strains (Figure 1).

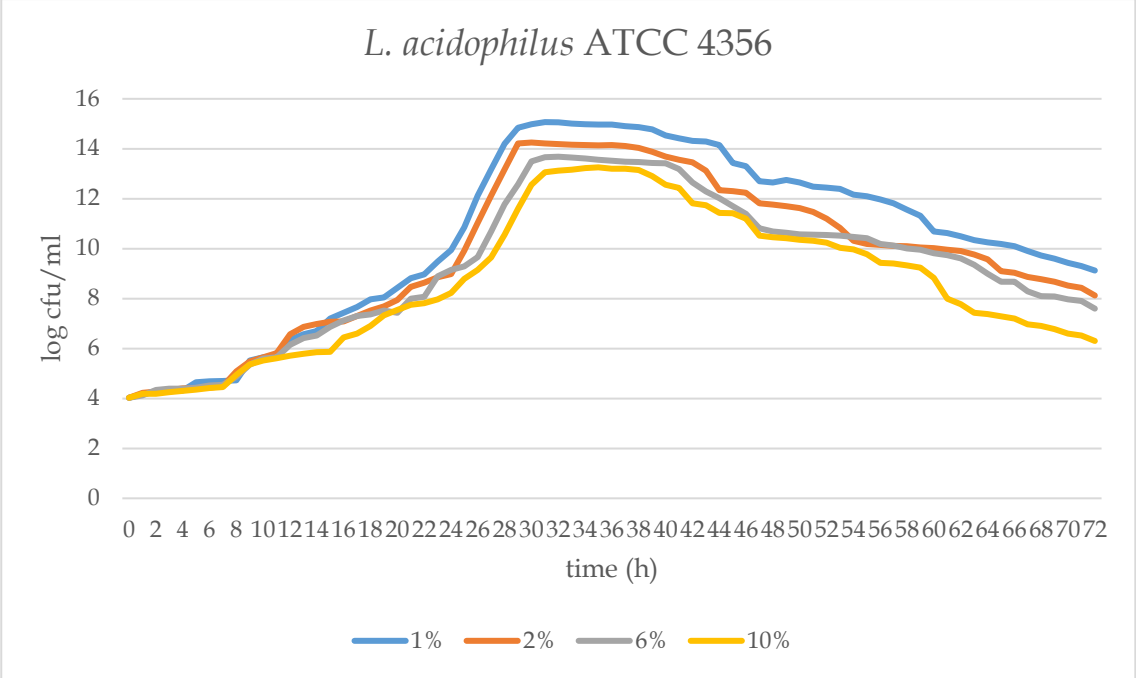

Figure 1. Growth curve of *L. acidophilus* ATCC 4356 at the different concentrations of NaCl.

L. kefiri 1a-A1 also showed good tolerance to NaCl, but it was weaker when compared to that of *L. acidophilus* ATCC 4356 (Figure 2).

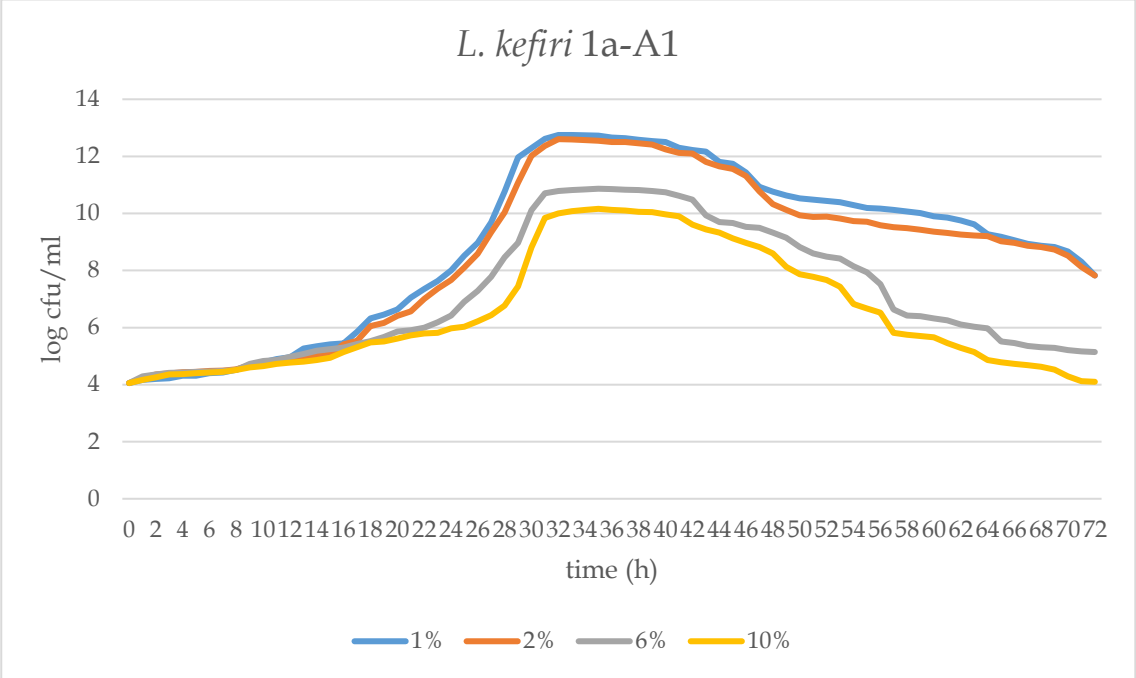

Figure 2. Growth curve of *L. kefiri* 1a-A1 at the different concentrations of NaCl.

All *L. plantarum* strains were unable to reach logarithmic phase at the 6% and 10% NaCl concentrations. However, *L. plantarum* strains survived for 72 hours. In the mean time, they successfully reached logarithmic phase and survived for 72 hours at the 1% and 2% NaCl concentrations (Figure 3, 4, 5).

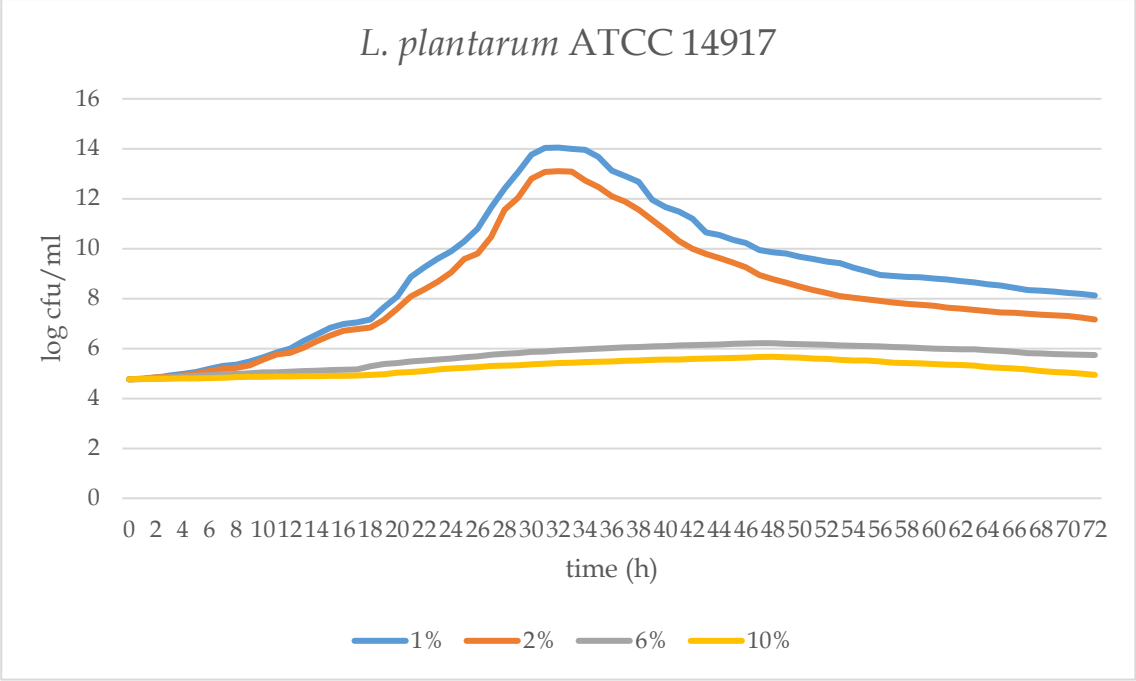

Figure 3. Growth curve of *L. plantarum* ATCC 14917 at the different concentrations of NaCl.

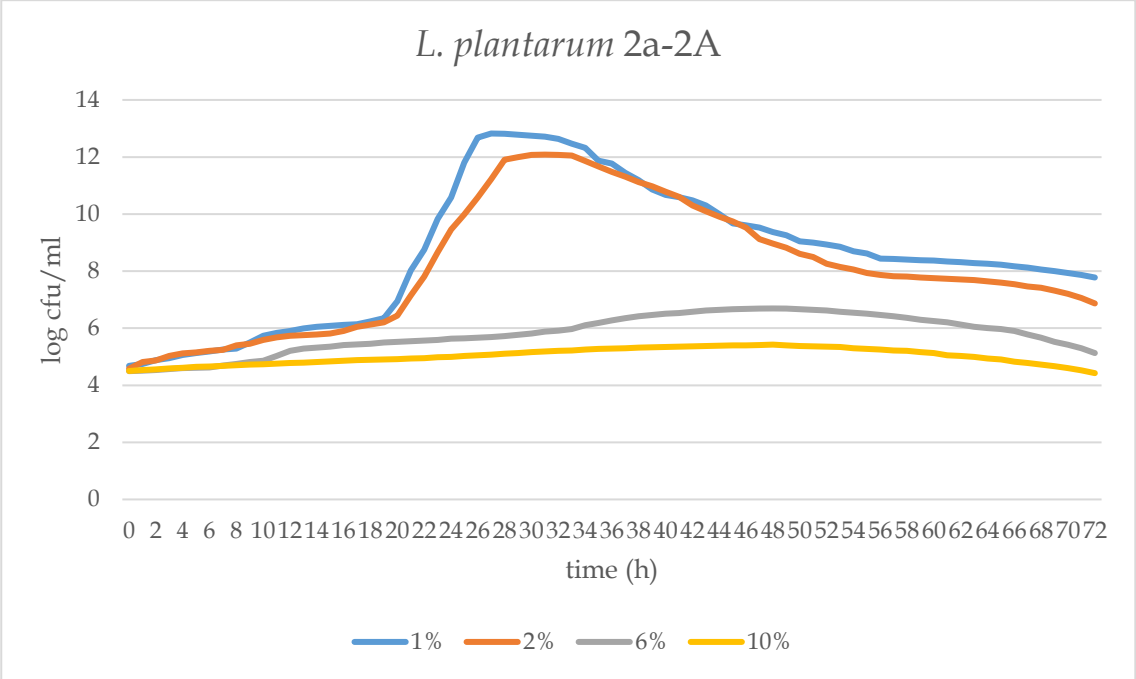

Figure 4. Growth curve of *L. plantarum* 2a-2A at the different concentrations of NaCl.

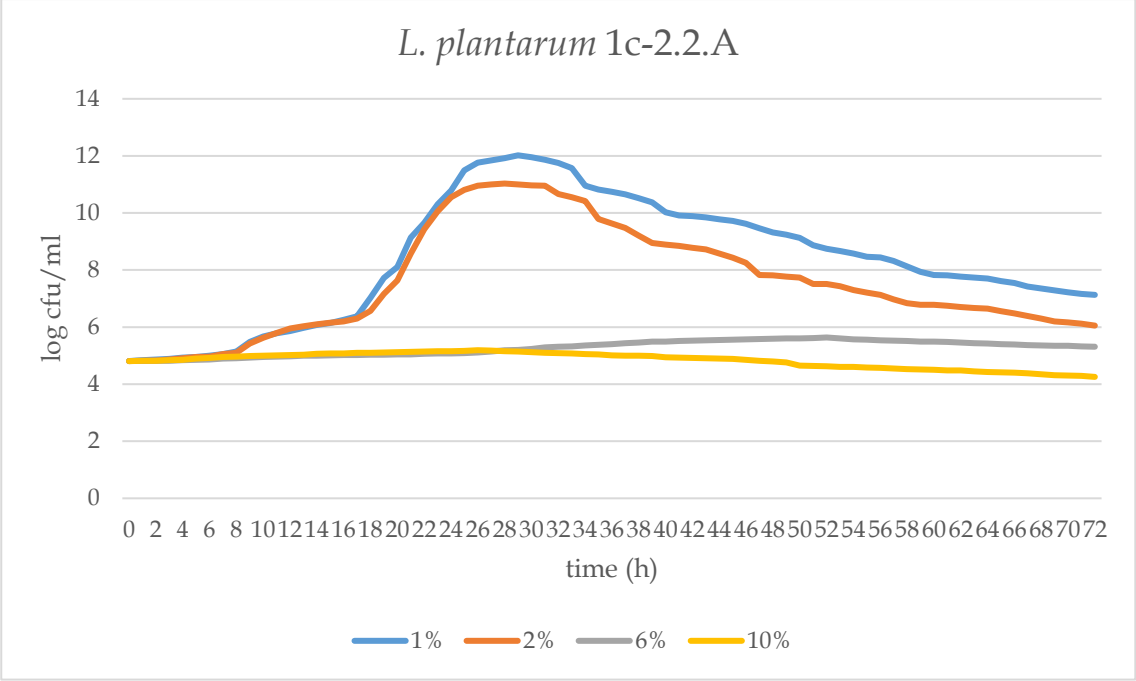

Figure 5. Growth curve of *L. plantarum* 1c-2.2.A at the different concentrations of NaCl.

3. DISCUSSION

The usage of probiotics, known as living microorganisms which have beneficial effects on human health when given in sufficient quantities, has recently increased worldwide. Similarly, the number of probiotic-containing foods, food supplements and medicines widely used all over the world has grown with studies reporting probiotic properties of microorganism species. Products containing probiotic microorganisms are also supplemented with various enzymes, vitamins and flavor components that have beneficial effects on the health of the host which are widespread used globally. Tablets, sachets, syrups and capsules containing probiotics are not used as substitutes in the treatment of diseases, but are marketed as health-supporting products [15].

In the present study, the tolerance of *L. kefiri*, various *L. plantarum* isolated from homemade whey and pharmaceutical product together with standard *L. plantarum* and *L. acidophilus* strains to sodium chloride was detected. There have been studies related with this topic in the literature. However, these studies were done with probiotic microorganisms different from the ones that took place in our study [16-21].

Nath et al. (2020) and Mulaw et al. (2019) measured the resistance of *L. plantarum* strains isolated from various sources to sodium chloride and observed tolerance at a maximum concentration of 6.5% NaCl [16,17].

Rocha-Ramírez et al. (2021) have investigated the effect of varying concentrations of salt on different *Lactobacillus* strains (*L. rhamnosus, L. helveticus* and *L. casei*) growth, the results of these investigators have shown that these strains were tolerant to sodium chloride between 1–4% NaCl concentrations [18].

In another study, El-Sayed et al. (2022) measured the tolerance of *L. plantarum* and *L. acidophilus* strains isolated from breast milk to NaCl and found that these microorganisms could grow at a maximum concentration of 7% NaCl [19].

Also, Halder et al. (2017) investigated physiological factors effecting different probiotics from various isolates and found that *L. plantarum* and *L. acidophilus* isolates were "weakly resistant" at the concentration of 6,5% NaCl while "resistant" at the concenteration of 4% NaCl [20].

In their study, Betancur et al. (2020) have found that *L. plantarum* and *L. acidophilus* isolates obtained from pig feces were resistant to NaCl between the concentrations of 2-10% [21].

We have concluded that standard and the other *L. plantarum* strains showed similar resistance to sodium chloride. Interestingly, the standard *L. acidophilus* strain in the present study was found to be more resistant to 10% NaCl concentration than the other strains. Additionally, *L. kefiri*, another microorganism isolated from homemade kefir, showed a similar tolerance with *L. acidophilus* to NaCl.

In our opinion, the difference of tolerance found in probiotic microorganisms to various levels of NaCl (1%, 2%, 6%, and 10%) is due to the their structural differences. As parallel to our hypothesis, Wu et al. (2016)

reported that the expression levels of the opuA, opuB, opuC, and qacT genes in *Lactobacillus plantarum* FS5-5 increased in parallel with the NaCl concentration, which was associated with an enhanced resistance level of the bacterium [22].

4. CONCLUSION

The ability of *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus kefiri* 1a-A1 to survive for extended periods under high sodium concentrations is promising. We believe that these strains could be utilized in addressing microbiome imbalances in patient groups with unstable blood sodium levels. Furthermore, we anticipate that the determination of NaCl tolerance profile of various standard and isolated probiotic microorganisms will be a guide in the development of new probiotic preparations as pharmaceutical formulations targeted the patient groups stated above.

5. MATERIALS AND METHODS

5.1 Probiotic Strains

In our study 2 standard and 3 probiotic strains from various products were used. Standard strains were *Lactobacillus plantarum* ATCC 14917 and *Lactobacillus acidophilus* ATCC 4356. Both strains were obtained from American Type Culture Collection®. Additionally, *Lactobacillus plantarum* 1c-2.2.A was isolated from homemade whey, *Lactobacillus plantarum* 2a-2A from pharmaceutical product and also *Lactobacillus kefiri* 1a-A1 from homemade kefir (Table 2).

Table 2. Probiotic strains and the sources they have been isolated from.

Probiotic Strains	Source
Lactobacillus plantarum ATCC 14917	American Type Culture Collection®
Lactobacillus plantarum 2a-2A	Pharmaceutical product
Lactobacillus plantarum 1c-2.2.A	Homemade Whey
Lactobacillus acidophilus ATCC 4356	American Type Culture Collection®
Lactobacillus kefiri 1a-A1	Homemade Kefir

5.2 Gram Staining

For the determination of Gram staining properties of the isolates; approximately a pinhead size was taken from the colonies formed on the solid medium with inoculation loop, suspended on a slide dripped with physiological saline (FTS) under aseptic conditions and spread on the slide surface. Firstly, the slide was dried on room temperature and then fixed by passing through flame 3 times. After that, crystal violet stain was dropped on the preparation, waited for 2 minutes and washed with water to remove the crystal violet. Lugol's solution was dropped onto the preparation, kept for 2 minutes and washed with water to remove the excess Lugol's solution. Then 96% ethyl alcohol solution was poured on the preparation and discoloured for 30 seconds. After that it was washed with water and safranin was dropped as a contrast stain and after waiting a minute, the preparation was washed again with water and left to dry. Finally, immersion oil was dropped on the preparation and examined under oil immersion objective and gram (+) bacteria stained with purple were included in the study for further identification [23,24,30].

5.3 Catalase Test

Bacterial colonies grown on MRS agar medium were taken and spread on a slide, then 3% hydrogen peroxide solution was dropped on slide surface. The formation of gas bubbles was evaluated as a positive result and cultures without gas bubbles were evaluated as catalase negative. *Staphylococcus aureus* ATCC 29213 was used as positive control and *Enterococcus faecalis* ATCC 29212 was used as negative control in catalase test [23,24].

5.4 Voges-Proskauer Test

The isolates were inoculated into MR-VP Broth medium and incubated at 37 °C for 48 hours. At the end of the period, α-naphthol (6 g α-naphthol/100 ml 95% ethanol) and KOH (16 g KOH/100 ml distilled water) solutions were added to the tubes and shaken vigorously (20 seconds) and the tubes were observed for colour change within 5 minutes. Tubes showing red colour formation were considered as positive and those without colour change were considered as negative. *Klebsiella pneumoniae* was used as positive control and *Escherichia coli* was used as negative control in Voges-Proskauer test [23].

5.5 Arginine Hydrolysis Test

The acidity developed in the colonies forming ammonia from arginine is neutralised by ammonia formation and the yellow colour caused by acidity disappears. Isolates that do not form ammonia from arginine change the colour of the acidity indicator dye (violet) to yellow and the colour of the medium does not change during incubation. Accordingly, all isolates in our study were inoculated on Reddy agar and left for incubation. The observation of bright clear yellow color (acid) or no change (nonfermenting rods) was considered as arginine hydrolysis negative, and the observation of turbid purple to faded-out yellow-purple color (alkaline) was considered as arginine hydrolysis positive [25].

5.6 Gas Production from Glucose

In order to determine whether lactic acid bacteria are heterofermentative or homofermentative, their gas production from glucose was investigated. Glucose phosphate broth medium containing inverted Durham tubes were inoculated with 24 h cultures and incubated at 37 °C for 7 days. Cultures in which gas was observed in Durham tubes after incubation were considered as positive [25].

5.7 Identification of Isolated Microorganisms

Single colony was taken from MRS agar and spread on slides with sterile swab and 0.9 µL VITEK MS CHCA (matrix solution) was dropped on it. All slides left to dry at room temperature. Then, the slides were analysed by MALDI-TOF MS. *Escherichia coli* ATCC 8739 is used to as calibrator. The growth of these bacteria in MRS broth containing different pH, temperature and different concentrations of bile salts was analysed.

5.8 Media

Probiotic strains were incubated in de Man Ragosa Sharp (MRS; Neogen®, MI, USA) broth at 37℃ for 72 hours and adjusted approximately 10^5 cfu/mL in saline (0,9% NaCl). Then, for each probiotic strain, four different MRS broth bottle including four different concentrations (1%, 2%, 6%, and 10%) of NaCl (Merck®, Darmstadt, Germany) prepared. Probiotic strains were inoculated into test tubes containing MRSB, each tube was considered as a stock and used to obtain hourly growth curve for 72 hours.

5.9 Serial Dilution Assay

In the present study, all of the microorganisms were seeded in the MRS broth containing various concentrations of NaCl (1%, 2%, 6%, and 10%) and incubated at 37°C for 72 hours.

Moreover, in order to find out the alive colonies on MRS agar containing plates 1 mL of probiotic microorganism solution was taken and poured into 9 mL saline (0,9% NaCl) tubes at each hour. Later on, ; 0,1 ml was taken from the test tubes where dilutions were made and spread on plates containing MRS agar media by drigalski spatulas. After that, the plates were incubated at 37℃ for 72 hours. Visible colonies between 50 - 300 was evaluated and then growth curves were generated. [27-29] (Figure 6).

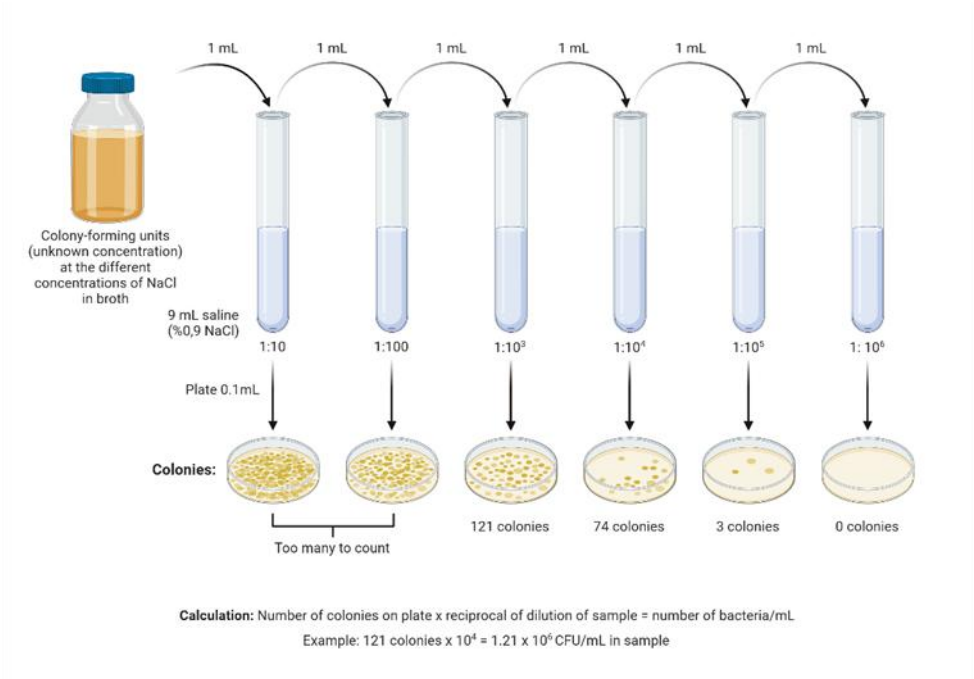

Figure 6. Serial dilution process of probiotic bacteria in saline for obtain visible colonies.

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