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Microencapsulation of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12 in alginate and pectin, and the effects on bacterial acid and bile resistance

Ayla Mumcu[1], Ayhan Temiz[2], Emir Baki Denkbas[3]

[1]Olive Research Institute, University Street No: 43, Bornova Izmir, Türkiye
[2]Hacettepe University, Faculty of Engineering, Department of Food Engineering, Ankara, Türkiye
[3]Baskent University, Faculty of Engineering, Biomedical Engineering Department, Ankara, Türkiye

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Corresponding Author
Tel.: +90 232 4627073
E-mail:
ayla.mumcu@tarimorman.gov.tr

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Abstract

This study aimed to encapsulate *Lactobacillus acidophilus* LA-5 (LA-5) and *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) in alginate and pectin, and to determine the effects of acid and bile resistance. The emulsion method was used; different gel and $CaCl_2$ concentrations and stirring rate were studied comparatively for the characterization of microcapsules. Alginate microcapsules were smaller and exhibited agglomeration behavior, while pectin microcapsules were fairly homogeneous and had a smooth shape. Encapsulation efficiency (EE) percentages of the bacteria cells encapsulated with alginate and pectin varied within a very narrow range of 83 to 85%. The size of microcapsules increased as the gel concentration increased, and decreased as the $CaCl_2$ concentration and stirring rate increased. Microencapsulated cells had more acid and bile resistance than free cells. No significant difference was found between alginate and pectin microcapsules of the two strains in terms of acid and bile resistance. The probiotic strains encapsulated with pectin and alginate maintained higher levels of viability under acid conditions at pH 2 and 3 than at pH 1 after 24 hours of incubation.

Introduction

Probiotic bacteria play an important role in promoting and maintaining human health. The viability and metabolic activity of probiotic bacteria in a food product is an important consideration for their beneficial efficacy. Probiotics have to survive during the processing and shelf life of foods, transit through the highly acidic conditions of the stomach and enzymes and bile salts in the small intestine, and finally reach the large intestine in sufficient viable cell numbers. Nevertheless, viability of probiotics can be undesirably affected by the external environmental conditions and factors during the food production processes, transportation, and storage (Kraesaekoopt et al., 2003; Chávarri et al., 2012; Cassani et al., 2020; Mendonça et al., 2022). Food related factors, such as the presence of oxygen, high temperatures, nutrients, pH, antimicrobials, and present microbiota, can also affect probiotic viability and stability (Cassani et al., 2020; Mendonça et al., 2022). Furthermore, there is an unfavorable condition in the gastrointestinal tract due to the low pH of the stomach and the presence of bile salts and enzymes in the small intestine (Marteau et al., 1997; Gbassi et al., 2011; Chavari et al., 2012). Probiotics exert a beneficial effect in the intestine when the concentration of viable cells is ~10^8–10^{10} CFU/day (considering 100 g or mL of ingested food), corresponding to ~10^6–10^9 CFU/g or mL in the product when ingested (Lee and Salminen, 1995; Champagne et al., 2011).

Encapsulation of probiotic cells is widely used to overcome these limitations by enhancing the survival of probiotic cells against harmful conditions, while ensuring their viability and functional characteristics (Chávarri et al., 2012; Xu et al., 2022). Different types of techniques are used for the microencapsulation of probiotics, such as emulsion, extrusion, spray-drying, fluid-bed agglomeration, and coating, freeze and vacuum-drying, coacervation, adhesion to starch granules, and compression coating (Chávarri et al., 2012). The particle size of the microcapsules is an important factor, since large grains may produce a sandy texture in the product, while smaller particles may not provide adequate protection for bacteria. Therefore, probiotics should be trapped in a limited range of particle sizes to minimize the problems associated with cell survival and food texture (Moghanjougi et al., 2021).

Alginate is the most widely used and investigated material for microencapsulation (Chávarri et al., 2012; Goh et al., 2012; Nezamdoost-Sani et al., 2023). It is a straight heteropolysaccharide composed of two basic units, D-mannuronic acid, and L-guluronic acid joined by glycosidic bonds. It is easy to handle, nontoxic, cost-effective, and biocompatible (Nezamdoost-Sani et al., 2023). Although alginate is suitable for encapsulation, its gel is porous and susceptible to extreme pH values (Mortazavian et. al., 2008, Chávarri et al., 2012). It degrades under low pH conditions, allowing the release of probiotics in stomach conditions (Amine et al., 2014; Sánchez-Portilla et al., 2020). Some studies revealed that alginate microcapsules protect probiotics during storage, but do not protect probiotics well in low pH conditions (e.g., in gastrointestinal fluids and in acid foods) compared to microcapsules containing a coating such as alginate-probiotic microbeads coated with chitosan (Hansen et. al., 2002; Oberoi et. al., 2021). Razavi et al. (2021) determined that high porosity of alginate microbeads leads to limitations such as rapid release of loaded molecules, low EE%, easy degradation in acidic environment, and poor transport of probiotics to the intestine. Therefore, chemical or physical modifications of alginate are needed to improve these limitations.

Pectin is an anionic heteropolysaccharide with a linear primary structural feature of α 1,4 linked D-galacturonic acid chains with varying degrees of methylation. It is plant-derived and non-toxic (Vincent and Williams, 2009), and it has been widely used as a delivery vector for colon-targeted medications (Liu et al., 2006; Bigucci et al., 2008; Jung et al., 2013). A recent interest has arisen in the commercial use of pectin due to its long-standing reputation for being non-toxic or generally considered safe, with relatively low production costs and high availability, and forms a gel structure in the presence of divalent metal ions such as calcium (Martău et al., 2019). Pectin has excellent biodegradability and biocompatibility. It is resistant to acidic conditions and enzymatic degradation by protease and amylase, and can be degraded by the intestinal flora in the colon (Wong et al., 2011). Pectin is also an emerging prebiotic that can more effectively regulate the composition of the intestinal flora and reduce the risk of colitis than some other commercial prebiotics (Gómez et al., 2016; Xu et al., 2022). These characteristics of pectin make it a promising carrier for encapsulating probiotics. Pectin microcapsules can be easily prepared by a simple ion-crosslinking process (Liu et al., 2006; Jung et al., 2013; Ribeiro et al., 2014; Belscak-Cvitanovic et al., 2015). Low methoxyl pectin can be cross-linked with Ca^{2+} to form microparticles or nanoparticles, and it is widely used as a carrier to deliver drugs to the colon (Nguyen et al., 2014; Ghibaudo et al., 2018). Several researchers focused on the protective effects of pectin on the survival of lactic acid bacteria in gastrointestinal tract conditions (Chen et al., 2020).

There are various studies in the literature on the encapsulation of probiotics, and these studies are still current and ongoing. Numerous studies are underway to preserve the viability of probiotics both in food and during transit through the gastrointestinal tract and to improve product quality, including diverse encapsulation techniques, the effects of processing parameters, and the use of different support materials. To maximize bacterial protection without compromising the final product's quality, it is crucial to select appropriate materials and microencapsulation techniques. The aim of this presented study was to encapsulate *Lactobacillus acidophilus* LA-5 (LA-5) and *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) in alginate and pectin wall materials by using the emulsion method, and determine the effects of acid and bile environments on the viability of the probiotic cells in alginate and pectin microcapsules. In our previous studies, LA-5 and BB-12 were found as the most acid- and bile-resistant probiotic strains among several *Lactobacillus acidophilus* and *Bifidobacterium* spp. strains tested (Mumcu and Temiz, 2014; Mumcu and Temiz, 2022). Therefore, these probiotic cultures were selected in this study. The effects of process parameters such as carrier material concentration, $CaCl_2$ concentration, and stirring speed on microcapsule formation were optimized, and the acid and bile resistance of probiotics encapsulated under appropriate encapsulation conditions were investigated.

Materials and Methods

Probiotic cultures

LA-5 and BB-12 purchased in lyophilized form (Chr. Hansen, Denmark) were used as probiotic test bacteria. LA-5 culture was activated in MRS (de Man, Rogosa and Sharpe, Merck) broth at 37 °C for 24 h, while BB-12 culture was activated in RCM (Reinforced Clostridial Medium, Fluka) broth under anaerobic conditions using anaerobic test kits (GENbox anaer, Biomérieux) at 37 °C for 24 h.

Preparation of alginate and pectin microcapsules and their characterization

The emulsion method was used for the preparation of alginate (sodium alginate, Fluka) and pectin (low methoxyl pectin; esterification degree of 35%, LM 12 CG-Z/200, CP Kelco, Germany) microcapsules (Krasaekoopt, 2003; Mortazavian et al., 2007). The certain concentration of alginate or pectin gel was transferred dropwise into 50 mL of the commercial sunflower oil containing 5% (v/v) Tween 80 (Sigma) by using a programmable syringe (Razel Scientific Syringe Pump, Model R99 EJM, Germany). The emulsion was continuously stirred with a mechanical mixer (Heidolp RZR 2021, Germany) at certain stirring speed during the dropping operation. The emulsion was then stirred for 10 min, with the mechanical mixer. A certain concentration of $CaCl_2$ (calcium chloride dihydrate, Riedel-de Haën) solution as cross-binding agent was transferred dropwise into the emulsion by using the programmable syringe and stirred for 2 h. Finally, alginate or pectin microcapsules were obtained in the emulsion. Different gel concentrations of alginate and pectin (0.5, 1, 2, 3, and 4%, w/v), $CaCl_2$ concentration (0.5, 1, 2, 4, 6, and 8%, w/v) and stirring speed (500, 1000, 1500, and 2000 rpm) of the mechanical mixer were studied for the characterization of alginate and pectin microcapsules. Morphology, size, and agglomeration behavior of alginate and pectin microcapsules were assessed using a simple light microscope with a 4x magnification objective lens. For this purpose, a small amount of sample taken from the pectin and alginate microcapsules was transferred to a clean slide and microscopic examination was carried out. At least 100 randomly selected beads were examined for each sample.

Microencapsulation of the probiotic bacteria in sodium alginate, and pectin gels

Microencapsulation of the probiotic bacteria was carried out at the selected parameters determined through characterization studies of the alginate and pectin microcapsules. Microencapsulation parameters for the alginate microcapsules were determined as 1% alginate, 2% $CaCl_2$, and 2000 rpm stirring speed; for the pectin microcapsules were determined as 2% pectin, 6% $CaCl_2$, and 2000 rpm stirring speed. At the beginning, LA-5 and BB-12 cultures were activated in the MRS broth and the RCM broth, respectively. Activated cultures were centrifuged at 3000 rpm for 10 min, and the precipitate was suspended in 2 mL of sterile distilled water to obtain a cell solution containing around 11 log CFU/g. The cell suspension was then added to 1% alginate gel or 2% pectin gel at a 5:1 ratio (gel solution: cell suspension). LA-5 or BB-12 cells in the gel solution were then microcapsulated by the emulsion method described above at the characterized $CaCl_2$ concentration and stirring speed for each gel microcapsule. The microcapsulated bacteria were frozen at -70 °C for 24 h and then freeze-dried in a lyophilizer (Christ, Alpha 1-4 LD plus, Germany) at 0.04 mbar for 24 h.

Observation of the location of LA-5 and BB-12 cells in alginate and pectin microcapsules by a fluorescence microscope

Fluorescent staining procedure was applied to detect the location of the probiotics in the microcapsules. SYTO®9 green-fluorescent nucleic acid stain (Invitrogen, Molecular Probes) was used at 0.5 µL/mL concentration for staining. One mL of SYTO® 9 stain solution was added to 10 mL alginate or pectin microcapsules and kept in the dark for 20 min. Microcapsules were washed with sterile distilled water and observed under a fluorescence microscope with a 20x magnification objective. SYTO® 9 stains both the DNA and RNA of bacterial cells, and the bacterial cells are observed as green fluorescent sparkle under a fluorescence microscope (Pereira et al., 2005).

Determination of LA-5 and BB-12 cell numbers in alginate and pectin microcapsules

At the beginning, the probiotic cells in the alginate or pectin microcapsules were released into buffer solutions. Phosphate buffer (pH 7.0) and citrate buffer (pH 4.5) were used for the microcapsules of alginate and pectin, respectively. To release the probiotic cells from the alginate microcapsules into the buffer solution, 0.1 g of freeze-dried alginate microcapsules was added into 9 mL of 1 M phosphate buffer (pH 7.0) and vortexed intermittently for 30 min (Chandramouli et al., 2004). Free viable bacteria cell number in the buffer solution was determined by the pour plating method using MRS agar and RCA (Reinforced Clostridial Agar, Sigma) for LA-5 and BB-12, respectively. For enumeration of encapsulated viable bacteria in pectin microcapsules, 0.1 g freeze-dried pectin microcapsules were added into 9 mL of 0.5 M citrate buffer (pH 4.5) and kept in a water bath at 40 °C until the solution temperature reached this temperature. Then, the pectinase (Sigma) enzyme preparation was added into the solution (2% v/v) and this solution was incubated at 40 °C (optimum temperature of this enzyme preparation) for 30 min. Free viable bacteria cell number in the buffer solution was determined by pour plating method by using MRS agar, and RCA for LA-5 and BB-12, respectively.

Encapsulation efficiency (EE%)

The efficiency of encapsulation, showing the number of viable bacteria cells during the microencapsulation process, was calculated using the equation below:

$$EE\% = \left(\frac{N}{N_0}\right) \times 100$$

Where EE% is the percentage of the efficacy of encapsulation; N denotes the number of viable bacteria cells released from microcapsules (CFU/g), and

N_0 represents the number of viable bacteria cells used for encapsulation (CFU/g).

Acid resistance of the microencapsulated bacteria

To determine the acid resistance of the probiotic bacteria microencapsulated in alginate, 0.1 g of freeze-dried alginate microcapsules was transferred separately to 10 mL 0.5% NaCl solutions with pH values of 1, 2, and 3 (pH values were adjusted by 1 M HCl). The same amount of freeze-dried alginate microcapsules was directly added to 0.5% (w/v) NaCl solution (pH 5.40) as a control. Each pH medium was incubated at 37 °C, and centrifuged at 3000 rpm for 10 min at 0, 1/2, 1, 2, and 3 h of incubation. Then, washing procedure was performed using distilled water and 10 mL of phosphate buffer (pH 7.0) was transferred onto the microcapsules. Each medium was vortexed intermittently for 30 min, thus allowing the bacterial content of the microcapsules to become free in the environment. Free viable bacteria cell number in the solution was determined by the pour plating method by using MRS agar and RCA for LA-5 and BB-12, respectively.

The acid resistance of the probiotic bacteria microencapsulated in pectin was determined by a similar method to those microencapsulated in alginate. The only difference in this case was the step at which bacterial cells were released from the pectin microcapsules. After the washing procedure that carried out using distilled water, 10 mL of citrate buffer (pH 4.5) was transferred onto the microcapsules. The solution, including the microcapsules, was kept in a water bath at 40 °C until the solution temperature reached this temperature. Then, pectinase (Sigma) enzyme preparation was added into the solution (2% v/v), and this solution was incubated at 40 °C for 30 min. Free viable bacteria cell number in the buffer solution was determined by the pour plating method by using MRS agar and RCA for LA-5 and BB-12, respectively.

Bile resistance of the microencapsulated bacteria

In order to determine the bile resistance of the probiotic bacteria microencapsulated in alginate, 0.1 g freeze-dried alginate microcapsules were transferred to 10 mL 0.5% bile (Oxbile, Merck) solution. The same amount of freeze-dried alginate microcapsules was directly transferred to 10 mL of water as a control. The solutions were incubated at 37 °C and centrifuged at 3000 rpm for 10 min at 0, 3, 6, and 24 h of incubation. Then, a washing step was performed using distilled water, and 10 mL of phosphate buffer (pH 7.0) was transferred onto the microcapsules. The solutions were vortexed intermittently for 30 min, thus allowing the content of the microcapsules to become free in the environment. Free viable bacterial cell content in the solutions was determined by the pour plating method by using MRS agar and RCA for LA-5 and BB-12, respectively.

The bile resistance of bacteria microencapsulated with pectin was determined by a similar method as for those microencapsulated in alginate. The only difference in this case was the step at which the bacteria were released from the capsules. After the washing procedure that was carried out using distilled water, 10 mL of citrate buffer (pH 4.5) was transferred onto the microcapsules. The solution containing the microcapsules was kept in a water bath at 40 °C until the solution temperature reached this temperature. Then, pectinase (Sigma) enzyme preparation was added (2% v/v) into the solution, and this solution was incubated at 40 °C for 30 min. Free viable bacteria cell number in the buffer solution was determined by pour plating method by using MRS agar and RCA for LA-5 and BB-12, respectively.

Statistical analyses

Each experiment weas carried out in triplicate (n = 3). The results were reported as mean values with standard deviation (±SD), and one-way analysis of variance (ANOVA) measurements at 5% significance level was conducted. Tukey's tests were performed to compare the differences among treatments ($P < 0.05$) using the Minitab statistical software (Minitab LLC, USA).

Results and Discussion

In this study, alginate and pectin were used as carrier materials for probiotic microencapsulation, and different process parameters were evaluated for their effects on microcapsule formation. Microcapsule formation was not achieved at the lowest alginate and pectin concentration (0.5%) studied. Stable and regular microcapsule formations could be obtained at the other gel concentrations studied. Simple light microscope images of alginate and pectin microcapsules are shown in Figure 1. Microscopic examination revealed that alginate microcapsules showed agglomeration behavior, while uniform and smooth pectin microcapsules were formed.

The effects of alginate or pectin concentration, $CaCl_2$ concentration, and stirring speed on the microcapsule size are shown in Figure 2-4. Only the selected gel and $CaCl_2$ concentrations and stirring speeds were proven. During the study with a certain parameter, only the values of the test parameter were changed while the other parameters were kept constant. Consequently, the values kept constant were 1% alginate, 2% $CaCl_2$, and 2000 rpm stirring speed in the alginate gel studies, whereas 2% pectin, 6% $CaCl_2$, and 2000 rpm stirring speed in the pectin gel studies.

The size of microcapsules increased as the wall material concentration increased (Figure 2). However, uniform and spherical exact pectin microcapsules could not be obtained at 1% pectin concentration. Therefore, the value related to 1% pectin concentration is not included in Figure 2. Sandoval-Castilla et al (2010) also determined that the diameter of *L. casei* microcapsules increased as the proportion of pectin and the total

biopolymers concentration increased. In general, pectin microcapsules are larger than alginate ones. Similarly, Sandoval-Castilla et al. (2010) determined that the size of alginate microcapsules was smaller than that of pectin microcapsules. On the other hand, at higher wall material concentrations, the beads became too viscous to be extruded from the needle, resulting in larger capsules. Smaller size microcapsules were obtained when alginate was used as a wall material. As the concentration increases, the viscosity of the polymer gel increases, and accordingly, it becomes more difficult for the gel to fall into the environment as drops, and as a result, larger capsules are formed. As a result, the larger size of pectin microcapsules than that of alginate is probably related to the higher viscosity of the pectin solution relative to the same amount of alginate solution.

It was observed that as the $CaCl_2$ concentration increased, the size of the microcapsules decreased (Figure 3). Despite the smaller size of the microcapsules at higher $CaCl_2$ concentrations, it is thought that the expected polymer degradation in the intestinal environment may not occur, and bacterial cells in the capsule may not be released into the environment due to the fact that each microcapsule is composed of a tightly bound polymer. In addition, numerous studies have shown that the count of the microencapsulated cells increases with increasing microcapsule size (Sheu et al., 1993; Lee and Heo, 2000; Chandramouli et al., 2004; Picot and Lacroix, 2004). It is considered sufficient that the microcapsules to be applied to the product should be large enough not to adversely affect the sensory and structural quality of the product and not to cause dissatisfaction in the consumer. Microcapsule particle size is an important factor regarding the survivability of probiotics. Sheu et al. (1993) found that the survival of entrapped *L. bulgaricus* in alginate bead, and added to frozen desserts was significantly higher when beads size fell in-between 30 and 102 µm, than when beads had a diameter of 15 µm. Holkem et. al (2017) reported that the microparticles of *Bifidobacterium* BB-12 obtained by internal ionic gelation with sodium alginate were 54.82 ± 0.54 µm. On the other hand, Hansen et al. (2002) reported that an alginate capsule should have a diameter of at least 100 µm to prevent a reduction in *Bifidobacterium* viability in simulated gastric juices. McMaster et al. (2005) also stated that an ideal diameter for microcapsules was in the range of 100–200 µm, as it represented a balance between probiotic viability and sensory perception. Considering the values reported in the literature, the size of the microcapsules can be considered to be within acceptable values when $CaCl_2$ concentration is used as 2% and 6% for alginate and pectin, respectively (Figure 3). The mean diameters of the alginate and pectin microcapsules produced at above conditions were within limits of 85 µm and 125 µm, respectively

It was observed that with increasing stirring speed, the size of the microcapsules decreased (Figure 4). It was mentioned that as the stirring speed increases, energy transfer to the medium increases, and the polymer gel disperses into the medium in smaller droplets, and the size of the microcapsules decreases (Denkbaş and Odabaşı, 2000). In the present study, it was also observed that alginate microcapsules tended to form more aggregates as the stirring speed decreased. As a result, it was observed that the smallest alginate and pectin microcapsule diameters were obtained when stirring at 2000 rpm in the reactor medium (Figure 4).

By evaluating the results obtained from the microcapsule characterization analyses, it was decided to use 1% alginate, 2% $CaCl_2$, and 2000 rpm stirring speed for alginate, and 2% pectin, 6% $CaCl_2$, and 2000 rpm stirring speed for pectin as the optimal encapsulation conditions.

Microencapsulation of probiotic bacteria in sodium alginate and pectin gels

The probiotic bacteria cells inside the microcapsules were observed as green fluorescence under a fluorescence microscope after staining the microcapsules with SYTO®9 dye (Figure 5). Viable cell levels of the probiotic bacteria in dry alginate and pectin microcapsules and encapsulation efficiency values (EE%) of the probiotic cells encapsulated with alginate and pectin are given in Table 1. Under microencapsulation conditions of 1% alginate, 2% $CaCl_2$, and 2000 rpm stirring speed for alginate, and 2% pectin, 6% $CaCl_2$, and 2000 rpm stirring speed for pectin, the viable cell counts of LA-5 and BB-12 in microcapsules and the EE% values varied in a narrow range. The EE% values of bacterial strains varied between 83% and 85% for alginate and pectin.

It was stated that the comparison between EE% values reported in the literature is complicated by the wide range of microorganisms studied, encapsulating techniques and carrier materials used (Gebara et al., 2013). Process parameters such as carrier material concentration, crosslinker concentration, and stirring speed significantly affect encapsulation efficiency. Low polymer concentrations result in poor capsule formation and may leach probiotic cells out. Conversely, high polymer concentrations can reduce porosity, limiting the diffusion of nutrients and oxygen necessary for probiotic survival. This can stress or kill probiotics within the capsule. Viscosity and gel density contribute to processing difficulties and possible cell damage. At low $CaCl_2$ concentrations, the number of Ca^{2+} ions may be insufficient to fully cross-link the polymer chains. Due to the weak gel network, capsule contents may leak out or the capsule may fail to form, resulting in poor probiotic retention and low encapsulation efficiency. At too high a concentration, excessive cross-linking can cause structural defects, and the rigid structure can limit nutrient diffusion necessary for probiotic cell survival during storage. Regarding stirring speed, low stirring speeds may cause probiotics to be unevenly encapsulated, leading to structure degradation during

separation and reduced microencapsulation efficiency. Excessive stirring speeds may generate excessive shear stress, and probiotic viability may be reduced due to mechanical stress.

Kong et al. (2003) showed that encapsulation in high viscosity alginate hydrogels decreased cell viability and they attributed this to the high shear forces required to mix cells with these solutions. They concluded that the lower, sufficient viscosity of alginate hydrogels may have contributed to higher cell viabilities and consequently higher encapsulation yields. A study by Mijanur Rahman et al. (2015) indicated the efficiency of homogenization for the bacterial load, the results showed a significant increase in homogenization efficiency as homogenizer speeds increased from 5000 to 10000 rpm. The homogenization of emulsion for bead development at 10000 rpm showed the highest bacterial count. Ali et al. (2024) showed that high homogenization speed at 12000 rpm significantly improved the beads surface and sphericity, and bacterial survivability in xanthan gum/alginate microbeads. The bacteria were unable to release properly from the beads due to an external thick protective gum coating at low homogenization speed, that is, 3000 rpm. Size of microbeads, wall material type, and its concentration, homogenization speeds, and pressure are considered as significant factors which are linked with the efficiency of homogenization (Saeed et al., 2022). The data suggested that the size of emulsion droplets reduced at high homogenization speed, which ultimately improved the surface area. Bacterial count was increased as the surface to volume ratio increased.

Studies have shown that the emulsification technique with sodium alginate has great potential for application to probiotic cultures, since it increases the viability of probiotic bacteria by approximately 80% (Goh et al., 2012; Amine et al., 2014; Holkem et al., 2017). Corbo et al. (2011) studied different microorganisms containing lactobacilli and bifidobacteria for encapsulation in alginate beads. They found EE varied from a minimum of 54.8% (*L. reuteri*) to a maximum level of 83.33% (*L. rhamnosus*) by using ionotropic gelation and 10% alginate concentration in the conditioning medium (2 g of Na-alginate+18 mL of cell suspension). In another study by Corbo et al. (2013), the encapsulation efficiency of *L. plantarum* loaded alginate beads was high, up to 93%. Gebara et al. (2013) determined a similar EE% value (on average 84.35±0.60%) for microencapsulation of *L. acidophilus* by ionotropic gelation using 2% low methoxyl amidated pectin as wall material, followed by coating with whey protein. On the other hand, Chávarri et al. (2010) found EE% values varying from 19.5 to 40.2% for encapsulation of *L. gasseri* and *B. bifidum* using chitosan-coated calcium alginate beads. Sandoval-Castilla et al (2010) stated that the use 2% pectin on its own or between 2 and 3% combined with 0.5% alginate tended to display optimal counts of *L. casei* after 20 days of storage at 4 ^{o}C. EE% value for pectin microcapsules containing *L. casei* was determined as 68.8 ± 2.5%. It was reported that EE% values of *L. casei* increased as the proportion of pectin and the total biopolymers concentration increased. Picot and Lacroix (2004) encapsulated *B. breve* and *B. longum* strains as freeze-dried or fresh cultures in water-insoluble food-grade microcapsules produced by emulsion and/or spray-drying, using milk fat and/or denatured whey proteins as immobilization material. The encapsulation yield differed significantly according to the method and the strain used. They obtained relatively low EE% values ranging from 0.03 to 25.67% for microencapsulation of *B. breve* and *B. longum* by the spray drying method. It was thought that low EE% values may be related to the sensitivity of microorganisms to high process temperatures used in the study, since the encapsulation by spray drying was carried out using an outlet air temperature of 80 °C.

Acid resistance of the microencapsulated probiotic bacteria

It was determined that free cell forms (non-microencapsulated) of LA-5 and BB-12 could not resist the pH 1 environment, and no viable bacterial cell was detected even after 30 min of incubation (Figure 6). LA-5 cells maintained the viability at certain levels until the 2nd h of incubation when microencapsulated with alginate and pectin. A decrease in viable cell number of the microencapsulated bacteria was observed over time in a pH 1 environment, and viable bacteria cells could not be determined after the 2nd h of incubation. Viable bacterial cell counts of microencapsulated LA-5 determined, after 30 min of incubation in a pH 1 medium, were 4.57 and 4.35 log (CFU/mL) for alginate and pectin microcapsules, respectively, while viable cell numbers in the same environment were 1.65 and 1.23 log (CFU/mL), respectively, after 1 h incubation. The decrease in the viable cell counts of LA-5 microencapsulated in alginate and pectin in a pH 2 medium was quite low compared to free cells (Figure 7). After 3 h of incubation, the viable cell count was determined as 5.80 and 5.92 log (CFU/mL) for alginate and pectin microcapsules, respectively. In a pH 3 medium, results very close to the initial cell number were obtained for microencapsulated LA-5 (Figure 8).

Similar results were observed in the assays in which acid resistance properties of microencapsulated BB-12 cells were tested. BB-12 maintained its viability at certain levels until the 2nd h of incubation when microencapsulated with alginate and pectin (Figure 9). A decrease in viable cell number of the microencapsulated bacteria was observed over time in pH 1 medium, and viable bacteria cells could not be determined after the 2nd h of incubation. Viable bacterial cell counts of microencapsulated BB-12 determined after 30 min of incubation in pH 1 medium were 3.84 and 4.12 log (CFU/mL) for alginate and pectin microcapsules, respectively, while the viable cell numbers in the same environment were 1.25 and 1.13 log (CFU/mL), respectively, after 1 h of incubation. The decrease in the

viable cell counts of BB-12 microencapsulated in alginate and pectin in pH 2 medium was considerably lower than that of free cells (Figure 10). After 3 h of incubation, viable cell count was determined as 6.45 and 6.33 log (CFU/mL) for alginate and pectin microencapsules, respectively. In pH 3 medium, results very close to the initial cell number were obtained for microencapsulated BB-12 (Figure 11).

Overall, the results presented here suggest that microencapsulation of LA-5 and BB-12 using both alginate or pectin protects bacterial cells against acid environments and therefore indirectly increases their resistance to acid environments. According to the statistical analysis, the difference between alginate and pectin microcapsules in terms of acid resistance of the probiotic strains was found to be insignificant ($p>0.05$). The viable cell counts of microencapsulated LA-5 and BB-12 determined at the end of the incubation periods were higher than the results obtained for free form LA-5 and BB-12. In many studies investigating the effects of microencapsulation processes on acid resistance of probiotic bacteria, it has been reported that microencapsulation applications increase the acid resistance of probiotics, and microencapsulated probiotics exposed to acid medium can survive at a higher level compared to their free forms (Lian et al, 2003; Chandramouli et al., 2004; Reid et al., 2005; Ding and Shah, 2007; Kim et al., 2008). Yeung et al. (2016a) reported that encapsulation of probiotics within alginate microgels could improve their viability during storage. It was also demonstrated that *Bifidobacterium longum* entrapped in alginate hydrogel beads showed an enhanced survivability after transit through simulated digestive conditions (Yeung et al., 2016b).

Bile resistance of the microencapsulated bacteria

The results obtained from the bile resistance analyses of the probiotic strains are shown in Figure 12 and 13. For microencapsulated LA-5 and BB-12, the viable cell levels determined in the presence of bile during the incubation periods were higher than the results obtained for their free forms. The viable cell counts of LA-5 and BB-12 in free form were determined as 4.1 log (CFU/mL) and 4.8 log (CFU/mL), respectively after 24 h of incubation in 0.5% bile solution. Under the same conditions, the viable cell counts of LA-5 microencapsulated in alginate and pectin were 5.9 and 5.6 log (CFU/mL), respectively. When BB-12 cells were microencapsulated in alginate and pectin, these values were 5.6 and 5.2 log (CFU/mL), respectively. Compared to the initial bacterial levels, there was an approximately 2-log decrease in the number of free LA-5 and BB-12 cells, while a less than 1-log decrease in the number of the microencapsulated cells occurred.

If the results are evaluated in general, it is seen that microencapsulation of LA-5 and BB-12 using alginate or pectin protects bacterial cells against bile and indirectly increases their resistance to bile. In the literature, there are many studies indicating that microencapsulation processes increase the bile resistance of probiotic bacteria (Lian et al., 2003; Reid et al., 2005; Ding and Shah, 2007; Kim et al., 2008). In terms of resistance to bile, there was no significant difference between alginate and pectin microcapsules.

The results indicated that LA-5 and BB-12 had similar resistance behavior in these gastrointestinal stress conditions. A considerable reduction in the survival of free cells in comparison with the encapsulated cells incubated in the gastrointestinal conditions was consistent with the results of Gebara et al. (2013). Holkem et al. (2017) reported that although free cells presented low resistance, encapsulated bacteria were resistant to the simulated gastrointestinal conditions, providing protection to *Bifidobacterium* BB-12. In the present study, free and microencapsulated cells of BB-12 were found to be more resistant to simulated gastric and intestinal juice than those of LA-5. This difference could be related to strain differences in tension conditions. It can be said that microencapsulation can provide better protection for LA-5 against acid and bile environments tested. These results are agree with those of de Lara Pedroso et al. (2012), Chandramouli et al. (2004), and Iyer and Kailasapathy (2005) who studied *L. acidophilus* strains.

Conclusions

Microencapsulation of probiotics aims to protect viable probiotic cells from environmental stresses and deliver them effectively to the gut. To maximize bacterial protection without affecting product quality, it is crucial to select appropriate materials and microencapsulation techniques. Both pectin and alginate are widely studied natural polysaccharides used for this purpose due to their gel-forming abilities, biocompatibility, and food-grade status. In this study, alginate and pectin were compared as carriers for the microencapsulation of LA-5 and BB-12. These probiotic strains were encapsulated using emulsion, and it was confirmed that microencapsulated probiotic cells had higher acid and bile resistance. While pectin microcapsules required more $CaCl_2$ for capsule formation and created a softer structure, they formed more uniform and spherical microcapsules with high EE around 85%. Additionally, there was no agglomeration problem with pectin microcapsules compared to the alginate. Pectin emerges as a highly promising carrier material for probiotic microencapsulation due to its ability to form protective hydrogels that enhance probiotic viability throughout the gastrointestinal tract, to provide targeted release in the colon, and its potential prebiotic effects, stimulating probiotic growth after release. On the other hand, pectin-based microcapsules may require formulation optimization to overcome mechanical limitations and to obtain microcapsules with a smaller size and higher EE%. Future research focusing on composites of pectin with other polymers and tailored gelation strategies could

enhance its potential in the targeted and controlled release of viable probiotics, ultimately improving the efficacy and commercial applicability of probiotic products.

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Author Contributions

Ayla Mumcu—conceptualization, investigation, methodology, writing original draft preparation, review and editing. Ayhan Temiz— supervision, writing, review, editing. Emir Baki Denkbas—methodology, writing, review, editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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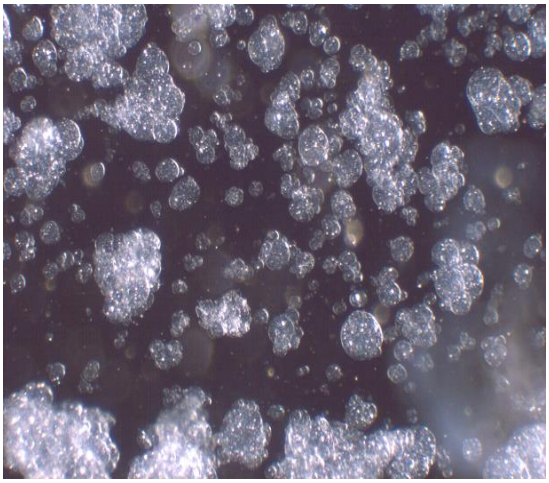
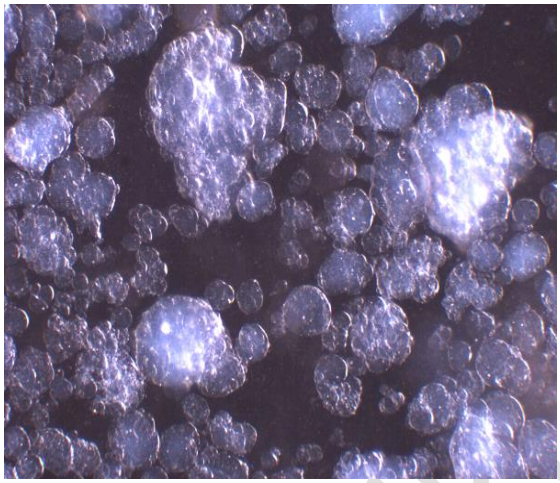
Table 1. Viable probiotic bacteria cell number level in dry alginate and pectin microcapsules and encapsulation efficiency (Values are mean ± SD, n=3)

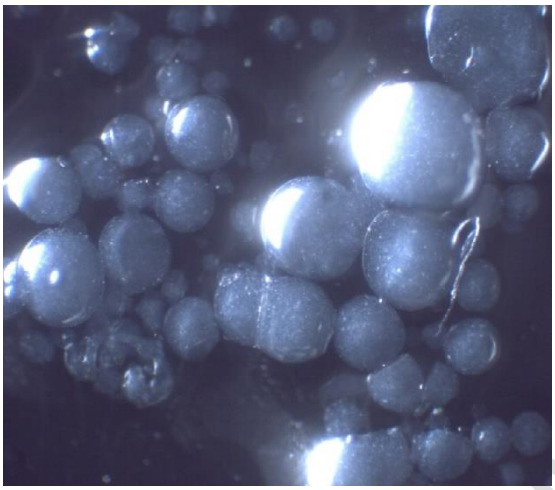
Probiotic bacteria	**Bacteria cell number (log CFU/g dry microcapsule)**		**Encapsulation efficiency (EE%)**	
	Alginate microcapsule	Pectin microcapsule	Alginate microcapsule	Pectin microcapsule
L. acidophilus LA-5	9.2±0.5	9.4±0.2	83.6±0.6	85.6±0.4
B. animalis subsp. *lactis* BB-12	9.3±0.3	9.2±0.6	84.6±0.2	83.6±0.6

a) b)





c) d)



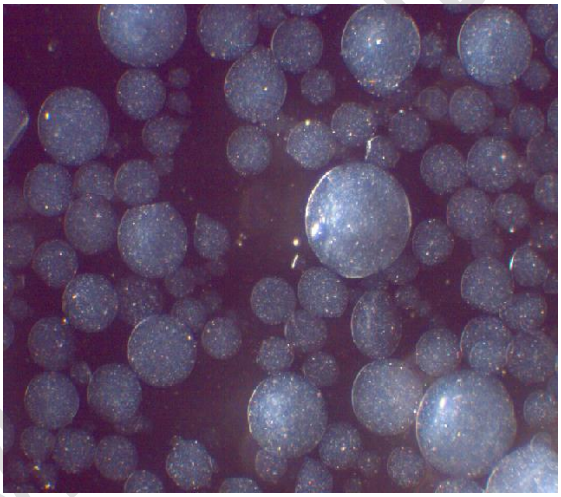


Figure 1. a-d. Simple light microscope images of alginate and pectin microcapsules with 4x objective: a-b; alginate microcapsules, c-d; pectin microcapsules.

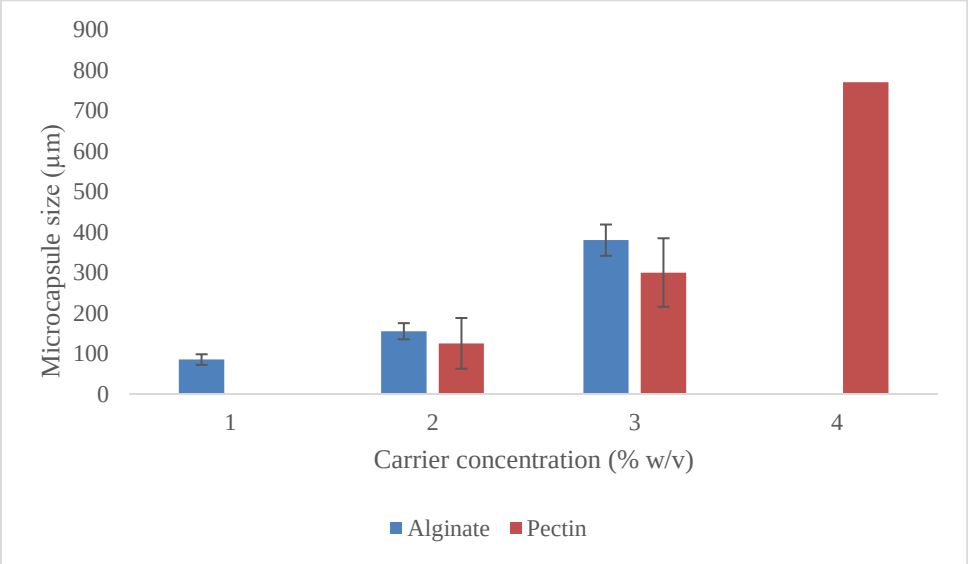


Figure 2. Effect of carrier (alginate or pectin) concentration on microcapsule size. (Values are mean ± SD, n=3)

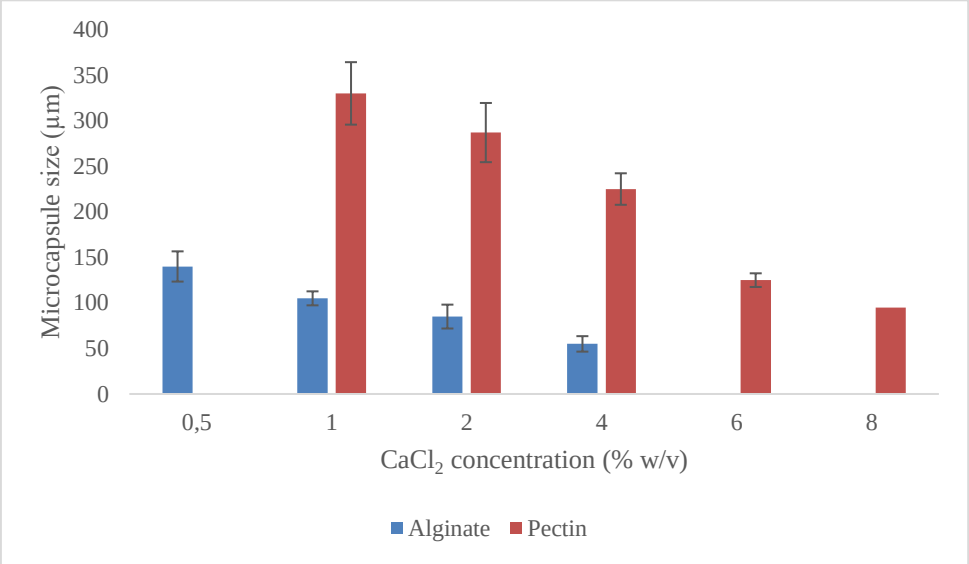


Figure 3. Effect of $CaCl_2$ concentration on alginate and pectin microcapsule size. (Values are mean ± SD, n=3)

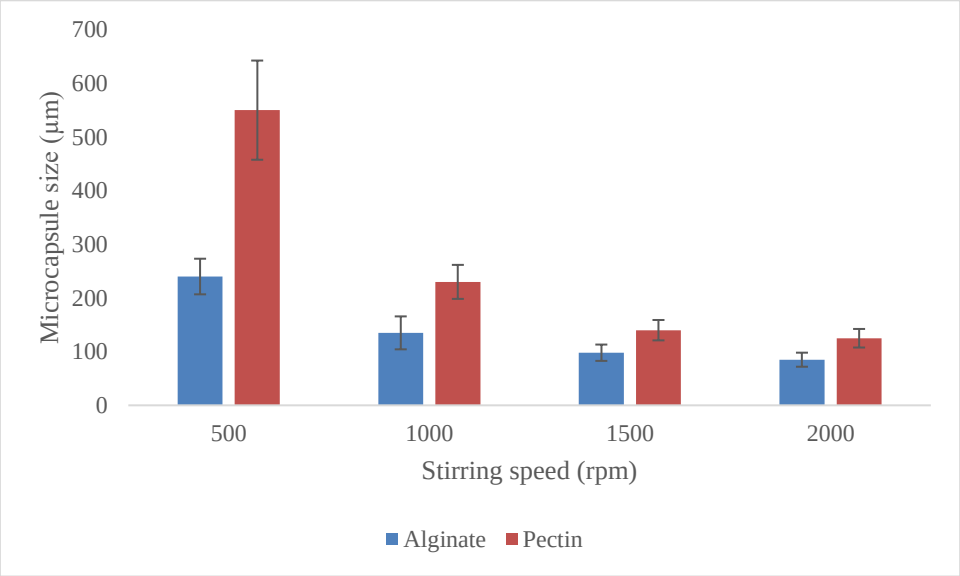


Figure 4. Effect of stirring speed on alginate and pectin microcapsule size. (Values are mean ± SD, n=3)

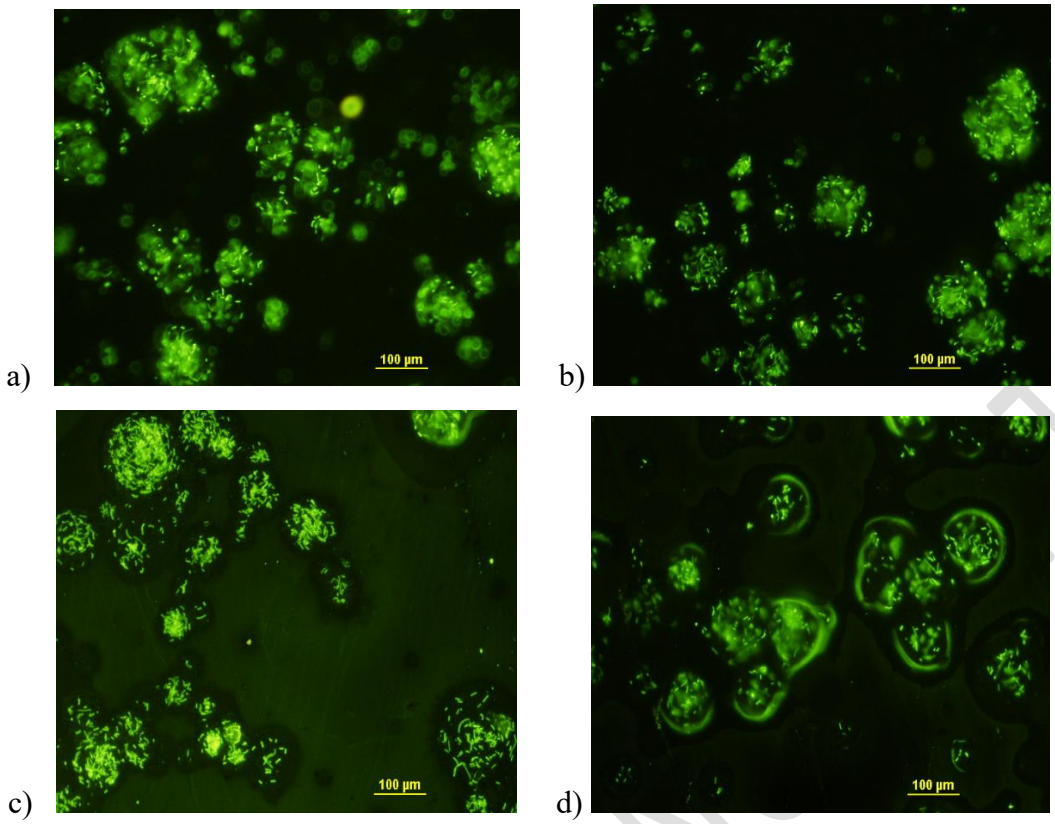


Figure 5. a-d. Fluorescence microscope images of alginate and pectin microcapsules with 20x objective: a) Microencapsulated LA-5 cells in alginate, b) Microencapsulated BB-12 cells in alginate, c) Microencapsulated LA-5 cells in pectin, d) Microencapsulated BB-12 cells in pectin

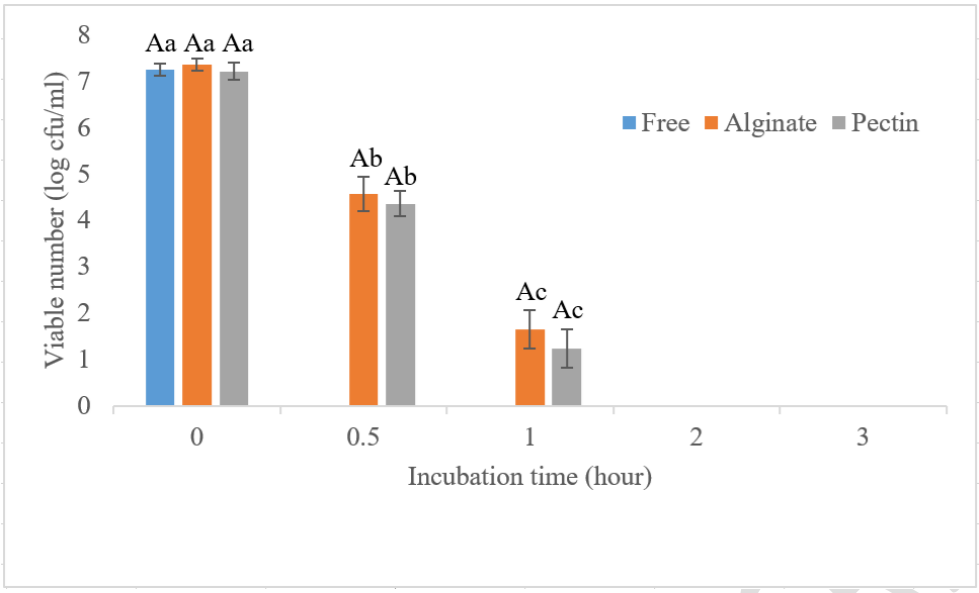


Figure 6. Effect of alginate and pectin microencapsulation on the viability of LA-5 in pH 1. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

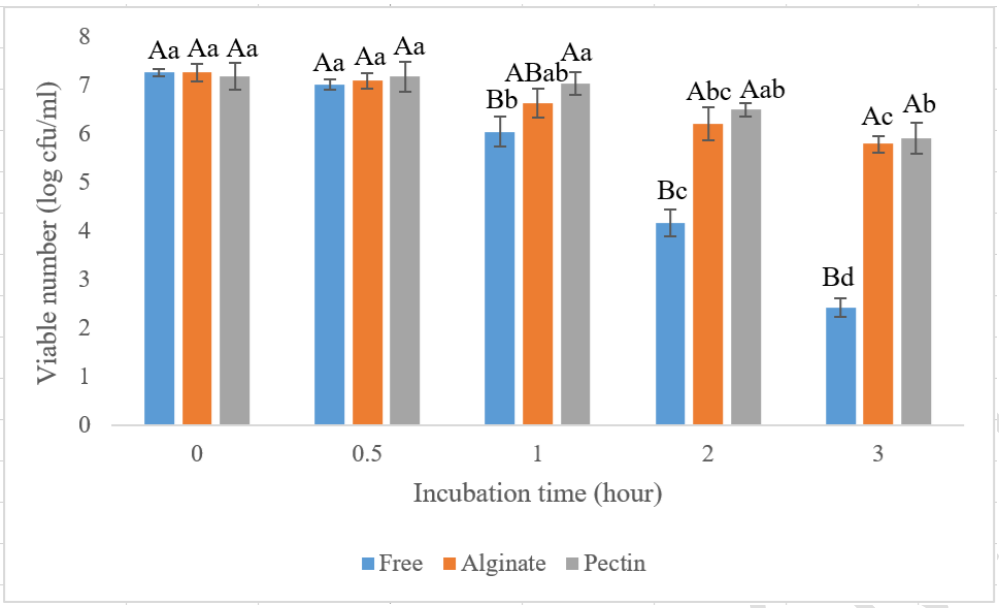


Figure 7. Effect of alginate and pectin microencapsulation on the viability of LA-5 in pH 2. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

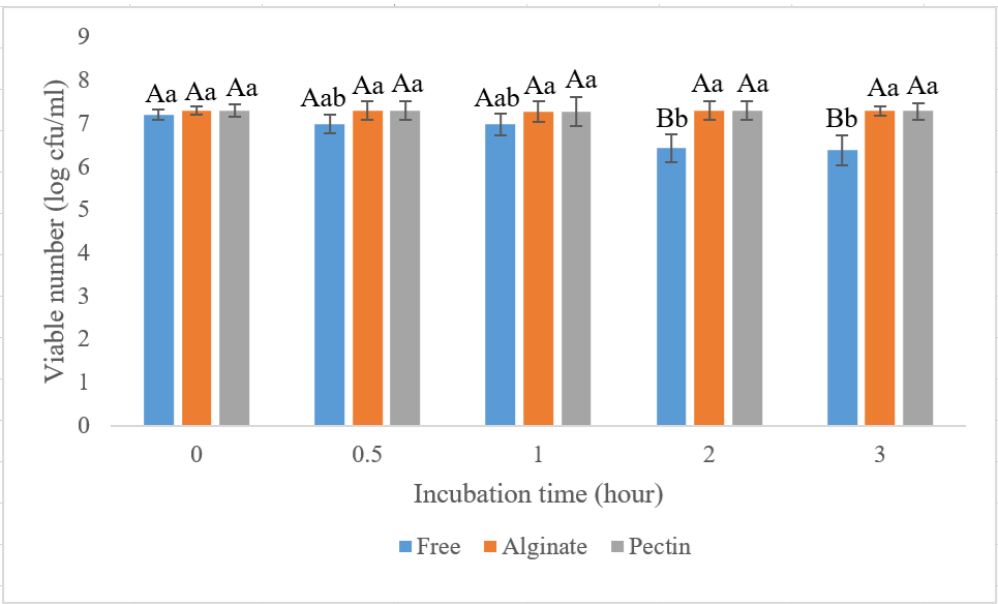


Figure 8. Effect of alginate and pectin microencapsulation on the viability of LA-5 in pH 3. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

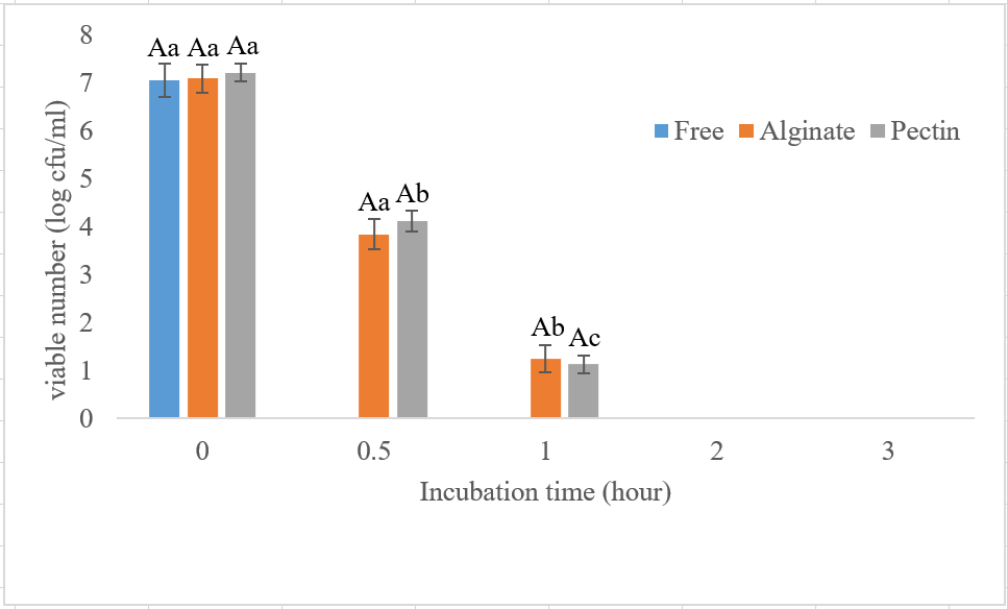


Figure 9. Effect of alginate and pectin microencapsulation on the viability of BB-12 in pH 1. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

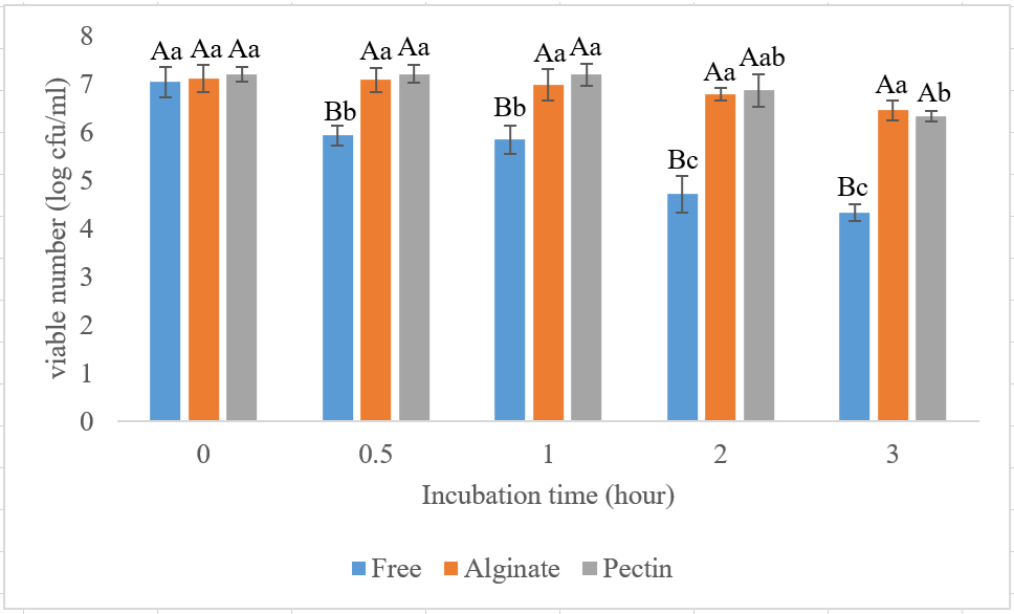


Figure 10. Effect of alginate and pectin microencapsulation on the viability of BB-12 in pH 2. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

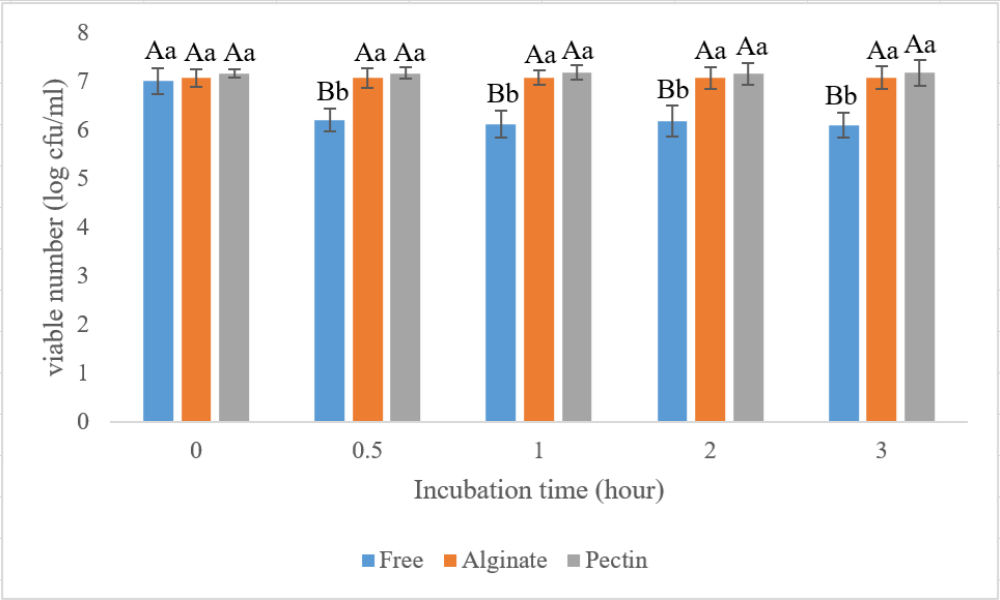


Figure 11. Effect of alginate and pectin microencapsulation on the viability of BB-12 in pH 3. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

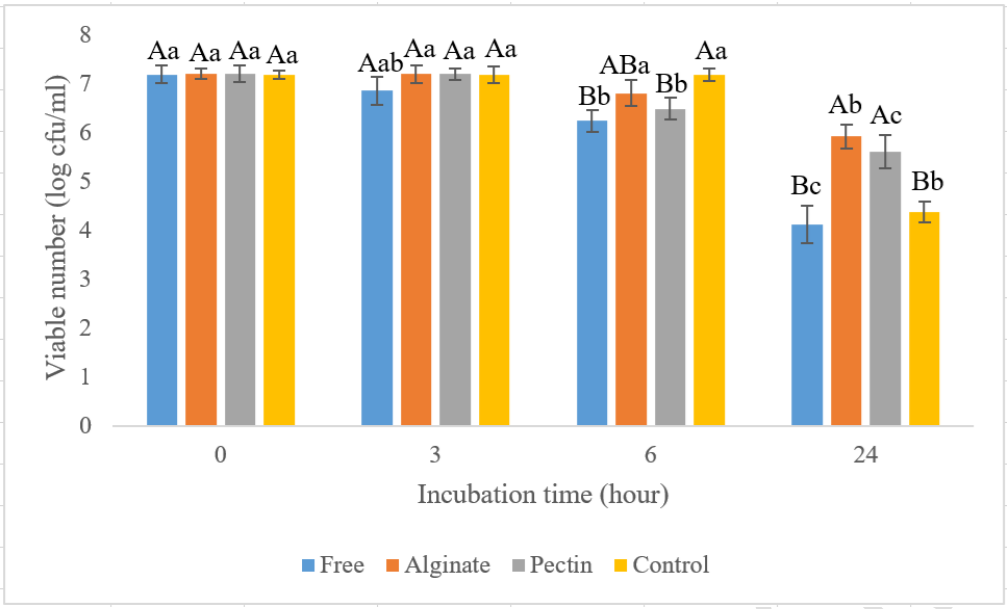


Figure 12. Effect of alginate and pectin microencapsulation on the viability of LA-5 in 0.5% bile solution. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).

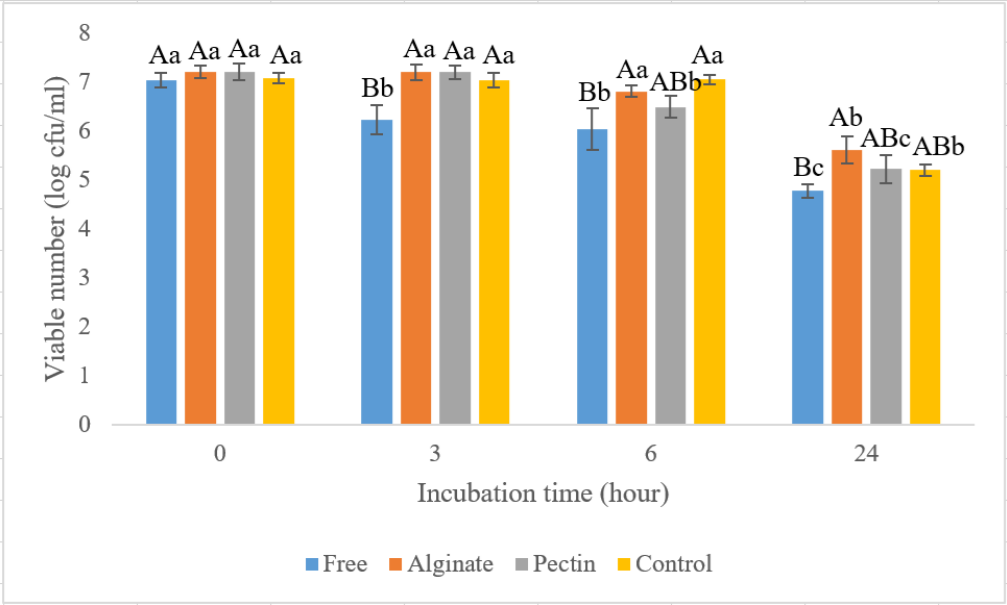


Figure 13. Effect of alginate and pectin microencapsulation on the viability of BB-12 in 0.5% bile solution. Different uppercase letters represent significant differences among samples at the same incubation time ($P < 0.05$); lowercase letters indicate the significant differences between the times of the same sample ($P < 0.05$).