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Microencapsulation containing *L. acidophilus* and *S. boulardii* for targeted release in the stomach and small intestine

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Abstract

In this study, a microencapsulation system was developed with a dual-layer structure comprising *Lactobacillus acidophilus* and *Saccharomyces boulardii* to enhance the survival rates of probiotic bacteria during transit through the stomach. Alginate and chitosan were identified as key factors influencing the shape, moisture content, and the number of encapsulated microorganisms in the microcapsules. The optimal concentrations of alginate and chitosan were determined to be 0.5 % and 3 %, respectively. The microencapsulated structure was clearly visualized through Scanning Electron Microscope images, and Infrared spectra confirmed the successful encapsulation. Experimental findings revealed that *S. boulardii* was released directly in the stomach, while L. acidophilus was released after a 2-hour delay, coinciding with the arrival of microcapsules in the small intestine. Subsequently, we proposed the release kinetics of microcapsules in the gastrointestinal tract. Although the viability of both *S. boulardii* and *L. acidophilus* in the microcapsules exhibited a steady decline over the storage period, a notable 10^9 CFU/g of bacteria persisted even after 120 days.

Keywords: probiotics, *L. acidophilus*, *S. boulardii*, viability, release.

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Introduction

Probiotics are living microorganisms that positively affect the host organism when given in suitable amounts. The International Scientific Association of Probiotics and Prebiotics (ISAPP) has upheld this idea since 2013. All probiotics are considered good companions for human and animal health and exert their effects usually in the gastrointestinal tract, where they may influence the intestinal microflora (Hill et al., 2014).There are some important probiotic microorganisms like strains of *Lactobacillus* (Jeong et al., 2022; Ravina, et al., 2023), *Bacillus* (Łubkowska et al., 2023; Luise et al., 2022) and *Bifidobacterium* (Li et al., 2023; Chen, Chen, and Ho, 2021) as well as yeasts *Saccharomyces boulardii* and *Saccharomyces cereviae* (Hedin, Kruse, Vazquez-Uribe, and Sommer, 2023).

Lactobacillus species are generally considered to be nonpathogenic and are widely used as probiotics and starter cultures by various companies for the production of a variety of functional, edible probiotic preparations (Bósquez et al., 2022). Among them, *L. acidophilus*, an important intestinal probiotic, has had a great deal of focus placed upon it in terms of research and development. *L. acidophilus* can inhibit the growth and reproduction of pathogenic bacteria by reducing the intestinal pH and producing metabolites (Tegegne and Kebede, 2022). An important mechanism for *L. acidophilus* to inhibit the function of pathogenic bacteria is competition for adhesion sites with pathogenic bacteria, thereby interfering with their invasion

into cells (Sigh et al., 2013). In addition, compared with many other probiotics, *L. acidophilus* can survive and multiply rapidly in the harsh environment of the gastrointestinal tract due to its good resistance against both acid and bile salts (Gao et al., 2022).

Saccharomyces boulardii is the first yeast that has been studied for use as a probiotic strain in human medicine. Currently, this yeast probiotic is used in most countries because of its efficacy and safety in the treatment or prevention of different diseases such as diarrhea, inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), candidiasis, dyslipidemia as well as gastrointestinal symptoms associating with small intestine bacterial overgrowth (SIBO) in multiple sclerosis (MS) patients (Pais, Almeida, Yılmaz, and Teixeira 2020; Le and Trinh, 2018). *S. boulardii* has several different types of mechanisms of action such as immunological and anti-toxin effects, pathogen-binding, as well as effects on digestive enzymes (Kaźmierczak-Siedlecka et al., 2020).

Many studies have shown that the combination of *S. boulardii* and *Lactobacillus* is harmless and has the ability to create synergistic beneficial bacterial effects on the digestive tract (Khoramdareh, Hamedi, and Sharifan, 2022; Naghibzadeh, Salmani, Nomiri, and Tavakoli, 2022; Ghasemi-Niri et al., 2012; Goktas et al., 2022). *S. boulardii* survives in gastric acid, so it can be released before the pyloric valve. *Lactobacillus* grows well in the small intestine, that means it needs to be released in the small intestine to have the best effect. In the stomach, *S. boulardii* can persist and prevent the growth of *H. pylori*. *Lactobacillus* thrives in the small intestine, making it essential to release it there for optimal effectiveness. *S. boulardii* can persist in the stomach, inhibiting the growth of *H. pylori*. On the other hand, *L. acidophilus*, if unprotected, faces the risk of cell death in the low pH environment of the stomach (below pH 2), but it flourishes in the intestine (Nezamdoost-Sani, Khaledabad, Amiri, and Khaneghah, 2023). The combination of *L. acidophilus* and *S. boulardii* in microencapsulation facilitates the encapsulation of more microorganisms. Microcapsules will release *S. boulardii* right in the stomach because *S. boulardii* can survive in the stomach, then *L. acidophilus* is released after 2 h when the microcapsules reach the small intestine, avoiding acidic pH in the stomach.

Probiotic preparations for the gastrointestinal tract must contain at least 10^7 CFU/mL of live microorganisms up until the expiration date to be effective (Zoghi et al., 2019). However, there are many factors that reduce the viability of probiotics during their passage through the gastrointestinal tract including pH, concentration of acetic and lactic acid, dissolved oxygen content, hydrogen peroxide, etc. Microencapsulation has been considered an efficient and novel technique for improving the viability of probiotics in the intestinal tract. Microencapsulation involves packing living cells and organisms into tiny cysts using film-forming agents (gels), such as polymers made of natural materials like gelatin, alginate, chitosan, and cellulose, or synthetic ones like polyester, polyamide, polystyrene, polyacrylate, and polyacrylamide (Wang, Li, Liu, and Wang, 2021). Alginate and chitosan are two natural polymers that are applied successfully as a pH-sensitive material for the microencapsulation of probiotic bacteria (Allan-Wojtas, Truelstrup Hansen, and Paulson, 2008). Alginate easily forms gel to cover bacteria cells, the resulting gel layer is highly stable. The process of microencapsulating probiotics with alginate is easy and simple, can be done at room temperature, so it has little effect on living cells. They are inexpensive, widely available, and microorganism-friendly. Therefore, we chose these two polymers to conduct research.

In this study, our primary objective was to pioneer the development of a microencapsulation two-layers system designed to synergistically release *S. boulardii* in the stomach, followed by a sustained release of *L. acidophilus* in the small intestine. The overarching stability goal of this system was to preserve a significant count of viable microorganisms approximately 8–9 log CFU/g ensuring their viability for an extended period of over three months during storage.

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Materials and methods

Bacterial strains and sample preparation

Lactobacillus acidophilus ATCC 4356 and *Saccharomyces boulardii* CNCM I-745 (National Institute of Drug Quality Control, Vietnam). Natri alginate (Alg) and chitosan (CHI) (Sigma-Aldrich, USA) were purchased. Other products used included Tween 80 (Sino-Japan Chemical, Taiwan), Sodium Citrate, Sodium Chloride, Calcium Chloride (Shanghai, China), Peptone (Himedia, India), Hydrochloric Acid, Monopotassium Phosphate (Sigma-Aldrich, U.S.A.), MRS agar and MRS broth (Merck, Germany), Starch (San Star, India).

Preparation of probiotic bacteria

ACTIVATION OF STRAINS

Activation from freeze-dried *L. acidophilus* in 100mL of liquid MRS (Casein pepton, Meat extract, Yeast extract, Glucose, Tween 80, K_2HPO_4, Na acetate (CH_3COONa), $MgSO_4$, $MnSO_4$) and *S. boulardii* in YPD (Yeast extract, pepton, and Dextrose), cultured for 24 h in a CO_2 cabinet at 37 °C for *L. acidophilus* and 30 °C for *S. boulardii.*

PROPAGATION

The culture was repeated in order to obtain the required number of bacteria. To get rid of any remaining traces of glucose, the cultures (Hettich Eba, Germany) were centrifuged at 8000 rpm for 20 min at 4 °C. A sterile 0.9 % NaCl solution was used for two cleanings.

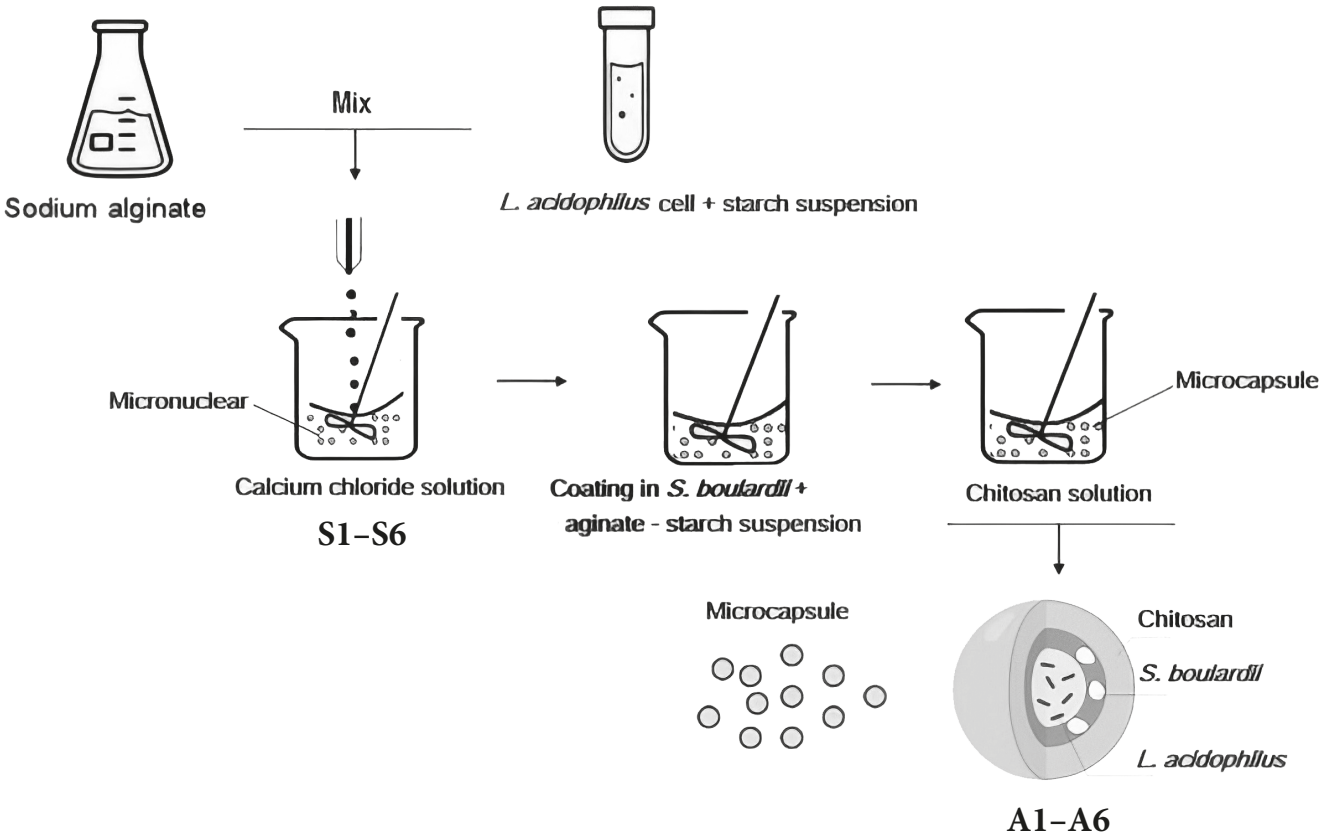


Fig. 1. Diagram of experimental stages for microcapsule preparation.

Microencapsulation procedure

Figure 1 showed experimental stages for microcapsule preparation.

MICROENCAPSULATION IN SODIUM ALGINATE

Probiotic bacteria were microencapsulated in sodium alginate using extrusion techniques, following the method outlined by Krasaekoopt et al. (2003) Holkem et al. (2016) with some modifications. A microbial biomass was obtained by adding 100 mL of the *L. acidophilus* propagation suspension. A uniform mixture of bacteria and alginate was dripped into $CaCl_2$ solution and stirred for 30 min (Daihan Scientific, Korea). Microcapsules were collected and washed in a 0.9 % NaCl solution. After that the microcapsules were added to the chitosan solution for 30 min. Microcapsules were then collected with a net and cleaned with 0.9 % NaCl to obtain *L. acidophilus*-containing microcapsules.

ENCAPSULATED SUSPENSION CONTAINING *S. BOULARDII*

A micronuclear contained *L. acidophilus* inside and *S. boulardii* in the layer coating would protect probiotics better. 15g micronuclear were stirred at 100 rpm for 30 min in 100 mL of encapsulated suspension. Added the microcapsules to the $CaCl_2$ solution for 30 min, washed in 0.9 % NaCl, then took out the seeds and washed with 0.9 % NaCl.

FREEZE-DRIED MICROCAPSULE

The bacterial suspension underwent freeze-drying by freezing at –40 °C for 24 h, followed by a 72 h freeze-drying process. Subsequently, the freeze-dried microcapsules were stored at two different temperatures (25 °C and 4 °C) for a duration of 8 weeks.

Probiotic cell count

Microcapsule cell counting was fulfilled by the method provided by Holkem et al. (2016) with some modifications. One mL of microcapsules was added to 9 mL of sterile sodium citrate solution (2 % w/v, pH 7), and it was homogenized by a stomacher (Circulator 400, Seward, UK) at 260 rpm for 4 min. During this process, beads were destroyed and bacterial cells were released. Serial dilution step with sterile peptone water solution (0.1 %) was performed using pour plate method in MRS. Finally, the number of bacteria was counted after 37 h of incubation at 37 °C. For free cell count, the pour plate technique was performed according to the method provided by de Lara Pedroso, Thomazini, Heinemann, and Favaro-Trindade (2012) with some modifications. It should be noted that *L. acidophilus* was inoculated in the MRS Agar and *S. boulardii* in YPD. All plates were done in two repetitions.

Encapsulation efficiency

The efficiency of encapsulation, showing the number of living microorganisms during the microencapsulation process, was calculated using equation (Maleki et al., 2020):

$$H\ \% = (N/N_0) \times 100$$

where H % is the percentage of the efficacy of capsulation; N denotes the number of cells released from capsules (CFU/g) and N_0 represents the number of live cells used for encapsulation (CFU/g).

Stability of microencapsulated bacteria during storage

The freeze-dried encapsulated microcapsules were stored at two different temperatures (25 °C and 4 °C) for a duration of 8 weeks, and the survival rate was assessed using the method outlined in the previous sections (McFarland et al., 2017) following the percentage of the survival in storage time.

Survival of probiotics after exposure in gastrointestinal conditions

The viability of free cells, Alg/CHI encapsulated microcapsules was assessed under simulated gastrointestinal tract conditions in vitro. Simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) were prepared using a modified method (Khosravi Zanjani, Ghiassi Tarzi, Sharifan, and Mohammadi, 2014; Yonekura, Sun, Soukoulis, and Fisk, 2014). The compositions of SGF, and SIF are detailed in Table 1. The components were dissolved in 100 mL of distilled water and thoroughly mixed. The volume was then adjusted to 500 mL with distilled water. Enzymes were introduced into the mixture, and all solutions were filtered using a 0.45-μm filter. All simulated fluids were stored at 4 °C and utilized within 24 h.

Table 1. The components of simulated gastrointestinal/ intestinal fluids

SGF (pH 1.5 ± 0.02)		SIF (pH 6.5 ± 0.01)	
NaCl	175.3	NaCl	175.3
KCl	89.6	KCl	89.6
NaH_2PO_4	88.8	$NaHCO_3$	84.7
NH_4Cl	30.6	$CaCl_2$	22.2
$CaCl_2$	22.2	NaH_2PO_4	8.0
Pepsin	2500	$MgCl_2$	5.0
—	—	Pancreatin	3000

Note: SGF, and SIF stand for simulated gastric fluid, and simulated intestinal fluid, respectively.

Characteristics of microcapsules

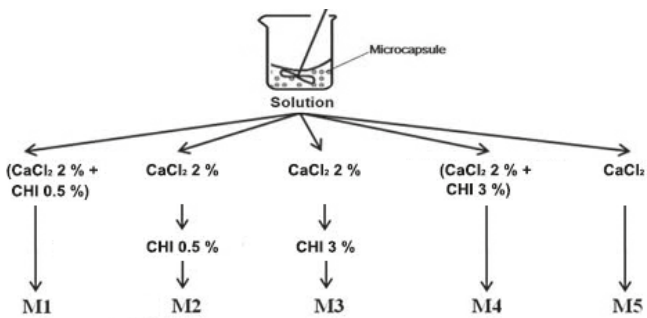
MORPHOLOGICAL CHARACTERISTICS

To observe the morphology of microcapsules, the surface of microcapsules with different magnifications was characterized by an SEM (Scanning Electron Microscope) (S-4800 Hitachi, Japan) at room temperature. The electron was reflected to the surface of the sample coated with gold in the vacuum environment, then collected by the detector and transformed into an optical photon to create a visible image.

IR ABSORPTION SPECTRUM

IR Absorption Spectroscopy (Agilent FT IR 630, USA) of chitosan, alginate-chitosan microcapsules after encapsulation containing microorganisms were recorded on an infrared spectrometer with 8 scans and spectrum recording range from 650 to 4000 cm^{-1}. The absorption spectra obtained were compared to evaluate the presence of chitosan on the microcapsule shell.

The process of preparation of different microcapsule samples depended on chitosan concentration following the diagram below:



(M1.1: scan M1 in the 1st IR sample).

EXPERIMENTAL DESIGN AND DATA ANALYSIS

All experiments were repeated 3 times (n = 3) and the average value was calculated, RSD < 3. Analysis of variance was done at α = 0.05, and the least significant difference test was used to confirm the difference between the means at $p < 0.05$ using Microsoft Excel 2016 software.

Results and discussion

Effect of alginate concentrations on microencapsulation

EFFECT OF ALGINATE CONCENTRATIONS ON MICRONUCLEAR AND MICROCAPSULES COATING

Alginate is a cheap, convenient, non-toxic, and safe raw material. Alginate gels quickly at neutral pH and room temperature, making it appropriate for sensitive bio-

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Fig. 2A. The micronuclear shape changed depending on the alginate concentration (0,1 %; 1 %; 2 %; 3 %; 4 %; 5 %).

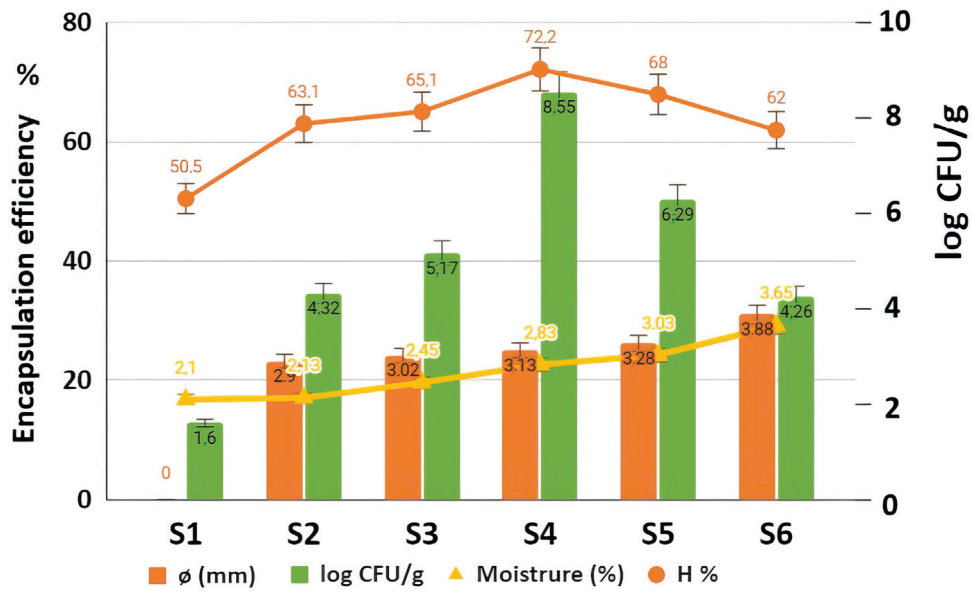

Fig. 2B. Microcapsule size, moisture content, and encapsulated microbial density when Alg concentration changes in Alg solution drops (0 %; 1 %; 2 %; 3 %; 4 %; 5 %).

molecules including proteins and nucleic acids as well as living cells. Alginate creates a thick, stable gel coating that easily covers bacterial cells (Holkem et al., 2016). Bacterial microencapsulation by alginate was a straightforward technique that can be carried out at room temperature with no impact on living cells. Reversibility of the gel's formation aids in the inner cells' liberation. The resulting microcapsules were exquisite and very homogenous. The diameter of microparticles was controlled by the concentration and viscosity of sodium alginate (Hansen, Allan-Wojtas, Jin, and Paulson, 2002). In different alginate concentrations, the changes in the viability of *L. acidophillus* inside micronuclear were compared with each other and were significant ($p < 0.05$).

As can be seen in Figs 2A, 2B when the alginate concentration was gradually increased, the alginate solution concentration below 2 % produced S1–S3 microcapsules, the shape of the microcapsules was uneven, the particles were soft and easily broken after the solidification process (Holkem et al., 2016). S4 micro-nuclear was uniform and beautiful. For S5–S6, the viscosity of the solution increased during preparation, the suspension for forming microcapsules became thicker, droplets were less likely to form, the dropper was more likely to clog, the preparation

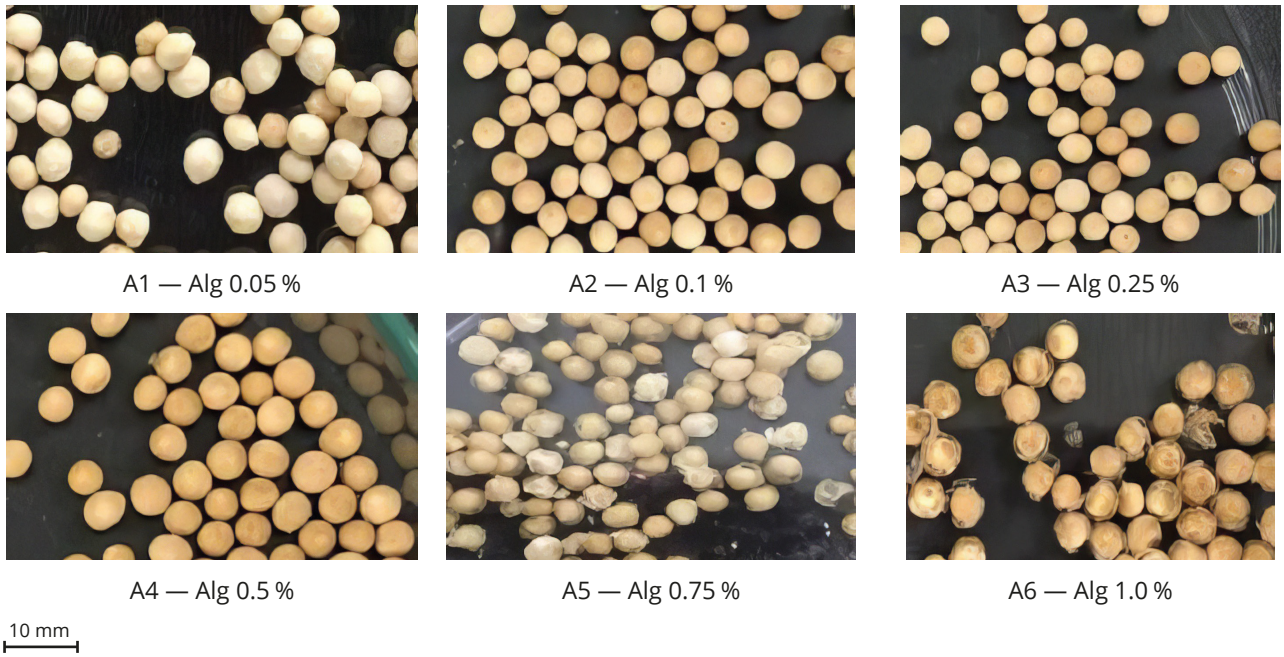

Fig. 3A. The shape of alginate beads when alginate concentration was changed in 1g microcapsules coating (0.05 %; 0.1 %; 0.25 %; 0.5 %; 0.75 %; 1 %).

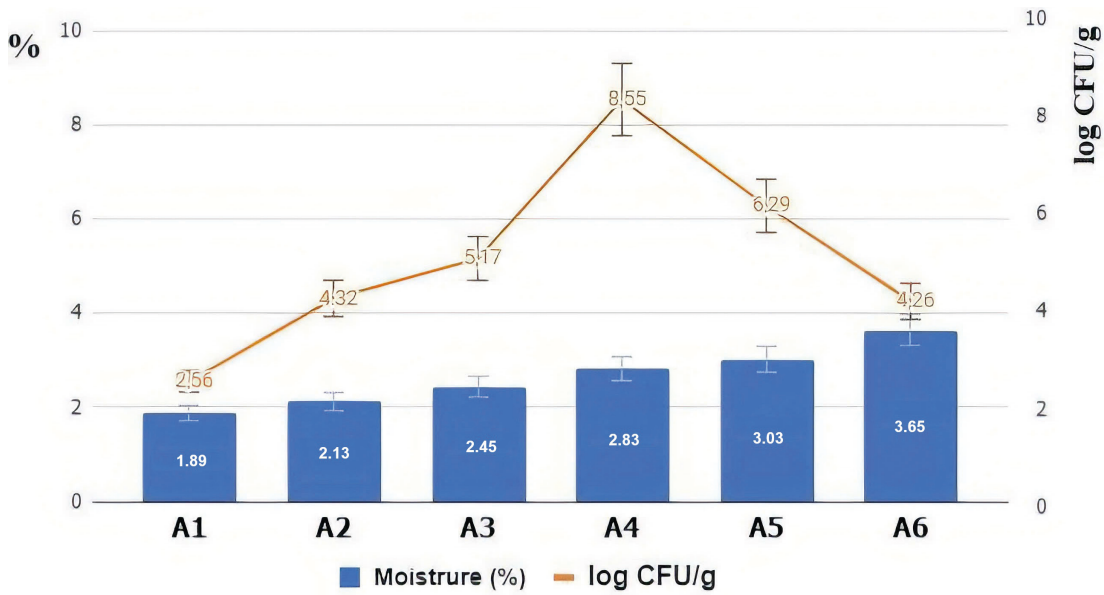

Fig. 3B. Moisture and viability of micronuclear when alginate concentration was changed in 1g microcapsules coating (0.05 %; 0.1 %; 0.25 %; 0.5 %; 0.75 %; 1 %).

process was more difficult, and irregular particles were formed (Gåserød, Sannes, and Skjåk-Braek, 1999).

In fact, the concentration of alginate solution affected the viability of *S. boulardii* in the layer coating. The difference between groups S1–S6 was statistically significant ($p < 0.05$).

Additionally, the moisture content rose steadily along with the alginate concentration. The micro-egg model's connection network was quite tight after freeze-drying, making it harder for the water within to evaporate. As a result, the moisture content also tended to rise. The S4 had the largest encapsulation capacity available. Microorganisms would occupy the empty cavities left by the broken bonds between the alginate and calcium ions during forming microencapsulation. The proportion of encapsulated microorganisms could not be increased or even decreased until the empty compartments were completely filled with bacteria (Lee et al., 2019). Therefore, it inferred that the alginate concentration impacts the micronuclear, microcapsules characteristics and microbial population.

From Figs 3A, 3B it can be seen that *S. boulardii* maintained in the envelope reduced from 8.55 to 4.26 log CFU/g as the alginate concentration rose to high levels (> 0.5 %). The coating suspension thickens and becomes more viscous at the same time, making preparation

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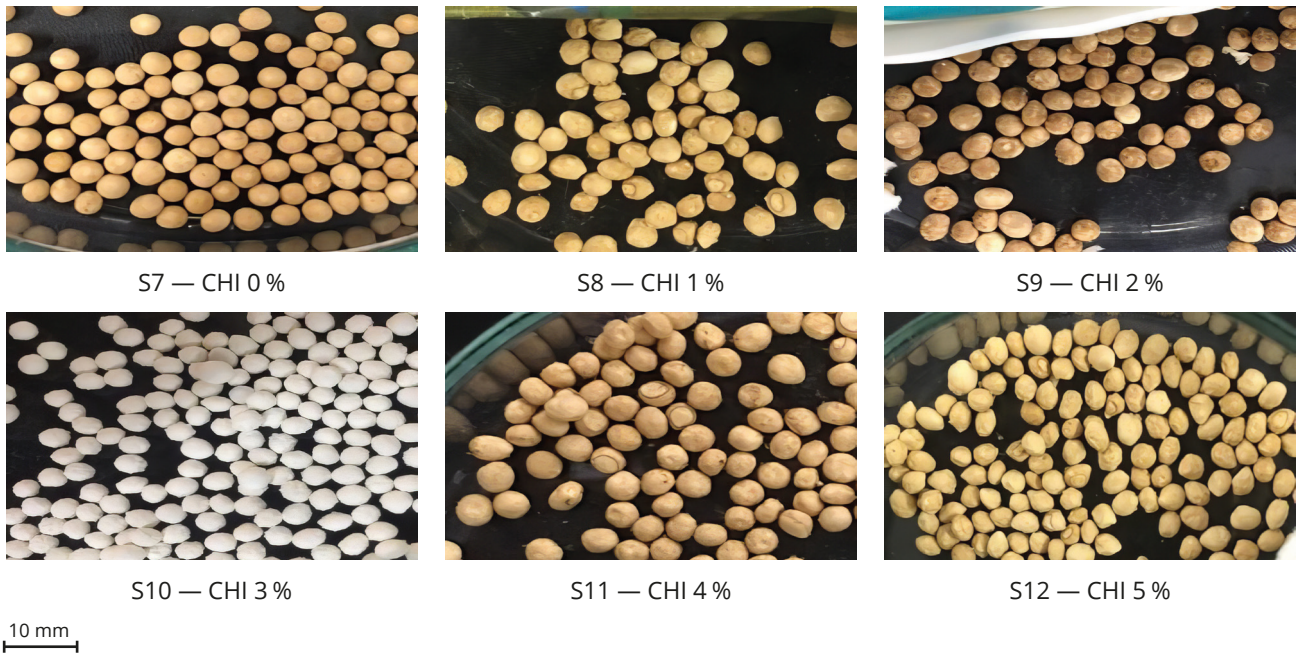


Fig. 4A. The image of encapsulated microcapsules changes with increasing CHI concentration in the coating solution.

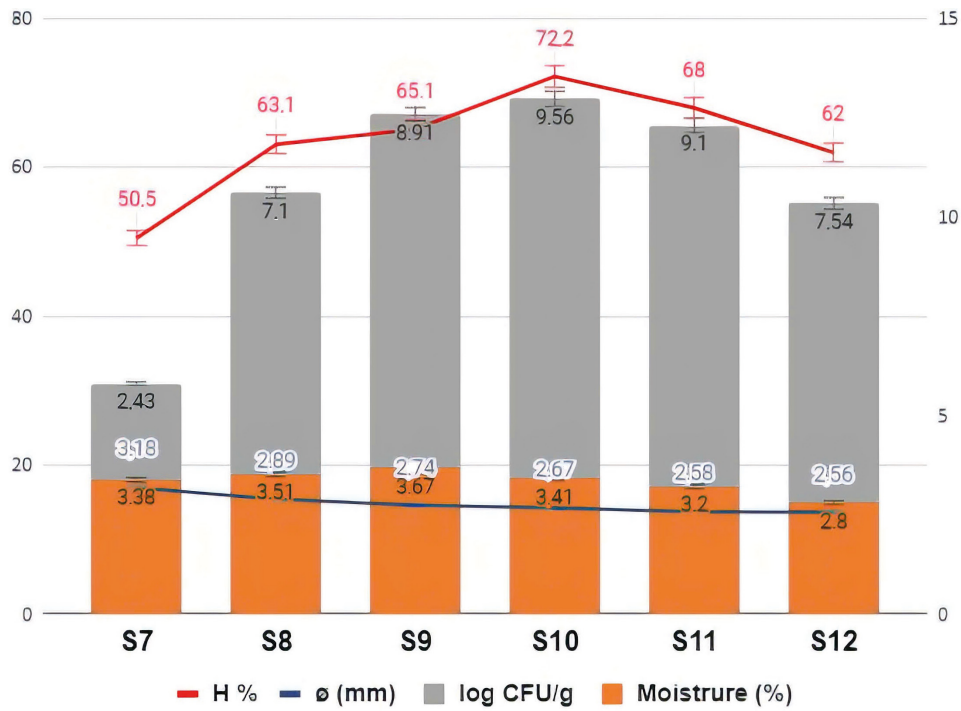


Fig. 4B. Microcapsule size, moisture, and encapsulated microbial density when CHI concentration changes (0 %; 1 %; 2 %; 3 %; 4 %; 5 %).

more challenging. The primary cause is that as alginate concentration rises, more linkages are added to the network, allowing more microorganisms to be captured in the vacant compartments. However, once the maximum number of bacteria is achieved, they are packed into the empty compartments and cannot increase. Starch and yeast biomass are difficult to disperse uniformly in the coating film due to the coating suspension's high viscosity, and the coating film is also easily ripped off after lyophilization (Holkem et al., 2016; Khosravi Zanjani, Ghiassi Tarzi, Sharifan, and Mohammadi, 2014).

Along with the increase in alginate concentration (from 0.1 % to 1 %), the moisture content also increased correspondingly, rising from 2.14 % to 3.65 %. Following is an explanation for this: alginate concentration rises in the envelope membrane, thickening the linkages and tightening the micro-egg model's network (Nezamdoost-Sani, Khaledabad, Amiri, and Khaneghah, 2023).

Water is difficult to evaporate, the freeze-drying process takes a long time, and the moisture content of the microencapsulation rises (Holkem et al., 2016).

EFFECT OF CHITOSAN CONCENTRATIONS ON MICROCAPSULES COATING

One of the biopolymers with a natural origin is chitosan. Chitosan serves as an efficient coating, safeguards bacterial cells during handling and storage, and ensures that microorganisms could survive the digestive process (acidity, stomach, bile salts, etc. and made it to the large intestine with enough microorganisms to exert its effects (Gåserød, Sannes, and Skjåk-Braek, 1999).

Figures 4A, 4B depicts the effect of chitosan coating on the alginate capsules. The analysis revealed that the size of the microcapsules tended to gradually shrink when the chitosan concentration was changed. Conversely, when the chitosan concentration rose, so did the moisture content. Only 2.43 log CFU/g, S7 did not have a low packing efficiency. The number of encapsulated microorganisms significantly increased to 7–9 log CFU/g and continuously increased with increasing chitosan concentration, reaching its greatest level in S10 (chitosan 3 %) at 9.56 log CFU/g. These results were also consistent with previous studies by research groups such as Cook et al. (2012), Chavarri et al. (2010). The S8-S12 had chitosan in the coagulation solution. These results could be explained as follows: Chitosan is an amine by nature and is extremely hygroscopic when kept in the air, which is relevant to the change in moisture content. As a result, the moisture content of the microcapsules increases together with the chitosan concentration. The $-NH_2$ in chitosan structure and the -COOH group of alginate linked chitosan and alginate together, creating a tight network that traps probiotics together with the network of Alg and calcium ions (Holkem et al., 2016). These links follow dimensions in space, forming a model network that captures microorganisms which liked microegg models. On the other hand, CHI aids in increasing packaging efficiency. In order to protect bacteria, nuclear microencapsulation created a double coat when CHI and Alg was used simultaneously. If the nuclear microencapsulation was contained by a single alginate, the monovalent ions present in the GI tract at low pH levels would quickly destroy it. Alginate microcapsules were enhanced with polymeric polycation coatings such as chitosan and poly-L-lysin to lessen the loss of packaging material. By generating a high charge density on their surface and boosting the stability of the microencapsulation, microcapsules with a chitosan-alginate double coat aid in the enhanced microorganism protection (Calinoiu et al., 2019).

The average diameter of particles was modest compared to the results obtained in other research for the microencapsulation by emulsion method due to the use of a low diameter syringe (Le and Trinh, 2018; Khosravi Zanjani, Ghiassi Tarzi, Sharifan, and Mohammadi, 2018). In addition, the size of the microencapsulated particles tends to gradually get smaller as the chitosan concentration rises. This is attributed to chitosan's involvement in both the coagulation process and the network's gel layer formation. The microcapsules often occupy less area than the typical network bonds with only Ca^{2+} and alginate because the entrance between the bonds makes the bonds tighter (Hansen, Allan-Wojtas, Jin, and Paulson, 2002). The survey results indicated that the most microorganism packages were produced at a chitosan concentration of 3 %, hence this concentration was chosen for the following studies.

MICROENCAPSULATED STRUCTURE

Micronuclear and encapsulated microcapsules have rather thick interior structures. This outcome is in line with the claims made by other researcher, who claim that during the gelation process, the microcapsules form a conjugated contour structure with cross links that are directed from the grain's outer core to its inner core, resulting in spherical microcapsules (He at al., 2021). Even though the hydrogel network generates thick concentric layers after lyophilization, the gaps in the microencapsulation lumen remain due to vapor sublimation. The microencapsulated structure has a layered appearance, as shown in the photographs of the samples taken under the scanning electron microscope (Fig. 5), and the inner layer is thick. The thick outer shell encloses the inner layers, which resemble concentric circles. The microcapsule is made up of a network of cells that are crisscrossed and connected to one another. The microcapsule is made up of a network of interconnected cells that resembles a sponge (Allan-Wojtas, Truelstrup Hansen, and Paulson, 2008).

The picture at 150X magnification reveals that the starch granules that make up the layers of the microcapsules are similarly organized in chains, are stable, and are not entirely dense. They are connected by an alginate gel. Microcapsules will therefore be quite hygroscopic if not properly protected after freeze-drying. *L. acidophilus* and *S. boulardii* were combined in microcapsules. Although, there were not any published studies showing inhibition or antagonism between *L. acidophilus* and *S. boulardii* strains. The synergistic effect of these two strains requires more extensive in vitro/vivo studies. However, in fact, multi-strain probiotics are composed of more than one species or strains of bacteria and sometimes, includue some fungal species with benefits to human and animals' health (Kwoji, Aiyegoro, Okpeku, and Adeleke, 2021).

It is feasible to discern the microbial cells sticking to the surface of the starch granules at magnifications of 5000–10,000X (Fig. 6). At 5000X magnification, yeast cells are readily visible. They are oval in form and packed closely together like a string of pearls. *L. acidophilus* cells appear as rod-shaped clusters at magnifications greater

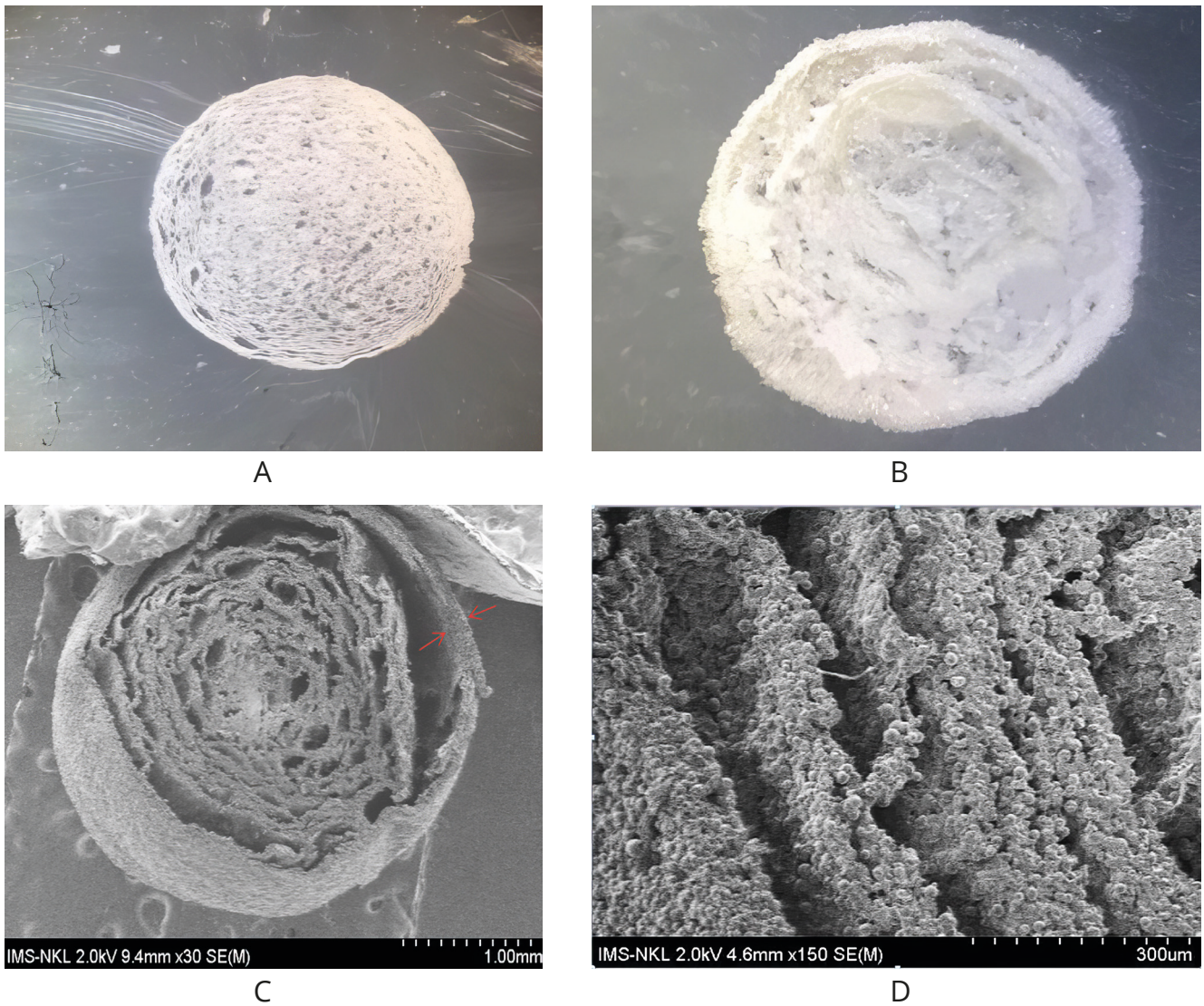


Fig. 5. Cross-sectional images of microcapsules. A — Micronuclear microsurgery; B — Microcapsule microsurgery; C — Cross-sectional image of the encapsulated microcapsules; D — Starch is interwoven between voids in the reticulum structure.

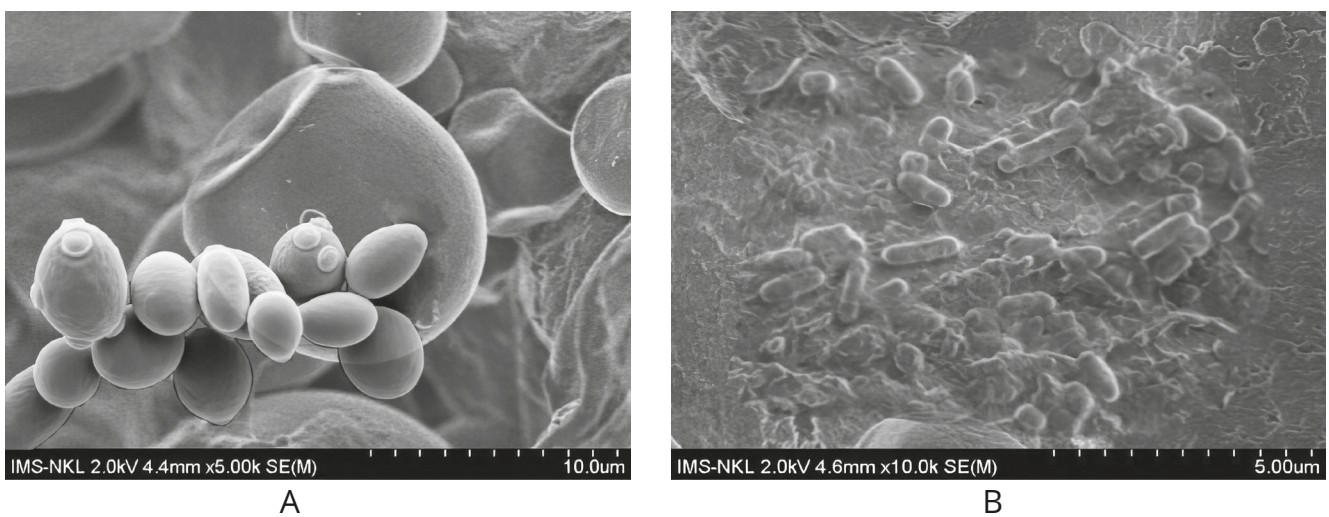


Fig. 6. SEM images of microbial cells. A — at 5000X magnification; B — at 10,000X magnification.

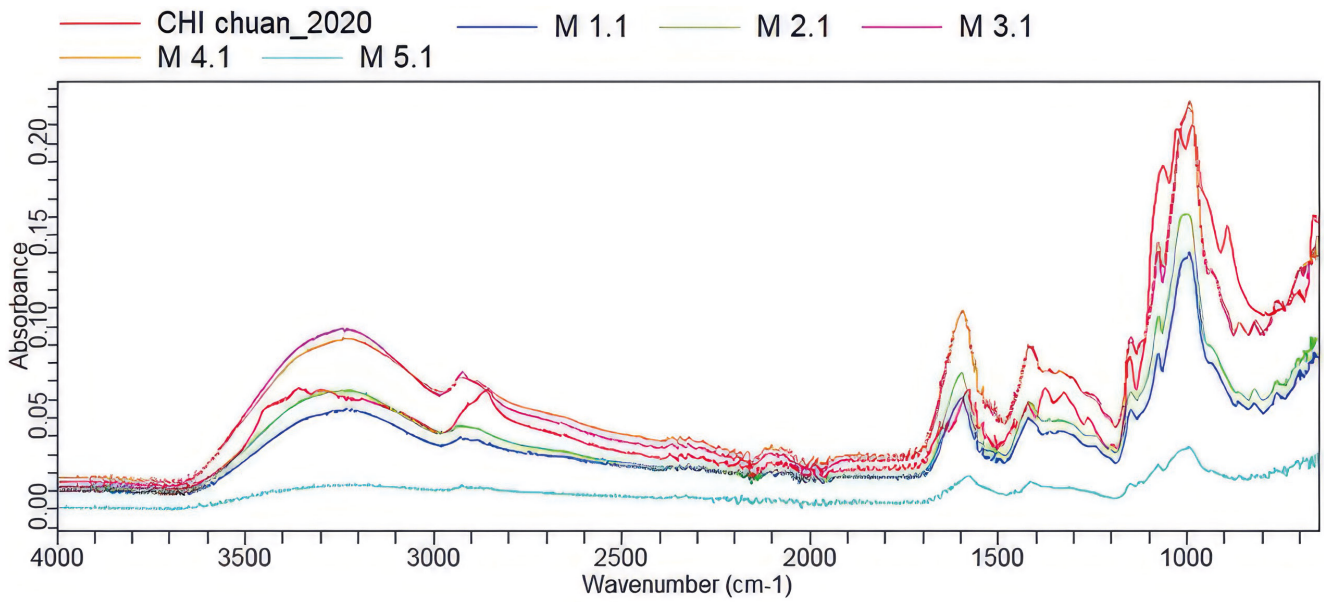


Fig. 7. IR spectra of microcapsules.

*Microcapsule in Chitosan: M1, M3 — 0.5 %; M2, M4 — 2 %; M1, M3 — mix calcium chloride and chitosan solution at the same time; M2, M4 — calcium chloride before; chitosan after.

**M1.1: scan M1 in the 1st IR sample.

than 10,000X on the surface of starch granules. As can be seen, probiotic organisms were able to bind to starch-alginate surfaces in stable and secure ways. It was also observed that the immobilization of cells in sodium alginate produced semispherical microcapsules with rigid surface and spongy structure (Albertini et al., 2010).

Figure 7 showed IR spectra of different microencapsulated samples depended on chitosan concentrations. Despite the fact that chitosan concentrations vary from 0.5 % to 2 % (M1, M2 — 0.5 %; M3, M4 — 2 %) and can be combined with calcium chloride solution and chitosan (M1, M4 — same time; M2, M3 — before, after) but the microcapsules still contain chitosan. Indeed, the infrared absorption spectra of microcapsules M1 to M4 all showed absorption peaks similar to those of standard chitosan, demonstrating that chitosan is present in all microcapsules. In addition, in sample M5, the characteristic absorption peaks in the range of 3000–3500 cm^{-1} of chitosan did not appear in the IR spectrum when comparing the stack of infrared absorption spectra of the M5 microcapsules, proving that chitosan is not present.

Survival of probiotics after exposure in gastrointestinal conditions

The release rate of *L. acidophilus* is different from that of *S. boulardii* since it is contained inside the microcapsule. Because in the early phases, the quantity of probiotics released was mostly due to leakage and loss through the network system, the viability of *L. acidophilus* released steadily grew but increased more slowly.

The membrane holding *S. boulardii* had nearly fully broken down by 90 min, at which point a huge release of *L. acidophilus* began. This release increased significantly between 90 and 120 min, peaked at 120 min (87.93 %), and then progressively dropped. Even yet, a significant number of *L. acidophilus* 7 log CFU/g (> 80 %) persisted in the SIF. The statistics indicate a progressive over time rise in the number of microorganisms discharged into the environment. The envelope totally broke down after 90 min, releasing a significant quantity of *S. boulardii* (88.73 %) into the SIF medium. Following this, the number of *S. boulardii* did not continue to rise, but instead tended to fall (Fig. 8). This indicates that the maximum amount of *S. boulardii* was released at this time, and that it may gradually disappear from the environment due to a shortage of food.

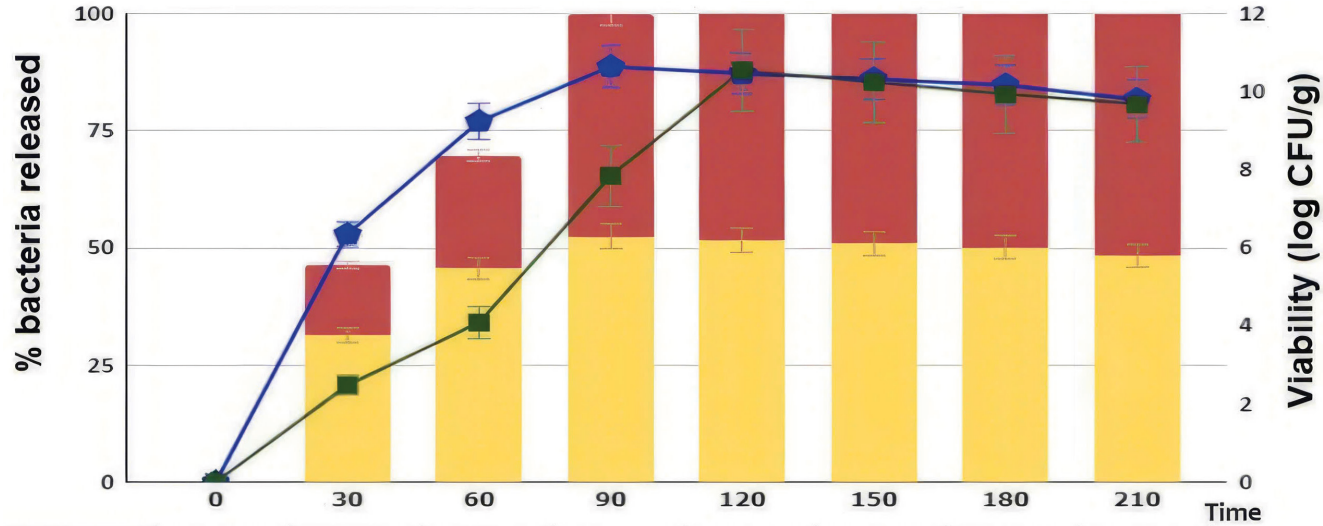
From the above results, the dissolution profiles of microcapsule model dependent (curve fitting), statistical analysis and model independent methods could be used. The most suitable model was that which had the smallest AIC and largest R^2. The results of R^2 and AIC coefficients are given in Table 2.

The Korsmeyer-Peppas model and the Higuchi model are the two that best describe the release kinetics of mi-

Table 2. Kinetic model of release mechanism of microcapsules

Kinetic model	Zero-order	First-order	Higuchi	Korsmeyer — Peppas
R^2	0.942	0.900	0.975	0.972
AIC	17.1079	18.4141	12.2721	7.8696

AIC: The Akaike information criterion.



S. b	0	3.76	5.47	6.3	6.2	6.11	6.02	5.8
L. a	0	1.8	2.9	5.69	7.65	7.43	7.21	7.02
% S.b	0	52.96	77.04	88.73	87.32	86.06	84.79	81.69
% L.a	0	20.69	34.05	65.4	87.93	85.4	82.87	80.69

Fig. 8. The survival of *L. acidophilus* and *S. boulardii* in the gastrointestinal tract.
*S. b: *S. boulardii*; L. a: *L. acidophilus.*

crocapsules in the gastrointestinal tract (Kwoji, Aiyegoro, Okpeku, and Adeleke, 2021). The release graph's first phase and zero-order kinetics are comparable. It is clear from the Peppas model matching value that microorganisms release is a result of a variety of anomalous diffusion processes, such as fusion, melt, swell, and corrosion. Alginate and chitosan are innately corrosive biopolymers that degrade through both swelling and dissolving, modes; thus, this conclusion makes sense as well (Witzler et al., 2021).

Stability of microencapsulated bacteria during storage

Figure 9 illustrates the viability of *S. boulardii* and *L. acidophilus* in the microcapsules over time. Results show that the viability of *S. boulardii* and *L. acidophilus* in the microcapsules steadily reduced over the course of 120 days while being monitored at room temperature (15 to 35 °C). Additionally, the moisture content steadily rises with time

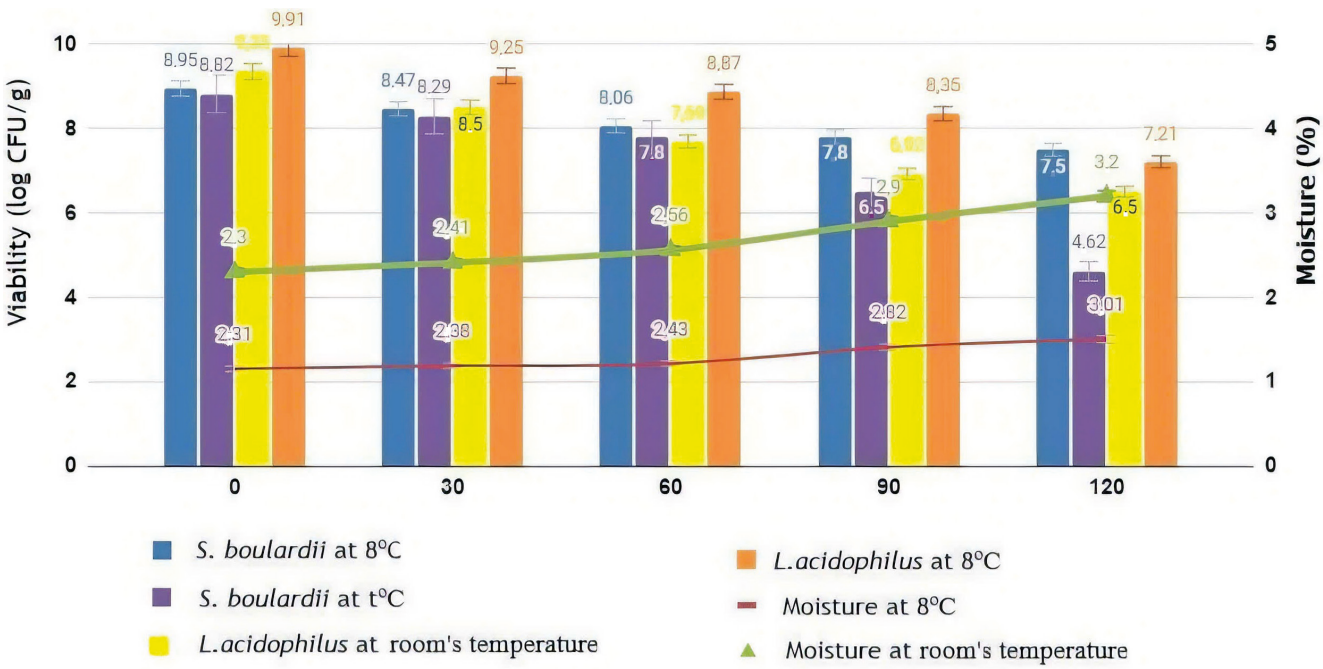


Fig. 9. Moisture, viability of *S. boulardii* and *L. acidophilus* in the microcapsules in 120 days storage.

from 2.31 % to 3.20 %. About 7.65 CFU/g of *S. boulardii* and 8.45 log CFU/g of *L. acidophilus* were still alive.

The fewer viable bacteria there are, the greater the moisture content is. Chitosan and alginate are both hygroscopic, which explains why the moisture level in the microcapsules rises. The probiotic bacterial strains become more active, mature, and eventually die as a result of high humidity. On the other hand, probiotic bacterial strains continued to maintain a significant proportion in the environment when temperatures between 2 to 8 °C, where they were less impacted by temperature and ambient humidity, increasing moisture content from 2.31 % to 2.71 %. *S. boulardii* microcapsules dropped from 8.95 log CFU/g to 8.37 log CFU/g, *L. acidophilus* CFU/g value dropped from 9.91 to 9.31. As a result, the data demonstrated that bacterium microencapsulation decreased acid inhibition, resulting in higher viability of microencapsulated cultures than that of free cells. In gastrointestinal stress circumstances, alginate has been shown to protect probiotic microorganisms from death (Holkem et al., 2016; Khosravi Zanjani, Ghiassi Tarzi, Sharifan, and Mohammadi, 2014). However, 10^9 CFU/g of bacteria were still present after 120 days of storage. Loss of survival during storage can be related to some factors such as the formation of free radicals in the presence of oxygen, the oxidation of fatty acids, and DNA damage (Shori et al., 2017).

Conclusion

In summary, this study showed for the first time that *L. acidophilus* and *S. boulardii* or other probiotics can be successfully combined in two-layers of microcapsulation by using the coagulation process. This potential microcapsule system released *S. boulardii* in the stomach it survived, inhibiting *H. pylori*, and *L. acidophilus* was released after 2 h, only when the microcapsules reached the small intestine, avoiding acidic pH in the stomach. Microcapsules improve the capacity to safeguard and release microorganisms in a model of the digestive system. After 2 h in SGF microcapsules had shielded > 80 % of the cell bacteria. More than 85 % of the microorganisms in the SIF were liberated. Two polymers such as alginate and chitosan were used to deliver probiotic microcapsules to particular areas in the digestive system was a viable method. The moisture content increased somewhat and the bacteria density slightly after storage, but both still met the specifications for probiotic goods. Subsequent research focusing on the development of systems incorporating multiple probiotics for programmed release, as well as the exploration of release models from microbial carriers, opens up new avenues in probiotics research. This promises to streamline dosing schedules, save time for preparation of microcapsulation and enhance convenience for users.

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