Jagannath University Journal of Life and Earth Sciences, **9**(1): 69-80, 2023 (June) pISSN 2414-1402, eISSN 2791-1845
DOI: https://doi.org/10.3329/jnujles.v9i1.72881

**Research Article** JnUJLES

# FORMULATION AND CELL VIABILITY ASSESSMENT OF MICROPARTICLES OF ISOLATED *LACTOBACILLUS* SP. WITH ALGINATE-CHITOSAN SOLUTION

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*Received: 18 January 2023, Accepted: 13 May 2023*

**ABSTRACT**

This study was performed to isolate the probiotic Lactobacillus sp. from dairy products available in Dhaka to form microparticles with isolated Lactobacillus probiotic bacteria and determine cell viability. Depending on their biological and physical traits and selective growth in MRS agar and MRS broth medium, the isolates were recognized as Lactobacillus spp. Major biochemical tests and growth at different temperatures were taken into account for the identification of the isolates. Low pH tolerance, homo- or hetero-fermentative activity, hydrolysis of arginine, and antibiotic susceptibility tests were conducted to assess probiotic activity. Among several isolates of Lactobacillus sp., four showed considerable tolerance to lower pH (pH 3.0) and growth at different temperatures (15 to 37°C). They were resistant to common antibiotics like penicillin (10µg), gentamicin (30µg), tetracycline (30µg), chloramphenicol (30µg), erythromycin (15µg) and streptomycin (30µg). The isolates were shown to have superior probiotic capabilities and may be employed as possible probiotic microorganisms after comparing all probiotic features evaluated in this experiment. The capacity of probiotic bacterial cells to survive when stored and in gastrointestinal transit is difficult to maintain. As a solution to these obstacles, microparticles were formulated with sodium alginate and chitosan. The cell viability check of the microparticles was continued for two weeks, and the response positively indicated increased stability. The results show promise for the encapsulation of the isolates.

**Keywords:** *Probiotic, Dairy, Microparticles, Alginate, Chitosan*

## Introduction

Probiotic organisms mostly consist of Lactic Acid Bacteria (LAB). The main food sector nowadays is dairy where probiotics are utilized to raise the standard of products (Gao, 2021). In Bangladesh, most of the fermented dairy products are richly contented with various species of *Lactobacillus.* One of the prerequisites for using probiotics is to ensure their survival, moreover, the factors related to manufacturing and fermentation, including pH, oxygen, temperature, and the appearance of other organisms negatively impact the efficacy and viability of probiotics.

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Microencapsulation is a method of encasing tiny capsules with liquid or gaseous particles or droplets that may discharge their contents at regulated rates or in certain situations (Favaro-Trindade, 2008). Microparticle formation protects these delicate cultures against high oxygen levels, and freezing (Shah, 2000), maintains the bacteria in heat and humidity, and increases the stability of the culture while the product is in storage (Champagne, *et al*., 1993), as well as improves flow characteristics during formulation development.

*Lactobacillus* is a phylogenetic, ecological, and metabolically diverse genus with about 260 species (Zheng *et al*., 2020). *Lactobacillus* isolated from dairy products can be selected for microparticle formulation based on some specific characteristics, resistance to low pH, fermentative activity, antimicrobial activity, and resistance to the colonization of non-native bacteria.

Extrusion, emulsion, and spray drying are popular for particle and capsule formation methods with probiotic cells. Probiotic microorganisms are captured in the gel matrix via various gel formation processes in these procedures (Cook, *et al*., 2012). Probiotics are living organisms, thus requirements for implementation infrastructure must be created to preserve the viability of cells, and the solutions used must be safer (Wang & Zhong, 2024; Gbassi *et al*., 2009; Champagne *et al*., 1993). As bacterial viability seems to be required for probiotics to operate in relation to gut and mucosal immunity, it poses an industrial challenge. Several studies have established the importance of living cells in relation to the functional attributes of probiotics; for example, antibacterial compounds and short chain fatty acids are suggestive metabolites generated by viable colonies (Wilkinson M.G., 2018; Lahtinen *et al*., 2005), and it demands that the probiotic fulfill requirements for purity and non-toxicity and be safe for the intended application (Vankerckhoven *et al*., 2008).

Extrusion is the ancient and prevalent method for creating hydrocolloid capsules (King, 1995; Nezamdoost-Sani *et al*., 2024). It comprises making a hydrocolloid solution and cell culture suspension administered into a syringe, and droplets produced by the syringe needle are allowed to fall naturally into a setting or a solution bath for hardening. The beads' size and form are determined by the span of falling and the needle's diameter. The most common approach is this one, because of its simplicity, cost-effectiveness, and gentleness formulation parameters ensuring maximum cell retention viability.

A cell culture suspension is combined with an alginate solution to create beads, which are subsequently injected into a $CaCl_2$ gel solution containing several cations (often $Ca^{2+}$), and spheres are formed by the droplets. Ionically cross-linked alginate is used in order to build a lattice that is three-dimensional, surrounding the cells immediately. The success of sodium alginate in this technique used for microparticles formation is due to the delicacy it offers the material, affordability, and biological compatibility (Qamar *et al*., 2023; Jurić *et al*., 2021; Tanaka *et al*., 1984; Klein *et al*., 1983; Schillinger *et al*., 1999; Martinsen *et al*., 1989). The stabilizing component utilized in extrusion is alginate. It is a simple hetero-polysaccharide obtained from many kinds of algae and contains both D-mannuronic and L-glucuronic acid (Smidsrod *et al*., 1972). The characteristics of alginate serve as a stabilizing material those substantially correspond with the content and order of D-mannuronic acid and L-glucuronic acid. Chitosan is deacetylated to produce chitosan, a positively charged linear polymer. It dissolves in water at a pH lower than 6, and similarly to alginate, produces a gel by ionotropic gel formation. A poly-cation with amine

groups called chitosan may be joined together by anions or poly-anions, for instance, polyphosphates $[Fe\ (CN)_6]^{4-}$, $[Fe\ (CN)_6]^{3-}$, poly-aldohydrocarbonate of carbonic acid. Different parameters are considered to evaluate the effectiveness of the probiotic microparticles, such as viability check at environmental conditions, cell release/recovery ability (Mortazavian *et al*., 2007; Reid *et al*., 2005).

In this research, the *Lactobacillus* present in dairy products was isolated, their probiotic properties were screened, and the approach for microparticle development was taken.

## Materials and Methods

### Sample Collection and Selection of Isolates

Four samples (curd, sweet yogurts from two sources, one is local and another is branded, and raw milk) were collected, and cultured on MRS agar plates (De Man, Rogosa, Sharpe medium), which favors the growth of *Lactobacillus* bacteria, following incubation at 37°C for 2 days. Four isolates were identified with morphological and biochemical tests as species of *Lactobacillus*.

### Morphological Characterization of Bacteria

Bacterial colonies were purified by continuous sub-cultures continuously on MRS media; the color and shape of the isolates' colonies were visibly observed.

The isolates were subjected to observation under a light microscope after performing Gram staining, to identify them as either Gram-positive or Gram-negative.

### Biochemical Test

Biochemical tests were conducted using the techniques recommended by Bulut *et al*., 2003. According to Holt *et al*., 1994, in the catalase test, bacteria produce catalase enzymes and gas bubbles by converting hydrogen peroxide ($H_2O_2$) into water and oxygen. The isolated organisms that could not produce gas bubbles were selected because lactobacilli do not produce catalase. The carbon dioxide generated from the glucose test was used to assess the fermentative characterization of isolates. MRS broth in the tubes containing flipped Durham tubes was inoculated and incubated for 5 days at 37°C. Gas formation in Durham tubes indicates $CO_2$ synthesis from glucose.

### Growth at Various Temperatures

To observe the ability of the bacteria to grow at various temperatures, five mL of MRS media with purple bromocresol indicators were taken into each tube. Then 100 microliters of the cell cultures were added into tubes and incubated for 5 days at 10°C, 15°C, 20°C, and 25°C. Throughout this stage of incubation, the color shift indicated cell development from purple to yellow as acidity increases.

### Carbohydrate Fermentation

Lactose, sucrose, ribose, and galactose are used for carbohydrate fermentation tests, using a modified protocol by Erkus, 2007. The carbohydrates were liquefied with a 5% (w/v) final concentration in distilled water and sterilized using 0.22-micrometer filter paper. The MRS broth was prepared in test tubes, each tube containing 9 ml of MRS broth. 1 ml of the sugar solutions was taken into the test tubes following inoculation and incubation for 36 hours at 37°C. Sugar fermentation was noticed as the acidity increased. The creation of gas in the medium changed its appearance from its initial color to yellow.

Hydrolysis of Arginine Test by *Lactobacillus*

This test was done following the method described by Kumar *et al*., 2017, the hydrolysis of arginine to ornithine is catalyzed in the presence of strongly acidic or alkaline conditions by L-arginase. Sugar (0.2 g) was dissolved in MRS broth (10 ml) which was taken in a tube for each of the isolates. Sugar lowers the pH of the media used by the bacteria and as a result, the enzyme arginine hydrolase became inactivated. Then 0.3% arginine and 0.2% sodium citrate were mixed into the broth, following inoculation and incubation for 36 hours at 37°C. After incubation, 0.004% purple bromocresol was used as an indicator, which becomes yellow at pH 5.2 but remains purple at neutral or alkaline pH.

Assessment of the Probiotic Characteristics of the Isolates

These key selection criteria were used to determine the probiotic characteristics of isolates.

Growth at Low pH in MRS Broth

To determine growth and development at various pH levels, the isolates were inoculated in MRS broth with a pH range of 2.5, 3.0, 4.0, 5.0, and 7.0 employing 100 µl cultures. After this, the broths were left to incubate for 36 hours at 37°C. The probiotic bacterial growth at low pH was assessed by comparison with the growth at neutral pH.

Acid Tolerance Study using PBS and Cell Count

In 10 mL of MRS broth, the isolates were cultured at 37°C for 36 hours. Then the suspension was centrifuged for 10 minutes at 5000 rpm. Thirty mL of neutral PBS (Phosphate buffer solution) was prepared and 20 mL was acidified with HCL addition. The pellets remained were re-suspended in 1 mL acidic PBS of pH 3, 3.5, 4, 4.5, and 5, individually and incubated for 24 hours, then centrifuged for 10 minutes at 5000 rpm. After being re-suspended in neutral PBS, the pellets were centrifuged for 15 minutes at 5000 rpm. Serial dilution of the pellets using normal saline was carried out until a dilution of $10^{-3}$ was achieved and plated on MRS agar through spread plating. Following incubation for 36 hours at 37°C, the cell colony was counted on a plate.

Homofermentative and Heterofermentative activity test

To recognize the hetero- or homofermentative activity of the bacteria, this test was performed with a Gibson semisolid medium. Skim milk is one of the ingredients of Gibson semisolid medium, which was sterilized on a heat stove in a glass beaker for 8-10 minutes.

After inoculation, the medium was incubated anaerobically at 37°C for about 2 days and then observed for gas formation. Lactic acid, acetic acid, and carbon dioxide ($CO_2$) are byproducts of the fermentation of glucose by heterofermentative *Lactobacillus,* while homofermentative *Lactobacillus* ferments glucose with lactic acid as the primary byproduct.

Antibiotic Susceptibility Test

The diffusion of agar overlay disc developed by Charteris *et al*., (1998) was used to determine antibiotic susceptibility. The isolates were cultivated under anaerobic conditions and then antibiotic discs were added on the plates. They were cultured for 36 hours at 37°C. All isolates were put through a series of tests to see if they were susceptible to penicillin (10 µg), gentamicin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15µg) and streptomycin (30µg).

Microparticle Preparation

The extrusion technique process referred to by Klinkenberg *et al*., (2001), was followed for microparticle formulation from chitosan and alginate with isolated *Lactobacillus.*

Alginate mixture and Chitosan solution preparation

The sodium alginate mixture solution was obtained by combining 2g sodium alginate, 5% MRS broth, 100 microliter tween 80, and 5 mL glycerol. Distilled water was added to attain a 100 mL volume, and vortexed to dissolve all of the components constructively. For the chitosan solution, 0.4 g chitosan and 0.4 mL glacial acetic acid were added to distilled water to make an acidic 100 mL solution, and 0.834 mL of 1 M NaOH was included to modify the pH between 5.5 and 6.5. Alginate mixture, Chitosan solution, and 0.5 M 500 mL $CaCl_2$ were sterilized through hot air sterilization at 120°C for 5-8 minutes. Then 20% fresh cell culture (which was prepared before) was added to the alginate solution. A syringe, four falcons, and two magnetic bars were taken under UV sterilization for 20 minutes.

The alginate mixture was instilled with a sterilized magnetic bar. The syringe was injected with an alginate mixture and sprayed through the 0.5 M 500 mL $CaCl_2$ solution contained in a glass beaker while stirring with another magnetic bar for about 15 minutes. The microparticles were hardened resulting from the cross-linkage of calcium ions with sodium alginate. After filtration using filter paper, the purified microparticles of alginate were washed twice with distilled water before being moved to the previously made 100 mL of chitosan solution, then stirred for 15-20 minutes with a magnetic bar, and then filtered again with filter papers, then stored at 4°C and checked for cell viability.

Cell viability check of the microparticles by CFU enumeration

Cell viability check of the microparticles was performed using the approach outlined by Klinkenberg *et al*., (2001) with modifications.

A 1% sodium citrate solution was made by adding 12.851g sodium citrate and 1.2105g citric acid. After serial dilution up to $10^{-8}$ with normal saline, the pour plating method was performed on MRS media and incubated for 36 hours at 37°C. Then the CFU (Colony Forming Unit) was counted at the dilution factor $10^{-6}$, $10^{-7,}$ and $10^{-8}$ to determine the survival of bacteria. This process was repeated two times at an interval of 1 week.

**Results and Discussion**

All the isolates were slender rods, purple in color, and non-motile when observed under a light microscope (100x), indicating them as Gram-positive bacteria (Figure 1A), and the appearance of the colony was yellowish, mucoid, and rounded on MRS agar (Figure 1B). As reported in prior research by Devi *et al*., 2023; Sheu & Marshall., 1991; Kebary *et al*., 1998; and Sheu *et al*., 1993, lactic acid bacteria (LAB) are Gram-positive as well as catalase-negative with an optimal growth temperature of 37°C. The selected isolates were affirmatively recognized as *Lactobacillus spp*. for being Gram-positive and catalase-negative and they did not produce carbon dioxide from glucose.

Growth at different temperatures of the bacterial isolates was represented in Table 1. All bacterial isolates showed optimal growth at 25°C and 37°C, but could not grow at 10°C.

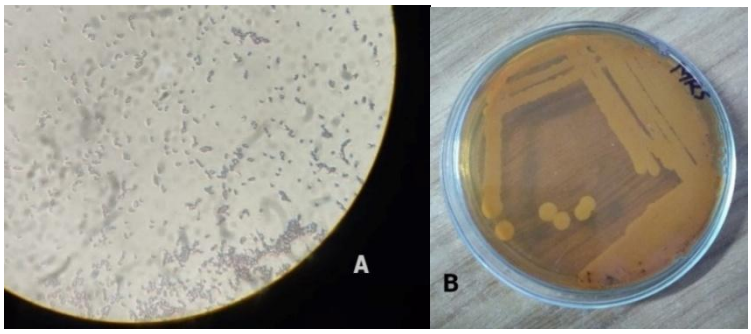


Fig. 1. (A) Microscopic image of Gram-positive *Lactobacillus* (100x), and (B) Bacterial growth of *Lactobacillus spp.* on MRS Agar.

Table 1. Growth at Different Temperatures of the Bacterial isolates

| Temperature (°C) | IS - 1 | IS- 2 | IS- 3 | IS- 4 |
|---|---|---|---|---|
| 10 | - | - | - | - |
| 15 | + | - | + | - |
| 20 | + | - | ++ | + |
| 25 | ++ | + | ++ | ++ |
| 37 | ++ | + | ++ | ++ |

**(**+ = poor growth; +++ = excessive growth; - = no growth)

In the evaluation of the fermentation of various carbohydrates presented in Table 2, all isolates confirmed the ability to ferment sucrose and lactose. IS-2 was unable to ferment galactose and IS-1 could not ferment ribose. The pH indicator purple bromocresol became yellow by IS-2, IS-3, and IS-4 but remained purple by IS-1 (Table 3) evincing that three of the isolates have arginine hydrolysis ability.

Table 2. Carbohydrate Fermentation Test

| Isolate | Lactose | Galactose | Sucrose | Ribose |
|---|---|---|---|---|
| IS-1 | + | + | + | - |
| IS-2 | + | - | + | + |
| IS-3 | + | + | + | + |
| IS-4 | + | + | + | + |

Table 3. Arginine Hydrolysis Activity by Isolates

| Isolate | Result |
|---|---|
| IS-1 | Negative |
| IS-2 | Positive |
| IS-3 | Positive |
| IS-4 | positive |

Probiotic Properties of Isolates

Growing each bacterial isolate in a different pH environment was used to determine its pH tolerance (Table 4). At pH 2.5, it was revealed that of all of them, only IS-1 could grow at such low pH. Isolates, IS-1 and IS-2, demonstrated their potential for acid tolerance by surviving at pH 3.0 and pH 4.0, respectively, while they were grown profusely at pH 5.0 to 7.0.

The cell count of isolated *Lactobacillus* bacteria at various acidic pH values and the acid tolerance studies are presented in Figure 2, in comparison with a standard *Lactobacillus* strain, *Lactobacillus plantarum* ATCC 8014. The horizontal axis represents the pH of the acidic PBS and the growth or acid tolerance of the IS-1 and IS-3 was represented on the vertical axis (log CFU/ml). The counted colonies of IS-2 bacteria on MRS agar was 16 which was too few to count; for IS-4, it was more than 300, too numerous.

The result of the homofermentative and heterofermentative activity test is shown in Table 5. When compared to the control of Gibson semisolid medium, IS-1, and IS-2 were noticed as heterofermentative with $CO_2$ gas formation.

In the antibiotic susceptibility test (Figure 3), the isolates were tested with penicillin (10µg), gentamycin (30µg), tetracycline (30µg), chloramphenicol (30µg), erythromycin (15µg), and streptomycin (30µg), however, none of the strains showed any sensitivity to the antibiotics.

Table 4. pH tolerance test result of isolates at different pH

| | Isolate Number | | | |
|---|---|---|---|---|
| pH | IS-1 | IS-2 | IS-3 | IS-4 |
| 2.5 | + | - | - | - |
| 3.0 | + | + | - | - |
| 4.0 | + | + | - | - |
| 5.0 | + | + | + | + |
| 6.0 | + | + | + | + |
| 7.0 | + | + | + | + |

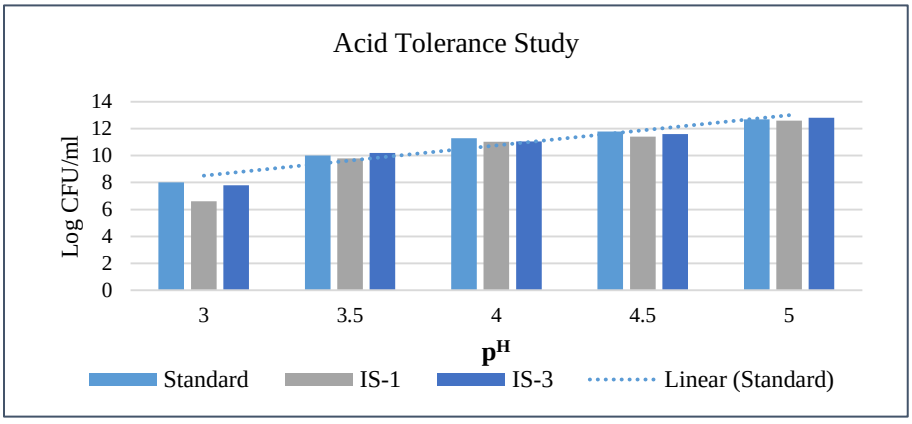


Fig. 2. Growth of isolated *Lactobacillus* (IS-1 and IS-3) at different pH values.

Table 5. Homofermentative/ Heterofermentative activity test result of the isolates

| Isolate | Appearance after incubation | Decision |
|---|---|---|
| IS-1 | The height of the medium was raised, color changed, and Durham tube was filled with gas. | Heterofermentative |
| IS-2 | Medium with a slight color change, the Durham tube was filled with gas. | Heterofermentative |
| IS-3 | Gas formation is absent | Homofermentative |
| IS-4 | Gas formation is absent | Homofermentative |

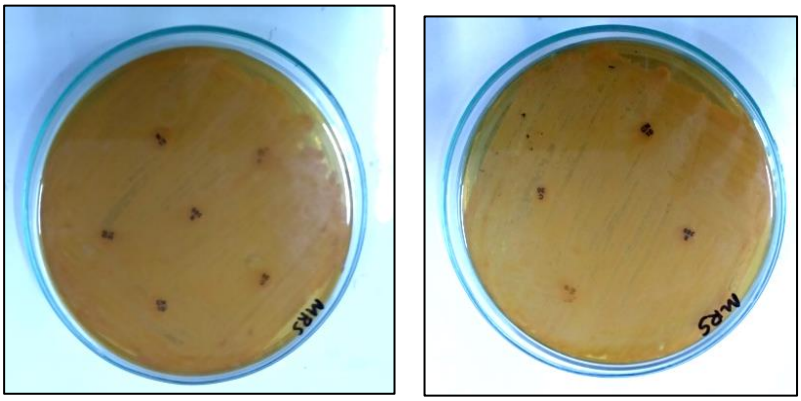

Fig. 3. Antibiotic susceptibility test

Microparticle preparation and Cell viability check by CFU enumeration

Alginate mixture and other steps to form microparticles are presented in Figure 4 and Figure 5. After the hardening and formation of the microparticles, the recovery was done by filtration.

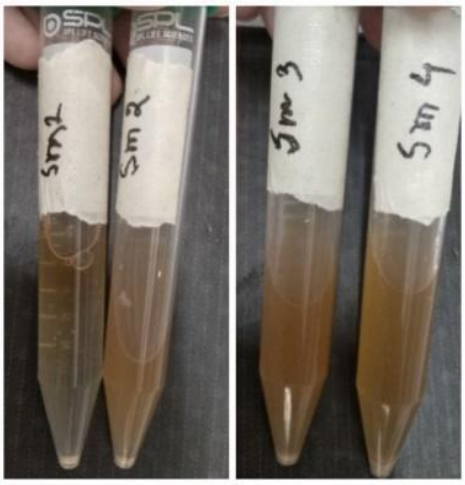


Fig. 4. Alginate mixture with cell suspension.

Fig. 5. Steps to form microparticles (clockwise: magnetic stirring, hardening and formation of microparticles, filtration of particles with filter paper, and microparticles in the $CaCl_2$ solution)

CFU counts of the isolates from microparticles on the MRS agar plate to measure the cell viability are presented in Table 6, at 1-week and 2-week time intervals, and the graph in Figure 6. It was observed that IS-1 and IS-3 were profusely present but after two weeks their numbers rapidly declined, compared to IS-2 and IS-4. The cell count in colony-forming units per ml was similar to a previous study which showed that the viability of entrapped *L. casei* was $2.6\times10^8$ cfu/g which was the highest after using the extrusion method (Djaenudin *et al*., 2020), the cell counts for these isolates after two weeks were closer to that of the study.

Table 6. The cell count of microparticles stored at 4°C

| Isolates | Cell count prior to 4°C storage (CFU/ml) | Cell count after preserving at 4°C for 1 week (CFU/ml) | Cell count following storage at 4°C for 2 weeks |
|---|---|---|---|
| IS-1 | $3.8 \times 10^{12}$ | $2.6 \times 10^{11}$ | $2.6 \times 10^{10}$ |
| IS-2 | $4.7 \times 10^{11}$ | $3.2 \times 10^{11}$ | $4.4 \times 10^{9}$ |
| IS-3 | $1.4 \times 10^{11}$ | $6.5 \times 10^{10}$ | $5.3 \times 10^{8}$ |
| IS-4 | $5.6 \times 10^{11}$ | $4.9 \times 10^{9}$ | $7.4 \times 10^{11}$ |

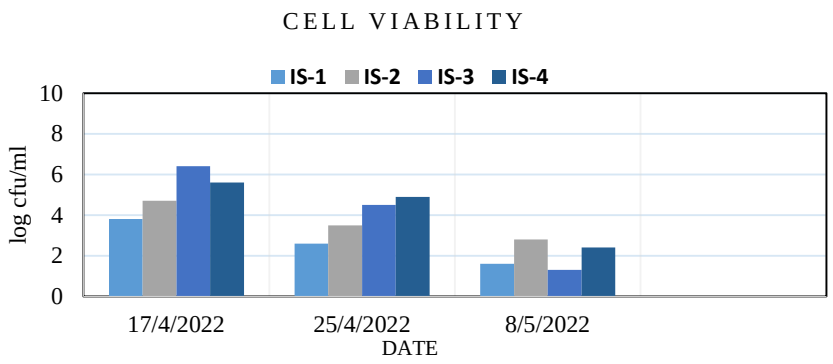


Fig. 6. The cell viability check of the microparticles of 4 isolates after storage.

According to Sheu and Marshall (1993), lactobacilli were around 40% more likely to survive when they became encased in calcium alginate than when they were not, which supports this study where the isolates were still alive after 14 days, despite the fact that their reduction rates varied.

## Conclusion

By taking an in-depth look into isolated probiotic species, the potential for their use as probiotics was investigated. In all in-vitro testing, four isolates showed significant probiotic attributes and constituted microparticles synthesized with 0.4 percent sodium alginate and chitosan were maintained at 4°C for shelf life testing. To build more sophisticated encapsulating approaches, a more extensive assessment is required.

## Acknowledgment

This research work was financially supported by the National Science and Technology Fellowship (NST) 2021-22 awarded by the Ministry of Science and Technology, Bangladesh.

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