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Survival and Viability of *Limosilactobacillus reuteri* Bacteria: A Comparative Study between Free and Microencapsulated Forms under Gastrointestinal and Thermal Stress Conditions

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Survival and Viability of *Limosilactobacillus reuteri* Bacteria: A Comparative Study between Free and Microencapsulated Forms under Gastrointestinal and Thermal Stress Conditions

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Abstract. The main aim of the current study was to assess how microencapsulation affects the survival and stability of *Limosilactobacillus reuteri* bacteria in conditions that simulate gastrointestinal digestion and thermal stress. *Lim. reuteri* bacteria were encapsulated within whey proteins and gum Arabic. The viability of *Lim. reuteri* was evaluated by exposing the bacteria to simulated gastrointestinal digestion and thermal stress. The utilization of microencapsulation showed a notable impact on the survival and stability of *Lim. reuteri,* as indicated by statistical significance ($p< 0.05$). In general, a distinct decline in the survival rate of *Lim. reuteri* was observed across all treatments. More specifically, a rapid decrease in numbers was noted when free *Lim. reuteri* cells were stored in refrigeration compared to the microencapsulated form. Moreover, in the simulated gastrointestinal test, the efficacy of microencapsulation was calculated to be 84.66% after 240 minutes, while non-encapsulated bacteria saw a decrease of 61.26%. The most prominent conclusion, the viable count analysis indicated that the microencapsulated cells maintained levels above the recommended threshold (10^6 CFU/g) both under thermal conditions and in simulated gastrointestinal conditions.

1. Introduction

Functional foods enriched with probiotics are considered to have a wide array of health benefits, as indicated by multiple sources. The popularity of these functional foods has been on the rise due to the growing demand for them in the global marketplace. According to the Food and Agriculture Organization/World Health Organization (FAO/WHO), probiotics are live microorganisms that, when consumed in adequate amounts (10^6-10^7 CFU/g), confer health advantages to the individual [1]. The health benefits associated with probiotics are closely connected to their capacity to endure and remain steady in carrier foods and the gastrointestinal environment. The survival and stability of these advantageous probiotics have caused apprehension within the scientific community. For probiotics to produce their desired effects, they must uphold an adequate level in the carrier food throughout storage and at the moment of intake. Various factors, whether intrinsic or extrinsic, contribute to influencing the survival of probiotics [2]. The factors influencing the viability of probiotic microorganisms encompass the availability of oxygen, the acidity levels in fermented goods, pH fluctuations, storage temperatures, and the generation of diverse antimicrobial agents during processing. Within the realm

of dairy items, issues like harm induced by freezing and contact with harmful substances pose substantial challenges to the survival of probiotic microorganisms during storage [3].
The increasing worldwide demand for probiotic microorganisms in the food and supplement sectors is a significant phenomenon. Specifically, there is a notable increase in the promotion of food items enriched with probiotics in the Asia-Pacific area [4]. This upsurge is linked to the expanding awareness among customers concerning the numerous health advantages related to the intake of probiotics. Nonetheless, it is essential to recognize that the levels of probiotics in food items may decrease during storage, processing, and other technological processes. As a result, the anticipated advantages of probiotics might not be fully realized upon the consumption of such items. The survivability of probiotic microorganisms is additionally compromised by the harsh conditions found in the stomach and intestines [5]. This poses a significant challenge for food producers since ensuring the survival and steadiness of probiotics emerges as a crucial element of product advancement. Consequently, a key focus for the sector lies in formulating approaches that can uphold the viability of probiotics in challenging conditions and across diverse food compositions. Conventional approaches such as utilizing resilient bacterial varieties, implementing dual-phase fermentation, incorporating micronutrients, regulating oxygen levels, and adjusting to stress have been employed to safeguard probiotic bacteria during their cultivation stage. Nonetheless, these methods have shown limited efficacy [6]. The utilization of microencapsulation is gaining traction as a viable solution due to its ability to provide a specified level of protection and precise delivery of probiotic core materials. This method not only shields probiotics from harsh environmental conditions but also maintains their therapeutic effectiveness in the end product. Employing suitable encapsulating materials for the microencapsulation of probiotics is a strategic approach to ensuring the survival of live cells in challenging acidic settings. Moreover, this technique guarantees that probiotics successfully reach the targeted area within the digestive system, thereby enhancing their overall functionality and health advantages [7]. In the field of probiotic stability and viability, microencapsulation is a noteworthy instrument that provides a dependable way to preserve the effectiveness and viability of these advantageous microbes in a range of food applications. Probiotics are presently being encapsulated using a variety of encapsulating materials in both laboratory and commercial settings, with an emphasis on those that have been granted Generally Recognized as Safe (GRAS) classification [8]. These substances mainly comprise proteins, fats, oils, carbohydrates, and related compounds. Key instances of efficient microencapsulating polymers are whey proteins, gum Arabic, sodium alginate, carrageenan, etc. Whey proteins are particularly notable for their widespread use in encapsulating probiotics, attributed to their advantageous traits like biocompatibility, versatility, resilience to high temperatures, and acidic conditions [9]. *Limosilactobacillus reuteri*, an obligately heterofermentative species found in the host's gastrointestinal tract, has been employed as probiotics in different functional foods and healthcare goods for over two decades. The use of specific strains of *Lim. reuteri* as probiotic bacteria has become a widespread practice in the industry. The probiotic attributes displayed by *Lim. reuteri*, whether *in vivo* or *in vitro* conditions, are partly associated with their ability to produce 3-hydroxy propionaldehyde (3-HPA). This particular compound, also referred to as reuterin, acts as a potent antimicrobial agent with a wide range of activity, which is synthesized by Lim. reuteri during glycerol metabolism. The synthesis of 3-HPA by this species plays a crucial role in its probiotic function, underscoring the significance of comprehending the mechanisms involved in the production of this antimicrobial substance by *Lim. reuteri*. The utilization of 3-HPA, or reuterin, as an antimicrobial agent, has attracted attention in the probiotics and functional foods field due to its potential health benefits and diverse applications in healthcare products [10]. The objective of this investigation was to microencapsulate *Lim. reuteri* using two distinct materials. The investigation also focused on evaluating the stability and lifespan of free and microencapsulated cells. To provide a comprehensive investigation, our evaluation involved subjecting the free and microencapsulated *Lim. reuteri* to simulated gastrointestinal tract conditions.

2. Materials and Methods

2.1. Bacteria strain and culture conditions

Lim. reuteri ATCC53608 was utilized in microencapsulation experiments and cultured in MRS Broth (hi-media, India) at 37 °C for 16-18 hours under anaerobic conditions. The growth of *Lim. reuteri* was harvested, subjected to centrifugation (at 5000×g for 15 min), and then rinsed with a sterile saline solution. Following this, the concentration of Lim. reuteri was adjusted to 11.74 log CFU/g. [10].

2.2. Bacteria microencapsulation

Lim. reuteri cells were encapsulated within a mixture of whey protein and gum Arabic utilizing a technique outlined by Eckert et al. [11]. A sterile solution containing 10% whey proteins from buffalo milk produced at the Agricultural Research Station/College of Agriculture/University of Basrah was used to disseminate the 5 g wet cell pellet that was obtained. Then, 3.25 g of gum arabic was mixed with the bacterial cell solution and agitated for 30 minutes at 500 rpm. Subsequently, the microencapsulation mixture needs to be dried at 500 MPa of pressure in a vacuum oven set to 50 ◦C. Samples of bacterium microcapsules were kept at 4 ◦C in storage.

2.3. Microencapsulated bacteria cells count

Encapsulated bacteria were released by immersing the microcapsules in a sterile phosphate buffer saline (0.1 M, pH 7.0) for 10 minutes with gentle agitation. The enumeration of viable cells within the encapsulated bacteria was conducted by serially diluting 1 mL of microcapsules, which were dissolved in 0.1% peptone water and then subjected to plate counting on MRS Agar. The count of bacterial colonies was recorded after 48 hours at 37◦C under anaerobic conditions. The efficiency of microencapsulation (ME) was calculated using the formula: $N_1/N_0 \times 100$, where N_1 represents the log. CFU/ml of viable bacteria cells post-microencapsulation and N_0 log. CFU/ml of viable bacteria of free cells was added to the microencapsulation solution [2].

2.4. Survival of free and microencapsulated bacteria in simulated stomach conditions

The primary focus of the study was to examine the survival of bacteria under conditions mimicking the stomach, whether they were in a free form or encapsulated within microcapsules. The viability of *Lim. reuteri* in both states was evaluated using a slightly modified protocol based on Afzaal et al. [12]. A sterile solution of NaCl with 3 g/L of pepsin enzyme was mixed with 1 N HCl to adjust the pH to 2± 0.2, creating a simulated stomach environment. This stomach solution contained one gram of microencapsulated bacteria, which were then cultured at 37°C and 110 rpm in a shaking incubator. Viable counts of *Lim. reuteri* were determined using MRS agar at time points of 0, 60, 120, 180, and 240 minutes. To ensure the accuracy of the results, the experiment was repeated three times.

2.5. Survival of free and microencapsulated bacteria in simulated intestinal conditions

The survival of *Lim. reuteri* in gastrointestinal conditions, particularly after passing through the stomach, is of utmost importance. An assessment of the viability of both free and microencapsulated bacteria was conducted following the methodology specified by Afzaal et al. [12]. The resilience of microencapsulated bacteria under conditions simulating intestinal transit was gauged by subjecting them to a simulated intestinal solution. This solution was comprised of 3 g/L bile salt and 10 g/L pancreatic enzymes dissolved in a pH 8.0 phosphate buffer, with the pH of the intestinal fluid adjusted to 7.5 using 0.1 N NaOH. A gram of microencapsulated bacteria was mixed into a simulated gastric solution. The enumeration of viable bacterial counts was carried out using MRS agar and log.CFU enumeration. Free and microencapsulated cells were separately introduced into the simulated solutions and incubated in a shaking incubator at 37°C and 80 rpm. The viability of both types of cells was assessed by plating them on MRS agar at specific time intervals (0, 60, 120, 180, and 240) minutes.

2.6. Thermal stress of free and microencapsulated Lim. reuteri

To assess the thermal resistance of microencapsulated *Lim. reuteri* cells, the viability of the bacteria was investigated across various time-temperature settings: 60°C for 3 minutes, 65°C for 3 minutes, 70°C for 2 minutes, 75°C for 2 minutes, 80°C for 1 min, and 80°C for 2 minutes. Samples were

formulated by diluting either free or microencapsulated cells in 0.1% peptone water and hermetically sealing them in test tubes. These tubes were subsequently immersed in a regulated water bath and exposed to the specified conditions. Post the stipulated duration, the tubes were extracted and rapidly cooled using flowing tap water. Portions were extracted both before and after the thermal treatments and quantified by diluting 1 mL samples of the substance, culturing them on MRS Agar, and then placing them in an incubator at 37°C for 48 hours. The outcomes were documented as the percentage of surviving cells, computed as $N_t/N_0 \times 100$, with N_0 and N_t denoting the cell densities in the log. CFU/mL pre- and post-the heat exposure, respectively. Three distinct trials were conducted [13].

2.7. The survivability of free and microencapsulated Lim. reuteri under refrigerated conditions.
Refrigerator temperatures were applied to both free and microencapsulated *Lim. reuteri* to evaluate their survival. Both kinds of bacteria were maintained at 6±2°C, and after 0,15,30,45, and 60 days of storage, the viability of the cells was assessed. The formula $\log N_t/N_0 \times 100$ was used to determine the survival percentage of *Lim. reuteri* under these conditions. N_t is the log. number of *Lim. reuteri* at a given point in time during storage, and N_0 is the starting count of Lim. reuteri at the beginning of the experiment [2].

2.8. Statistical analysis
Statistical analyses of the various treatments mentioned above were carried out using the SPSS Statistics V22.0 software (Statistical Package for Social Sciences, San Antonio, TX, USA). An examination of the variance (ANOVA table) of the data was conducted, and the means comparison of treatment values by the Least Significant Difference test (LSD) method at $p \leq 0.05$.

3. Results and Discussion

3.1. Microencapsulation efficiency of Lim. reuteri
The results of microencapsulation experiments and the percentage of microencapsulation efficiency (ME%) for *Lim. reuteri* bacteria can be found in Table 1. Initially, the count of viable *Lim. reuteri* bacteria and the efficiency of the microencapsulation process were recorded in the log. 10.53 CFU/mL and 88.69%, respectively. Subsequently, following the drying process, the numbers of viable bacteria and ME% decreased, resulting in values of log. 9.22 CFU/mL and 87.55%, respectively. The components composing the microencapsulated blend function to uphold the viability of probiotic bacteria within acceptable parameters and shield them from the harsh environment of the digestive tract. Furthermore, the technique employed for drying the microencapsulated solution (Vacuum oven) did not impact the survivability of the bacterial population. The process of complex coacervation involves microencapsulation by inducing phase separation in an aqueous solution containing two or more biopolymers with opposing electrical charges [14]. As a result, the coacervate layer that surrounds the bioactive component is created and aggregates. A wide range of protein-polysaccharide combinations have been used to create complicated coacervates in recent years. Examples include the combination of whey protein with gum arabic (GA) or sodium caseinate and low methoxyl pectin. [15]. GA is regarded as the oldest type of natural gum and is widely used in many different industrial goods all over the world. It has been demonstrated by several studies that GA serves as a prebiotic and improves host health. Moreover, GA functions as a bifidogenic dietary fiber that is fermented by gut microbes to produce short-chain fatty acids [16].

Table 1. Effect microencapsulation and drying process on the survivability of *Lim. reuteri.*

The microencapsulation steps	Log. CFU/ g or mL	ME (%)
Number of Fee cells in microencapsulation	11.74±0.21	-
Number of viable cells in microencapsulation solution after mixed	10.53±0.51	89.69±2.11

Number of viable cells in microencapsulation powder after drying	9.22±0.37	87.55±1.99

3.2. Stability of the microencapsulated Lim. reuteri in simulated stomach conditions

The viability and stability of *Lim. reuteri*, whether free or microencapsulated, were evaluated under simulated stomach conditions. Results revealed a rapid decline in ME % for free cells, whereas encapsulated cells with whey proteins and gum Arabic displayed higher stability (Figure 1). The ME % of free cells decreased from 100.00% to 58.96% after an exposure period of 240 minutes. In the case of microencapsulation with whey proteins and gum arabic decreased from 100.00% to 82.11%. For probiotic bacteria to provide the desired effects, cell survival in the stomach and intestinal environments is essential. The findings of this investigation are consistent with those of Frakolaki et al. [17], who demonstrated that the use of polymers in probiotic bacteria microencapsulation contributes to the protection and maintenance of the intended viability of probiotic bacteria in acidic environments. However, to express probiotic activity as best as possible, these bacteria need to survive the circumstances of the gastrointestinal tract (GI) in large quantities, reaching a count of 10^6–10^7 CFU/g or mL at the end of the product's shelf life. The bacterial cells are microencapsulated with components of proteins and polysaccharides that increase their stability and capacity to survive in low pH settings. [18].

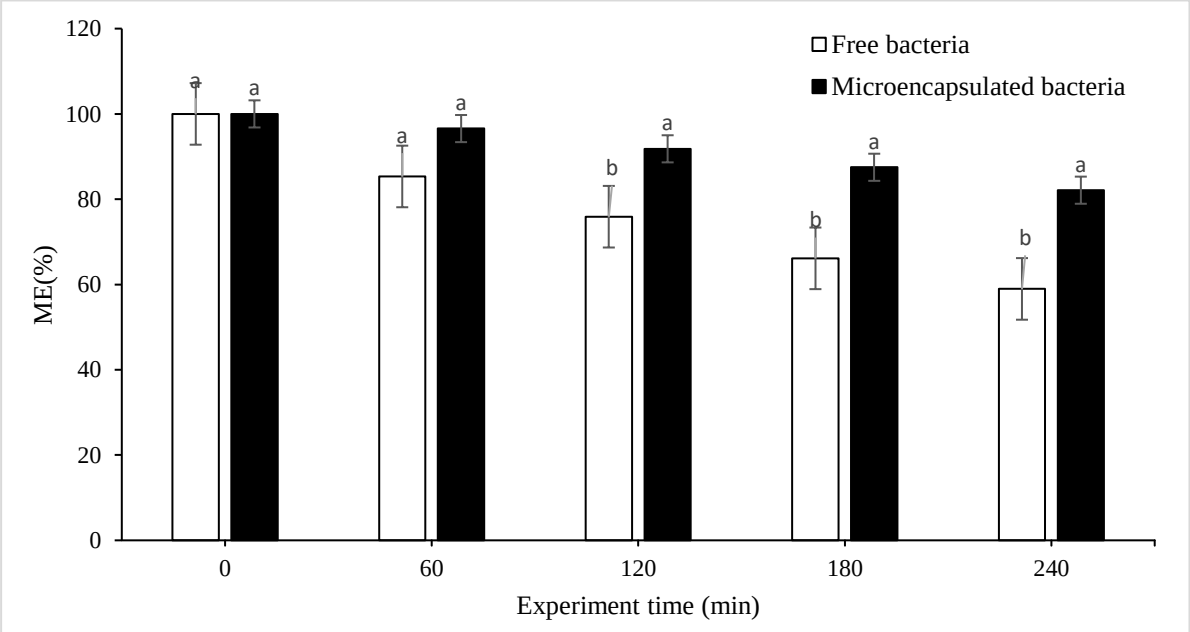

Fig 1. Microencapsulation efficiency and numbers and ME (%) of free and microencapsulated *Lim. reuteri* under the stomach conditions at 0, 60, 120, 180, and 240 minutes.

3.3. Stability of the microencapsulated Lim. reuteri in simulated intestinal conditions

The results illustrated that microencapsulation offers shielding to probiotics when exposed to simulated conditions in the intestines. A rapid decline in the number of free bacteria cells was noted in comparison to the microencapsulated *Lim. reuteri* under simulated intestinal conditions with a pH of 7.5 (Figure 2). The microencapsulation of the *Lim. reuteri* cells with whey proteins and gum arabic had a statistically significant effect ($p< 0.05$) on Microencapsulation efficiency (%). ME (%) of microencapsulation with whey proteins and gum Arabic decreased from 100.00% to 84.66% over an exposure of 240 minutes. While ME (%) of free bacterial cells was 61.26% after 240 minutes. Achieving the desired benefits of probiotics requires maintaining cell viability in a variety of stomach and intestinal environments. The release of microencapsulated cells may be impacted by a high pH in

the intestinal environment. Bacterial cells were kept at a high pH during the microencapsulation procedure, which caused the bacteria to release from the microencapsulation powder gradually under intestinal circumstances that were mimicked. When compared to free cells, the probiotic starting culture's encapsulation into protein-carbohydrate carriers resulted in a significant increase in cell viability throughout both digestion and storage. Regarding cell protection during manufacture and storage, the use of the microencapsulation approach showed promise [9]. When compared to the non-microencapsulated bacteria, the microencapsulated bacteria were marginally affected by an extended exposure duration to the simulated stomach environment. The methodical procedure of microencapsulating probiotic bacteria is essential for preserving their viability and protecting them from environmental stresses outside of their cells. This approach helps to ensure that the population of bacteria reaches sufficient numbers to colonize the host's small intestine, improving the intestinal ecology as a whole.

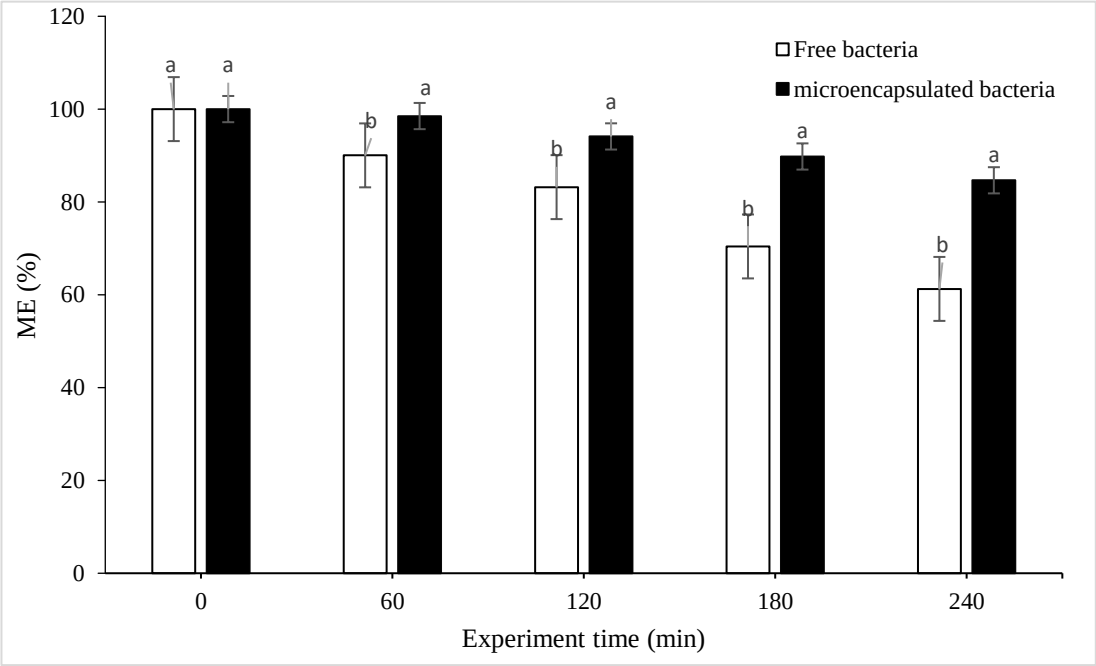

Fig 2. Microencapsulation efficiency and numbers and ME (%) of free and microencapsulated *Lim. reuteri* under the stomach conditions at 0, 60, 120, 180, and 240 minutes.

3.4. Stability of free and microencapsulated Lim. reuteri in thermal stress

The viability of probiotic bacteria is crucial when they are exposed to heat treatments. Liquid food products such as milk and juices typically undergo rigorous heat treatment processes (such as pasteurization and ultra-high temperature treatment) to eliminate pathogens. However, the effectiveness of probiotic bacteria may be compromised during heat treatment. According to a study by Ding & Shah [19], microencapsulation can enhance the survival of probiotics in high-temperature conditions. The results of the present research showed that high temperatures were detrimental to the survivability of free *Lim. reuteri*. After being exposed to a particular temperature for a predetermined amount of time, microencapsulated *Lim. reuteri* cells showed improved survival. Probiotics that were microencapsulated saw less heat transfer from the surrounding environment, which allowed them to remain viable even at very high temperatures. The percentage of viable cells of the microencapsulated bacteria reached 80.49%, 76.98%, 65.74%, 62.06%, 62.45%, and 53.79% for 60°C for 3 minutes, 65 °C for 3 minutes 70°C for 2 minutes, 75°C for 2 minutes, 80°C for 1 minutes, and 80°C for 2 minutes, respectively. While The percentage of viable *Lim. reuteri* cells of the free bacteria was 52.12%, 47.97%, 36.90%, 30.15%, 30.55%, and 15.09%, respectively, (Figure 3). Numerous variables, including bacterial strain, temperature, microcapsule size, gum arabic content, and others, affect the

heat tolerance results. Previous studies by many authors have demonstrated that microencapsulation can improve a cell's ability to survive at a certain temperature. The earlier study's conclusions suggest that heat treatments did not affect the microencapsulated cells' increased capacity for survival. More specifically, microencapsulated *Lactiplantibacillus plantarum* 48M cells showed increased susceptibility. After treatments for three minutes at 60°C and two minutes at 70°C [11].

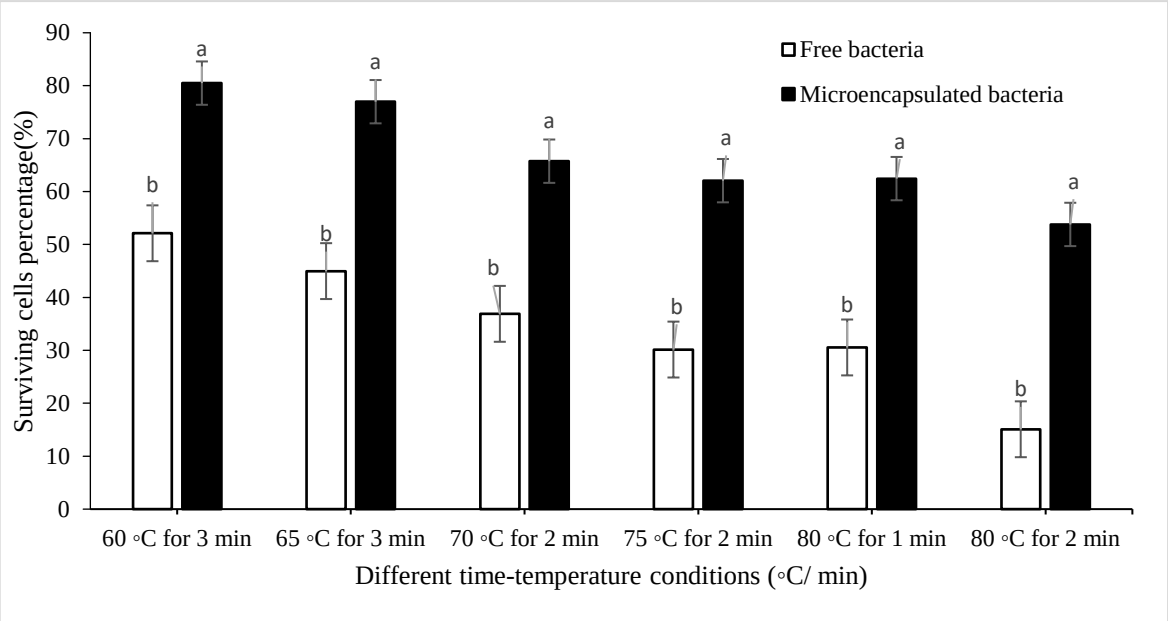

Fig 3. The percentage of surviving cells of free and microencapsulated *Lim. reuteri* under thermal stress.

3.5. Stability of free and microencapsulated Lim. reuteri under refrigerated conditions

The impact of a lower temperature on the survival of *Lim. reuteri* whether it is encapsulated or not is substantial and remarkable. Low temperatures have been shown to interfere with the enzymatic functions that occur inside bacteria's cellular structure, making it more difficult for the organism to absorb vital nutrients that are necessary for its development and nourishment. The results of the study indicate that the technique of microencapsulation serves a pivotal role in bolstering the ability of *Lim. reuteri* bacteria to withstand the adverse effects of cold environments, as visually depicted in Figure 4 of the research report. It was noted that the viability percentage of microencapsulated *Lim. reuteri* exhibited a decline to 97.61% following a storage period of 45 days in refrigerated conditions, and a further decrease to 95.14% after 60 days. In stark contrast, the survival rate of free-floating bacterial cells stood at 95.22% after 15 days, followed by reductions to 90.38%, 88.63%, and 86.91% at the 30-day, 45-day, and 60-day marks, respectively. These results highlight the vital role that encapsulation plays in maintaining *Lim. reuteri* viability for a prolonged length of time under cold storage settings, underscoring the significance of these preservation techniques in the field of probiotics and biotechnology research. The study's data provides important insights for future research endeavors aimed at improving the stability and efficacy of probiotic formulations in various applications by illuminating the relative resilience of encapsulated versus free *Lim. reuteri* cells when exposed to extended periods of refrigeration. The results of this investigation corroborated those of Dianawati et al. [20], who discovered that probiotic bacteria in carrier food may be preserved using microencapsulation and kept viable for up to 28 days at 4°C. When refrigerated (4ºC) for more than 21 days, microencapsulated *Lacticaseibacillus rhamnosus* bacterium with alginate gum coating showed better vitality than free cells. [21].

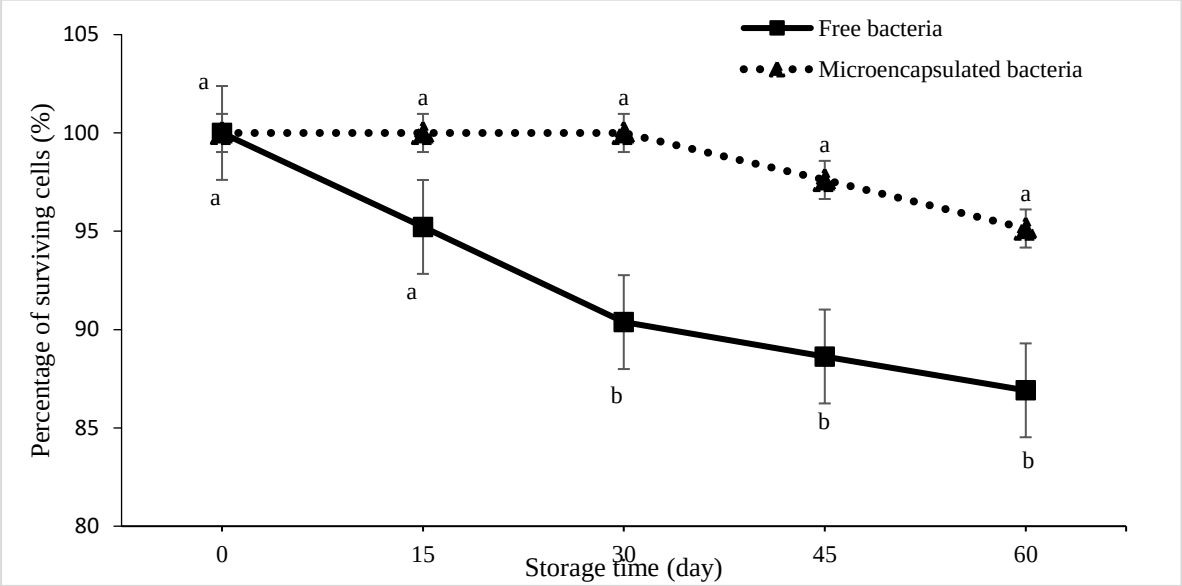

Fig 4. The percentage of surviving cells of free and microencapsulated *Lim. reuteri* under refrigerated storage conditions**.**

4. Conclusion

The results of the study indicated the vital function that microencapsulation performs in improving *Lim. reuteri* vitality and stability under harsh manufacturing conditions and during in vitro digestion. Gum arabic and whey proteins seem to be extremely effective polymers in ensuring *Lim. reuteri* viable and accurate distribution. Furthermore, under circumstances that mimic the stomach, whey proteins and gum arabic were proven to have better protective qualities. When exposed to higher temperatures, the microencapsulated bacteria performed better than the loose cells, demonstrating improved resilience. Moreover, a 60-day trial period of low-temperature storage produced no discernible changes in the bacteria's survival rate. These results highlight the possibility of microencapsulation as a workable strategy for enhancing bacterial resistance in harsh environmental settings. The delivery of health benefits is ensured by maintaining the appropriate amounts of *Lim. reuteri* by microencapsulation, even in unfavorable health situations.

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