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Microencapsulation of Probiotic *Streptococcus salivarius* LAB813

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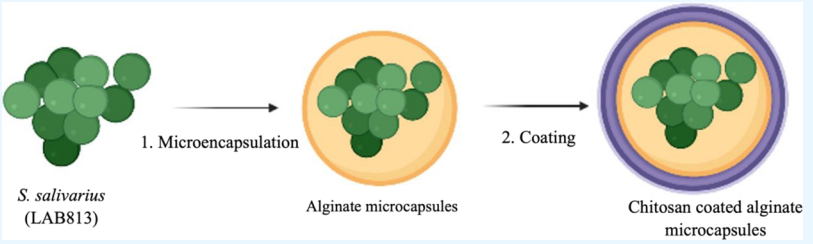

ABSTRACT: Probiotics are living microorganisms that confer a health benefit on the host when administered in adequate amounts. *Streptococcus salivarius*, a commensal bacterium found in the oral cavity, has been shown to secrete antimicrobial peptides and can be used as probiotics. This study aimed to develop a delivery system for the probiotic LAB813, a novel *S. salivarius* strain first identified in the laboratory. Probiotics can be delivered and protected through the encapsulation of biomaterials such as polysaccharides. Their biocompatibility, biodegradability, user-friendliness, and ease of access make polysaccharides useful for encapsulating probiotics. Alginate (Alg) and chitosan (Ch) are naturally obtained polysaccharides and, hence, tested for LAB813 encapsulation. An extrusion method of encapsulation was performed to form Alg microcapsules (Alg-LAB813), some of which were coated with Ch (Alg-LAB813-Ch) to provide dual-layered protection. Inhibitory assays of the Alg-LAB813 and Alg-LAB813-Ch microcapsules were assayed against an indicator strain. Alg-LAB813-Ch microcapsules showed superior antibacterial properties compared to Alg-LAB813 microcapsules over 24 h and when subject to temperatures ranging from 4 to 68 °C. In addition, Alg-LAB813-Ch microcapsules retained antibacterial activity for up to 28 days of storage at 4 °C. The strong and sustained inhibitory activities of Ch-coated Alg encapsulated LAB813 signify the potential for their use to improve oral health.

1. INTRODUCTION

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".[1] Currently, antibacterial effects, stabilization of the flora, and modulation of the host's immune system are thought to provide these benefits. A range of bacteria, most of which are acidogenic like *Lactobacilli*, *Bifidobacteria*, or *Streptococci*, have been suggested to exert one or more of these effects.[2] Probiotic bacteria might also be able to produce antimicrobial substances (e.g., hydrogen peroxide, bacteriocins, adhesion inhibitors) that directly inhibit pathogenic oral bacteria.[3] Indeed, many probiotics produce proteinaceous bacteriocin-like inhibitory substances (BLIS) or antimicrobial peptides capable of killing other bacteria. While membrane permeabilization is the main mechanism of action of antimicrobial peptides against pathogens, additional mechanisms have been described, including membrane destabilization, inhibition of macromolecular synthesis, intracellular translocation of the peptide, and inhibition of DNA/RNA/protein synthesis.[4] An important group of probiotics is the lactic acid bacteria (LAB) among which are the widely used lactobacilli and streptococci.[5] Studies have shown that these bacteria can inhibit the growth of other closely related bacteria[6] by competing with potential pathogens for nutrients or adhesion sites and/or modulating the immune system toward anti-inflammatory action.[7]

The commensal oral bacterium, *Streptococcus salivarius*, appears to be a promising species for probiotic use. *S. salivarius* is a pioneer predominant colonizer of the native microbiota of the oral cavity and persists throughout the human lifespan[8] and is a prolific antimicrobial peptide-producing species.[9] We identified a novel strain of *S. salivarius*, LAB813, from the oral cavity of a healthy child.[10] LAB813 possesses potent antibiofilm properties and strong antimicrobial activity against one major oral microorganism associated with poor oral health, *Streptococcus mutans*.[11] LAB813, therefore, has the potential to be developed for use as a probiotic to improve oral health.

For a probiotic to exert any beneficial effects on the host, live cells of adequate dosage (to be determined in dosage efficacy studies for each specific probiotic; International Scientific Association for Probiotics and Prebiotics) should be able to reach the target site and survive and function. Microencapsulation is widely used to package the probiotics in

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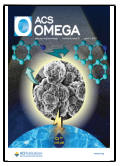

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protective shells, thereby providing physical barriers to improving the viability and bioavailability of the probiotics.[12] Natural polymers are very common delivery carriers for probiotic microencapsulation, with alginate (Alg) being one of the most popular.[13] Alg is a linear polysaccharide consisting of β-(1−4)-linked D-mannuronic acid and α-(1−4)-linked L-glutaronic acid residues.[14] Due to their derivation from brown algae, they exist in abundance in nature and are generally recognized as a safe and nontoxic material for bacterial encapsulation. When Alg interacts with an ionic cross-linking agent such as calcium chloride, a hydrogel matrix is formed. The permeability of the hydrogel matrix makes it easy for air and nutrient exchange, and survival of the probiotics, thereby making Alg hydrogels an excellent and popular carrier for delivering probiotics.[13,15] One disadvantage associated with the Alg hydrogels is their high porosity which can be improved by combining them with other materials including chitosan (Ch).[16] Ch, a nontoxic, biocompatible, and biodegradable cationic polysaccharide with partly acetylated (1−4)-2-amino-2-deoxy-β-d glucan obtained from chitin. It is commonly used as a coating material for Alg beads and has been shown to increase survivability of probiotics as compared to uncoated Alg beads.[17−19] The mechanism involves a negatively charged Alg that, upon interacting with positively charged Ch, generates a smoother surface with smaller porosity and less permeability to water-soluble molecules.[20,21]

The aim of the work was to analyze the antimicrobial activities and the survival of encapsulated LAB813. The inhibitory activities of LAB813 upon encapsulation with Alg, and either coated with Ch or not, were tested against *Micrococcus luteus*, a sensitive target indicator strain, under different temperatures, length of time, and in the presence of saliva. The release of BLIS from LAB813 upon encapsulation was also studied.

2. MATERIALS AND METHODS

2.1. Materials, Buffers, and Stock Solutions. The sodium form of Alg and Ch (medium molecular weight) was purchased from Sigma-Aldrich (Oakville, Canada). Calcium chloride ($CaCl_2$), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium phosphate (KH_2PO_4), and all other chemicals were purchased from BioShop (Burlington, Canada) and used as stated.

A stock solution of 3.0% Alg was made by mixing 3 g of sodium alginate in 100 mL of de-ionized water and autoclaved. A stock solution of 1.5% Ch was made by dissolving 1.5 g of Ch in 100 mL 1% (v/v) glacial acetic acid solution. The pH was adjusted to 5.3 by adding 0.1 M NaOH after which the solution was filtered and sterilized using UV light for 30 min.

Artificial saliva was prepared by dissolving 1.6 g NaCl, 0.3 g NH_4NO_3, 0.6 g KH_2PO_4, 0.2 g KCl, 0.3 g potassium citrate, 0.02 g uric acid sodium salt, 0.2 g urea, 0.5 g lactic acid sodium salt, 3 g mucin-type II in 1 L of distilled water and autoclaved at 121 °C for 15 min. 20 mM sucrose was UV sterilized and added to the autoclaved artificial saliva and pH adjusted to 6.8.

2.2. Bacterial Cultures. Microbial strains were frozen at −80 °C in nutrient-rich liquid broth supplemented with 15% (v/v) glycerol. LAB813 was cultivated in BAC-7 broth medium (10 g tryptone, 10 g yeast extract, 10 g peptone, 10 g neopeptone, 0.2 g NaCl, 0.25 g magnesium sulfate, 0.2 g sodium acetate, 0.5 g ascorbic acid, 2.5 g disodium phosphate per liter) supplemented with filtered-sterilized 0.5% (v/v) sucrose added after autoclaving the rest of the medium. *M. luteus* (kindly provided by Dr. Jeremy Burton, University of Western Ontario), used as a sensitive indicator strain for the antimicrobial assays, was cultured in Brain Heart Infusion (BHI) broth (Becton Dickenson; 6.0 g Brain Heart infusion, 6.0 g peptic digest of animal tissues, 3.0 g dextrose, 5.0 g $NaCl_2$, 14.5 g pancreatic digest of gelatin, and 2.5 g Na_2HPO_4 in 1 L). For semisolid agar medium, the preparation was supplemented with 0.3% (w/v) agar.

2.3. Alg Encapsulation and Ch Coating of LAB813 Microcapsules. A loop culture from LAB813 was inoculated in BAC-7 broth containing 0.5% (v/v) sucrose and incubated overnight at 37 °C. 1 mL of overnight grown LAB813 cells were centrifuged at 12,000 rpm for 10 min, resuspended in separate 1, 1.5, 2, 2.5, and 3% sodium alginate solutions (prepared from 3.0% Alg stock solution). After mixing thoroughly, the Alg-LAB813 solutions were released into the suspension bath of 1% $CaCl_2$ solution. The microcapsules were kept in the 1% (w/v) $CaCl_2$ solution for 30 min and rinsed with PBS buffer and stored at 4 °C until further use (summarized in Figure S1, Supporting Information).

Concentrations of Ch at 0.1, 0.2, 0.3, and 0.4% (v/v) were prepared from the 1.5% stock solution of Ch. Alg-LAB813 microcapsules were then immersed in different concentrations of Ch solution for 40 min followed by rinsing with PBS buffer and stored at 4 °C until further use.

2.4. Spot-on-Lawn Inhibitory Assays. The antimicrobial activity of the encapsulated LAB813 was studied via spot-on-lawn assays as described by Dufour et al.[22] and quantitated as outlined in Figure S2 (Supporting Information). Briefly, ~10^7 CFU/mL of *M. luteus* was overlayed on the BHI agar plate. Next, wells of 0.9 mm diameter were created in the agar plates into which Alg-LAB813, Alg-LAB813-Ch microcapsules, and any controls were placed. The plates were incubated overnight at 37 °C and inhibition of growth was examined and quantitated. Quantitation of inhibitory activities was performed by averaging the zones of inhibition (ZOI) of each well (detailed in Figure S2, Supporting Information).

2.5. Encapsulation Efficiency and Cell Survival Study. The percentage of LAB813 survival over a period of 5 days and the encapsulation efficacy of LAB813 in the Ag and Ch were determined as described by Oberoi et al.[23] Alg-LAB813 and Alg-LAB813-Ch microcapsules were disrupted using 2% (w/v) sterile trisodium citrate solution at pH 6.0, and gently shaken at room temperature for 10 min, followed by sonication on ice for 1 min. The released LAB813 cells were then serially diluted and plated on the BHI agar plate and incubated at 37 °C for 24 h and enumerated. Percent encapsulation efficiency and cell survival were calculated as follows:

$$\text{Encapsulation efficiency } (\%) = (N/N_o) \times 100$$

$$\text{Cell survival } (\%) = (N/N_{zero}) \times 100$$

where N is the number of viable encapsulated cells (CFU/mL) released from the microcapsules, and N_o is the number of the free viable bacterial cells (CFU/mL) added during the microcapsule preparations for the calculation of encapsulation efficiency. For cell survival, the percentage of LAB813 survival was determined by comparing the number of viable bacterial cells, N, released from the microcapsules on days 1, 2, 3, 4, and 5 against N_{zero} (the number of cells released at day 0).

2.6. Characterization of Alg-LAB813 and Alg-LAB813-Ch Microcapsules. FTIR spectra of the Alg-LAB813 and Alg-LAB813-Ch microcapsules were recorded on the PerkinElmer

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A

Alginate		Chitosan*	
% w/v	mm	% w/v	mm
1	5.1 ± 0.1	0	5.6 ± 0.1
1.5	5.5 ± 0.2	0.1	6.6 ± 0.5
2	4.7 ± 0.2	0.2	5.3 ± 0.6
2.5	4.1 ± 0.5	0.3	4.8 ± 0.2
3	3.5 ± 0.1	0.4	4.4 ± 0.3

*Coated on LAB813 encapsulated in 1.5% alginate

B.

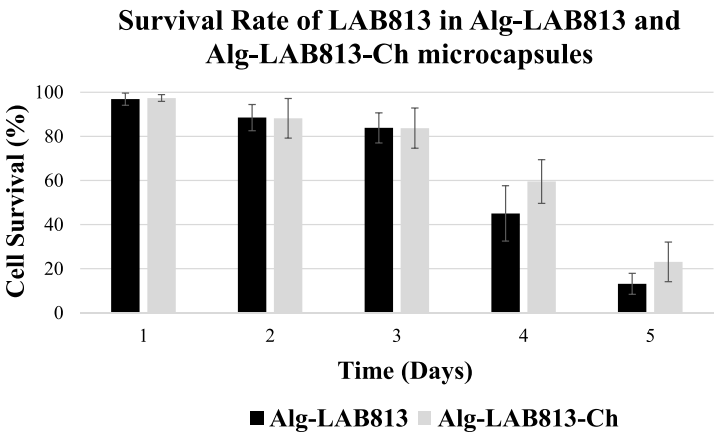

Figure 1. (A) Table of values of the zones of inhibition (mm) of different concentrations of Alg and Ch used in the Alg-LAB813 and Alg-LAB813-Ch. (B) Cell survival of Alg-LAB813 (black) and Alg-LAB813-Ch (gray) at days 1, 2, 3, 4, and 5.

FTIR spectrometer from 1000 to 4000 cm^{-1} at a resolution of 4 cm^{-1}.

The surface morphology of Alg-LAB813 and Alg-LAB813-Ch microcapsules was characterized using a scanning electron microscope (FlexSEM1000, Hitachi SU1000). Prior to analysis, the microcapsules were air-dried and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C. The microcapsules were then dehydrated in graded ethanol solutions (30, 50, 70, 90, 95, and 100%) for 15 min. The microcapsules were dried using a critical point drying (CPD) chamber and sputter coated with gold for 2 min using a sputter coater after which they were analyzed using SEM at an accelerating voltage potential of 20 kV.

2.7. Antimicrobial Activities of Alg-LAB813 and Alg-LAB813-Ch Microcapsules at Different Temperatures, Time Periods, and in the Presence of Artificial Saliva. A series of experiments were performed to test the antimicrobial activities, as assayed by spot-on-lawn assays, of Alg-encapsulated LAB813 either coated or not coated with Ch under the incubation conditions described below:

2 h at 4 different temperatures, i.e., 4, 15, 37, and 68 °C for 2 h.

4 °C for 0, 7, 14, 21, and 28 days.

37 °C for 0, 4, 12, 24, 48, and 72 h.

In artificial saliva at 37 °C for 0, 2, 4, 6, 12, and 24 h.

Inhibitory activities of these experiments were assayed by spot-on-lawn as described above.

2.8. Antimicrobial Activities of PBS and Saliva Incubated with LAB13 Microcapsules. Triplicates of 7 tubes of Alg-LAB813 and Alg-LAB813-Ch microcapsules were incubated at 37 °C for 0, 4, 6, 12, 24, 48, and 72 h in 1 mL of either PBS or artificial saliva. Following the defined periods of incubation, 100 μL of PBS or artificial saliva was removed and tested for antimicrobial activities.

2.9. Statistical Analysis. All the experiments were repeated five times and data represent an average of five independent experiments ± SD (standard deviation) shown by an error bar.

3. RESULT AND DISCUSSION

3.1. Encapsulation of LAB813 in Alg and Alg-Ch Microcapsules. Alg encapsulated *S. salivarius* LAB813 formed microcapsules that were approximately 4. 5 ± 0.5 mm (n = 10) mm in diameter. Alg microcapsules coated with Ch to form Alg-LAB813-Ch microcapsules did not look different from the uncoated ones upon visual inspection (Figure S3, Supporting Information).

To determine the percentage of Alg that would give the highest antimicrobial activities, five different concentrations of Alg, i.e., 1.0, 1.5, 2.0, 2.5, and 3.0% (w/v), were used to encapsulate LAB813. The inhibitory activities of Alg encapsulated LAB813, Alg-LAB813, were highest with microcapsules encapsulated in 1.5% Alg concentration, with ZOI of 5.5 (±0.2) mm, that decreased with increasing concentrations of Alg such that when 3.0% Alg was used, the ZOI was only 3.5 (±0.1) mm (Figure 1A). We next tested whether the 1.5% Alg encapsulated LAB813Ch microcapsules, Alg-LAB813-Ch, coated with different concentrations altered its inhibitory activities. Of the four tested concentrations of Ch, i.e., 0.1, 0.2, 0.3, and 0.4%, the inhibitory activities of LAB813 decreased with increasing concentration of Ch (Figure 1A). Ch at 0.1% concentration showed the biggest inhibitory zones (6.6 ± 0.5 mm) and was thus used for the coating of 1.5% Alg encapsulated LAB813 microcapsules. It was interesting to note a decrease in the inhibitory activities of LAB813 encapsulated in concentrations of Alg greater than 1.5%. This could be related to the increased viscosity with increasing Alg concentrations that made it harder to allow proper mixing of the bacteria prior to cross-linking, hence resulting in an overall decrease in encapsulation efficiency. Furthermore, Alg gels have been documented to be typically nanoporous with pore size of ∼5 nm.[24] Indeed, one study testing the distribution of pore size in 0.8% and 1.4% w/v Alg showed that pores <10 nm corresponded to approximately 50% and 60% in 0.8% and 1.4% w/v microcapsules, respectively.[25] At concentrations lower than or higher than 1.5% w/v, it is possible that the overall pore sizes were either too big for adequate retention of the bacteria or, when the Alg concentration increases, too viscous to enable proper mixing. In comparing the LAB813 encapsulated microcapsules with or without Ch coating, it was noted that the optimized concentrations of 1.5% Alg microcapsules with 0.1% Ch coatings of LAB813 exhibited an inhibitory zone of 6.6 (±0.5) mm compared to the 5.5 (±0.2) mm of 1.5% Alg alone, suggesting that the presence of Ch resulted in greater antimicrobial efficacy of Alg encapsulated LAB813 microcapsules. The improved antimicrobial ability with Ch coating of different probiotics

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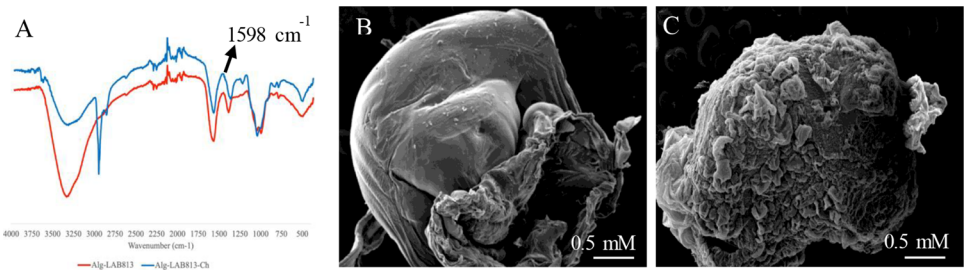

Figure 2. (A) FTIR analysis of Alg and Ch coating of LAB microcapsules. Scanning electron microscope images of (B) Alg-LAB813 and (C) Alg-LAB813-Ch microcapsules.

Temp (^{0}C)	Alg-LAB813	Alg-LAB813-Ch
4	5.1 ± 0.3	6.0 ± 0.2
15	4.9 ± 0.3	6.1 ± 0.1
37	5.1 ± 0.1	5.9 ± 0.1
68	1.6 ± 0.2	3.6 ± 0.5

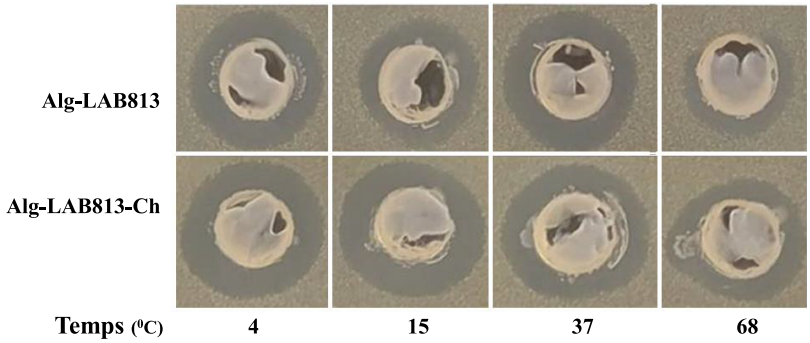

Figure 3. Antimicrobial activities of Alg-LAB813 and Alg-LAB813-Ch microcapsules at 4, 15, 37, and 68 °C temperatures for 2 h. Representative results of inhibitory spot-on-lawn assays are shown below the tabulated numbers.

encapsulated in Alg has been documented[26] and believed to be due to the reduction of leakage of the encapsulated materials and formation of a semipermeable membrane from the interactions of the negatively charged Alg with the positively charged Ch.[27] As expected, Alg- and Ch-coated Alg microcapsules without LAB813 did not show any inhibitory effect (as can be observed in Figure S2-C). The antimicrobial activities from LAB813 were most likely through its release of BLIS with antimicrobial activities, as has been demonstrated in numerous other *S. salivarius* strains.[28] Indeed, the analysis of the sequenced genome of LAB813[10] revealed the presence of a bacteriocin locus in LAB813 and attempts are currently directed at identifying the bacteriocin(s).

Encapsulation efficacy of Alg-LAB813 and Alg-LAB813-Ch microcapsules showed no statistically significant differences (p value > 0.05) of 90.55 (±2.05)% and 94.75 (±1.25)%, respectively, in line with other reported encapsulation efficacy data.[21,29] Next, we determined the survival of LAB813 in Alg-LAB813 and Alg-LAB813-Ch microcapsules over a period of 5 days (Figure 1B). LAB813 cells retained similar cell viability for at least 3 days, with Alg-LAB813 and Alg-LAB813-Ch showing 93.55 (±4.9)% and 96.52 (± 3.15)% cell survival, respectively, at Day 1. By Day 4, however, LAB813 cell viability was reduced to 42.17 (±12.5)% and 57.5 (±9.9)% in Alg-LAB813 and Alg-LAB813-Ch, respectively. A log reduction in cell count was seen a day later (Day 5), at 12.34 (±4.7)% and 22.34 (±8.9)% in Alg-LAB813 and Alg-LAB813-Ch, respectively. No significant difference (p value > 0.05) was observed in the cell survival in Alg-LAB813 and Alg-LAB813-Ch microcapsules, suggesting that Ch coating did not improve the cell viability of LAB813, a finding corroborated by Yeung et al.[30]

3.2. Characterization of Alg-LAB813 and Alg-LAB813-Ch microcapsules. To understand the structural changes and functional groups of the Alg- and Ch-coated Alg LAB813 microcapsules, Fourier transform infrared (FTIR) spectroscopy was conducted (Figure 2A). The FTIR spectrum of Alg showed characteristic peaks at 3367 cm^{-1} corresponding to the −OH stretching vibrations, and 2323 and 2286 cm^{-1} that corresponded to the −CH stretching vibrations. The peaks located at 1603 and 1420 cm^{-1} were associated with the symmetric and asymmetric vibrations of the carboxylate group (COO−), respectively. However, the band at 1030 cm^{-1} indicated an asymmetric elongation of C−O−C.[31] FTIR spectra of Ch-coated Alg microcapsules showed a band at 3346 cm^{-1} associated with −NH or −OH stretching vibration. The appearance of peak at 1598 cm^{-1} signified an effective ionic interaction between the $-NH_2$ group of Ch and the −OOH group of Alg.[32,33] The peak at 1394 cm^{-1} corresponded to the CH_2OH of C-6 position of the sugar group of Ch. The band at 1074 cm^{-1} was associated with −OH bending of Ch.[34] This set of data therefore confirmed the presence of functional groups of Alg and Ch in the microcapsules.

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A. 4°C

Days	-	-LAB813-
0	5.6 ± 0.2	6.6 ± 0.3
7	5.4 ± 0.1	6.3 ± 0.2
14	5.1 ± 0.2	6.1 ± 0.1
21	4.5 ± 0.1	5.9 ± 0.2**
28	4.1 ± 0.2	5.5 ± 0.2***

B. 37°C

	-	-LAB813-
0	5.5 ± 0.2	6.1 ± 0.3
4	5.4 ± 0.2	6.0 ± 0.1
12	3.5 ± 0.1	5.9 ± 0.3
24	2.9 ± 0.3	5.2 ± 0.2**
48	2.5 ± 0.1	4.7± 0.2
72	1.3 ± 0.2	4.0 ± 0.2

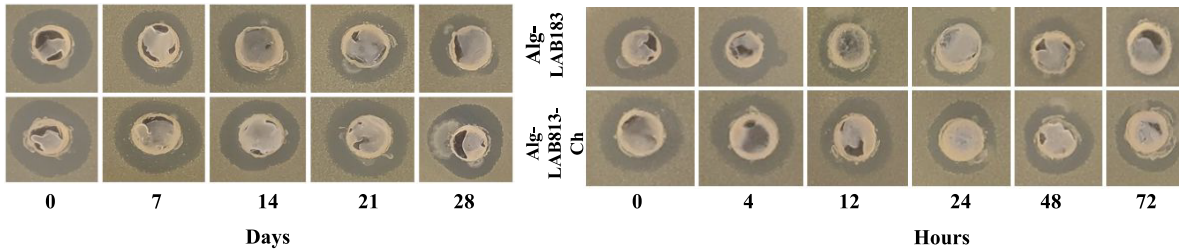

Figure 4. Antimicrobial activities of Alg-LAB813 and Alg-LAB813-Ch microcapsules at (A) 4 °C for 0, 7, 14, 21, and 28 days (**p value < 0.03 and ***p value < 0.007), and (B) at 37 °C for 0, 4, 12, 24, 48, and 72 h (**p value < 0.008). Representative results of inhibitory spot-on-lawn assays shown below the tabulated numbers.

Time (h)	Alg-LAB813	Alg-LAB813-Ch
0	5.8 ± 0.2	6.6 ± 0.2
2	5.6 ± 0.2	6.4 ± 0.3
4	5.5 ± 0.1	6.3 ± 0.2
6	5.5 ± 0.2	5.9 ± 0.2
12	2.2 ± 0.1	2.8 ± 0.2
24	1.4 ± 0.2	1.8 ± 0.2

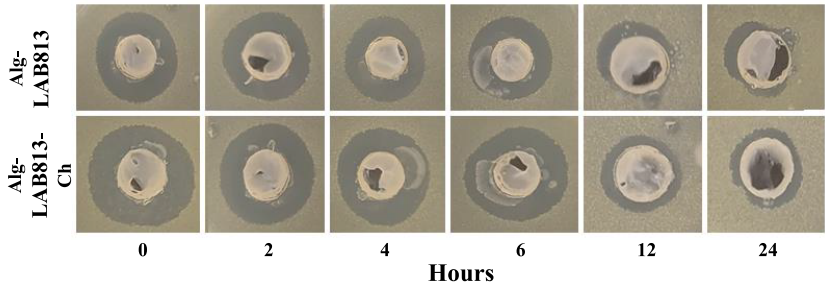

Figure 5. Antimicrobial activities of Alg-LAB813 and Alg-LAB813-Ch microcapsules in artificial saliva for 0, 2, 4, 6, 12, and 24 h at 37 °C. Representative results of inhibitory spot-on-lawn assays shown below the tabulated numbers.

Scanning electron microscopy (SEM) was used to investigate the overall smoothness of the surfaces of the Alg-LAB813 and Alg-LAB813-Ch microcapsules. Compared to the smooth surface of the Alg microcapsules (Figure 2B), that of the Ch-coated Alg microcapsules appeared irregular with jagged edges (Figure 2C), suggesting that the Ch layer has been successfully deposited onto the external surfaces of the Alg microcapsules.

3.3. Efficiency of Antimicrobial Activities of Alg-LAB813 and Alg-LAB813 Microcapsules at Different Temperatures. The effect of temperature on the antimicrobial activity of Alg-LAB813 and Alg-LAB813 microcapsules was next determined. Four different temperatures were chosen to test the stability of the inhibitory properties of the encapsulated LAB813 ranging from temperatures used in storage conditions, at room temperature, body temperature, and in the oral cavity during the ingestion of hot beverages, i.e., 4, 15, 37 and 68 °C, respectively. At temperatures up to 37 °C, both Alg-LAB813 and Alg-LAB813-Ch microcapsules retained similar activity showing zones of inhibition ranging from 4.9 (±0.3) to 5.1 (±0.1) mm and 5.9 (±0.1) to 6.1 (±0.1) mm in Alg-LAB813 and Alg-LAB813-Ch microcapsules, respectively (Figure 3). However, at 68 °C, the inhibitory zone in Alg-LAB813-Ch microcapsules decreased significantly to 3.6 (±0.5) mm and to a greater degree, i.e., 1.6 (±0.2) mm (**p value < 0.004), in Alg-LAB813 microcapsules, suggesting the protective effect of Ch coating on the inhibitory property of the LAB813 microcapsules at 68 °C.

Next, the stability of Alg-LAB813 and Alg-LAB813-Ch microcapsules was tested over a period of 28 days at 4 and 37 °C temperatures that represent storage conditions of the final consumer product and the human body temperatures, respectively. The inhibitory activities of Alg-LAB813 and Alg-LAB813-Ch microcapsules were retained over a period of 28 days at 4 °C, albeit with decreases in the activity over 28 days, e.g., from 5.6 (±0.2) to 4.1 (±0.2) mm from day 0 to 28 for the Alg-LAB813, and 6.6 (±0.3) to 5.5 (±0.2) mm from days 0 to 28 for the Alg-LAB813-Ch (Figure 4A). Alg-LAB813-Ch micro-

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Time (h)	Alg-LAB813	Alg-LAB813-Ch
0	5.4 ± 0.1 (5.5 ± 0.1)	6.3 ± 0.1 (5.9 ± 0.1)
4	5.3 ± 0.1 (5.3 ± 0.3)	6.2 ± 0.2 (5.5 ± 0.1)
6	5.1 ± 0.1 (4.9 ± 0.1)	5.8 ± 0.1 (4.1 ± 0.1)
12	3 ± 0.2 (<1.0)	3. 2 ± 0.1 (<1.0)
24	2.6 ± 0.2 (0)	3.0 ± 0.2 (0)
48	1.9 ± 0.3 (0)	2.0 ± 0.1 (0)
72	1.5 ± 0.2 (0)	1.9 ± 0.2 (0)

Orange numbers denote saliva buffer

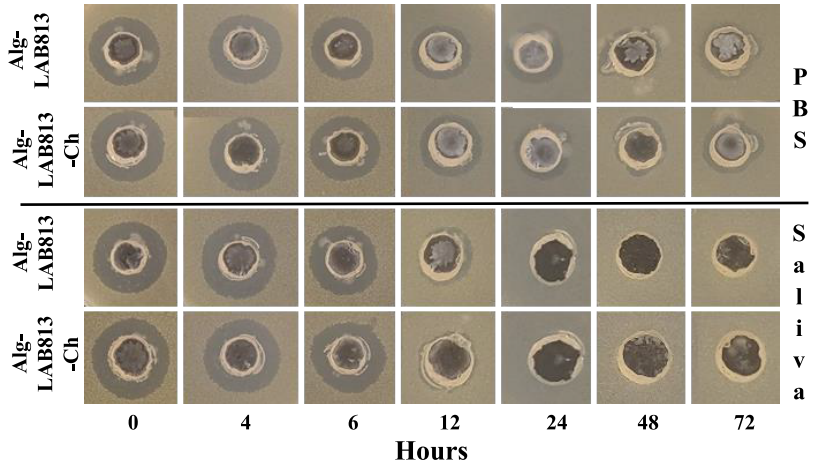

Figure 6. Antibacterial activity of BLIS released in PBS buffer and artificial saliva from the Alg-LAB813 and Alg-LAB813-Ch microcapsules. In the tabulated form above, values indicated in black colors are those of the microcapsules incubated in PBS, and of orange, incubated in saliva. Representative results of inhibitory spot-on-lawn assays shown below the tabulated numbers.

capsules showed no significant difference (**p value < 0.03) from days 0 to 21; however, a significant difference (***p value < 0.007) was observed on day 28.

At 37 °C, the presence of the Ch coating had an obvious protective effect on the loss of antimicrobial activities that was observed in the Alg-LAB813 microcapsules after only 12 h of incubation (Figure 4B). For the Alg-LAB813 microcapsules, the 5.5 (±0.2) mm inhibitory zone observed at the beginning (0 h) decreased to 3.5 (±0.1) in 12 h and reduced to 1.3 (±0.2) mm at 72 h, showing a loss of inhibitory activity of ∼75% in 72 h. The inhibitory activities of Alg-LAB813-Ch microcapsules had a smaller decrease: from 6.1 (±0.3) mm at 0 h to 5.2 (±0.2) mm at 24 h, followed by a significant difference (**p value < 0.008) from 0 to 24 h, that continued downward at 48 and 72 h (Figure 4B).

LAB813 is to be used in the oral cavity for its antimicrobial activity. Hence, the antibacterial efficacy of encapsulated LAB813 was tested in the presence of saliva, a liquid that bathes the oral cavity to keep it in a moist condition, and at the body temperature of 37 °C. Human saliva contains about 99% water and about 1% of organic and inorganic molecules, of which one is the glycoprotein, mucin, that coats all oral cavity surfaces and acts as an important lubricant during oral functions.[35] Alg-LAB813 and Alg-LAB813-Ch microcapsules were incubated with saliva for 0, 2, 4, 12, and 24 h at 37 °C (Figure 5). The inhibitory activities of Alg-LAB813 and Alg-LAB813-Ch microcapsules remained relatively constant up to 6 h that dropped substantially at 12 h. That is, the inhibitory activity decreased from 5.5 (±0.2) mm at 6 h to 2.2 (±0.1) mm and from 5.9 (±0.2) mm to 2.8 ± 0.2 mm for Alg-LAB813 and Alg-LAB813-Ch microcapsules, respectively, and tapering to a minimal by 24 h. (Figure 5).

A consistent result obtained in all the experiments described thus far was the improved antibacterial efficiency and increased stability of the killing ability when the Alg LAB813 microcapsules were coated with Ch. Others have found similar findings, e.g., microencapsulation of *S. boulardii* in Ch-coated Alg beads.[36,37] A study by Juntarachot et al.[38] showed that encapsulation of dextranase in Alg beads increased the stability for up to 3 months without any significant change in their activity. Koo et al.[20] speculated that coating of Alg beads with Ch improved the chemical and mechanical strength of the beads and allowed for longer-term storage. Nualkaekul et al.[26] demonstrated that the presence of an anion group on Alg also played a significant role in its interaction with the cation compounds such as Ch and provided a strong membrane on the surface of the microcapsules resulting in the prevention of leakage of entrapped cells, thus resulting in enhanced antibacterial efficacy. Santos and Machado[36] also explained that external ionic gelation along with a Ch coating showed better results regarding the survival of *S. boulardii* in gastrointestinal fluids.

3.4. Activity of BLIS Released from the Encapsulated LAB813. The antimicrobial activities of encapsulated LAB813 were believed to be from secreted antimicrobial products, most likely BLIS, as has been documented in other *S. salivarius* strains.[28] We thus tested whether the PBS buffer or artificial saliva in which the microcapsules were immersed over 72 h had antimicrobial activities. Whether in PBS or saliva, the inhibitory activities remained quite constant from 0 to 4 h in Alg encapsulated LAB813 coated with Ch or not, then decreasing slightly at 6 h (Figure 6). By 12 h, however, there was a noticeable decrease in the inhibitory zones in all groups, whether in PBS or saliva (Figure 6). Whereas the antimicrobial activities in the PBS buffer exhibited a progressive but gradual decrease

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from 6 to 72 h, those of the saliva microcapsule groups showed a dramatic decline by 12 h, with no inhibitory activities, especially of the Alg-LAB813-Ch, by 72 h (Figure 6). Interestingly, in comparing the antimicrobial activities of Alg-LAB813 microcapsules (Figure 4B) and those of the PBS buffer in which the microcapsules were immersed (Figure 6), not much difference in the antimicrobial activities was observed, suggesting that diffusion of antimicrobial substances occurred easily from the capsules to the PBS buffer. The presence of Ch coating appeared to reduce the release of these peptides at 12 h and after, as observed in the 3.2 (±0.1) mm inhibitory zone by 12 h in the buffer as compared to the 5.9 (± 0.3) mm in the Alg-LAB813-Ch microcapsules. This finding is important in terms of the ability of the 0.1% Ch to control the release of the antimicrobial substances from this probiotic into the surrounding buffer, as previously documented.[39] Another interesting finding is the ability of saliva to cause a reduction in the antimicrobial activities and in the release of the antimicrobial substances into the immersed saliva, whether Ch was present or not. For example, by 12 h, the antimicrobial activities of both Alg-LAB813 and Alg-LAB813-Ch microcapsules were almost all gone (Figure 6), indicating that the Alg and/or Ch may be interacting with the chemicals or reagents used in the preparation of the saliva, an area that needs further investigation.

4. CONCLUSIONS

The study presents the encapsulation of an oral probiotic bacterial strain, *S. salivarius* (LAB813), in a microcapsule form. Alg microcapsules were designed with special attention such as the preservation of bacterial viability during preparation and storage and preservation of their antibacterial activity. Ch coating to Alg microcapsules resulted in a significant increase in the survival rate and enhanced antibacterial activity of LAB813. In addition, this encapsulating agent enhanced the tolerance of LAB813 to different temperatures without disturbing their antibacterial activity. Considering the promising result achieved, the developed technique can be an attractive approach in the efforts to improve oral health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07721.

Schematic representations of the microcapsule preparation (Figure S1) and quantitative inhibitory (spot on the lawn) assays (Figure S2); a color image of the Alg-LAB813-Ch microcapsules (Figure S3) (PDF)

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Notes

The authors declare no competing financial interest.

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