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The Production of Synbiotic Bread by Microencapsulation

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Summary

Bread is a global staple food. Despite attempts to develop functional breads containing viable microorganisms, this has not been done yet because of the high temperature during baking. The aim of this study is to obtain synbiotic bread, hence hamburger bun and white pan bread were selected. *Lactobacillus acidophilus* LA-5 and *L. casei* 431 were encapsulated with calcium alginate and Hi-maize resistant starch *via* emulsion technique and coated with chitosan. The morphology and size of microcapsules were measured by scanning electron microscopy and particle size analyser. Inulin was added at 5 % wheat flour mass basis for prebiotic effect. The encapsulated probiotics were inoculated into the bread dough and bread loaves were baked. The survival of encapsulated probiotics was determined after baking; also sensory evaluation was performed. Both types of bread met the standard criteria for probiotic products. The probiotic survival was higher in hamburger bun. *L. casei* 431 was more resistant to high temperature than *L. acidophilus* LA-5. A significant increase in probiotic survival was observed when the protective coating of chitosan was used in addition to calcium alginate and Hi-maize resistant starch. Storage for 4 days did not have any effect on the viability of encapsulated bacteria. The addition of encapsulated bacteria did not have any effect on flavour and texture; however, 5 % inulin improved the texture of bread significantly. Results show that microencapsulation used in the production of synbiotic bread can enhance the viability and thermal resistance of the probiotic bacteria.

Key words: probiotic, microencapsulation, alginate, chitosan, inulin, bread

Introduction

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit to the consumer (*1*). In order to provide health benefits of probiotic bacteria, they should be present at a minimum level of 10^6 CFU/g of food product or 10^7 CFU/g at point of delivery or be eaten in sufficient amount to yield a daily intake of 10^8 CFU/g (*2*). Prebiotics are nondigestible substances that contribute to the well-being of their host by selectively stimulating the favourable growth or activity of a limited number of indigenous nonpathogenic bacteria (*1,3*). Fructooligosaccharides and inulin are among the most famous prebiotic compounds (*4,5*). Synbiotic foods are synergistic combinations of pre- and probiotics.

The development of nondairy probiotic products is a challenge to the food industry in its effort to utilize the abundant natural resources by producing high-quality functional products (*6*). Bread is a staple food in many countries and it constitutes a dominant portion of a standard diet, supplying a large fraction of the needs for energy, carbohydrates, proteins and micronutrients. In recent years, there is an increased interest in the role of food with health benefits. The priority of the industry today is innovative approach in satisfying consumer needs. However, functional bread containing viable microorganisms has not been developed yet because of the high temperature during baking (*7*). A new approach to improve the probiotic survival is by physical protection by microencapsulation, which can help protect the bacterial cells

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from the hostile conditions such as those present within gastrointestinal tract, thus potentially preventing cell loss (*3*).

Encapsulating lactobacilli in calcium alginate has been found to improve their heat tolerance and increase the survival by up to 80–95 % (*8–10*). Alginate is an approved food additive and the benefits of its use as an encapsulating agent include: non-toxicity, formation of gentle matrices with calcium chloride to trap living microbial cells, simplicity and low cost (*8,10*). However, the use of alginate is limited due to its low stability in the presence of chelating agents and in acidic conditions below pH=2.0 (*2,11,12*). Combination of calcium alginate with prebiotics such as resistant starch improves both the viability of probiotics and structures of capsules (*12,13*). The coating of alginate beads and its effectiveness in protecting probiotic bacteria has been extensively studied. Previous researchers have reported that coating alginate microcapsules with chitosan improves the stability of the alginate beads, increasing probiotic viability even further (*2,8*). Little research has been carried out with an aim to incorporate probiotics into bakery products, due to destruction of live culture during heat treatment. The aim of this study is to obtain synbiotic bread, hence hamburger bun and white pan bread were selected.

Materials and Methods

Preparation of cell suspension

Pure freeze-dried *Lactobacillus acidophilus* LA-5 and *L. casei* 431 probiotic cultures were obtained from CHR-Hansen (Horsholm, Denmark) and were activated by inoculation in the MRS (de Man-Rogasa-Sharpe) broth at 37 °C for 24 h. The probiotic biomass was harvested in late log phase by centrifugation at 600×*g* for 10 min at 4 °C (3–18K; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), then washed twice in sterile 0.9 % saline solution under the same centrifugation conditions, and used in the microencapsulation process (*3*).

Encapsulation procedure

All glassware and solutions used in the protocols were sterilized at 121 °C for 15 min. Alginate beads were produced using a modified encapsulation method originally reported by Sheu and Marshall (*10*) and Sultana *et al.* (*12*). A 3 % alginate (batch number 71238; Sigma-Aldrich, London, UK) mixture in 100 mL of distilled water containing 2 % Hi-maize resistant starch (Hi-maize® 260; Ingredion, London, UK) and cell suspension (0.1 %, by mass per volume) was prepared. The mixture was heated slightly (50 °C) until complete dissolution before cell suspension (0.1 %, by mass per volume) was dispersed into the solution. The mixture was added into 500 mL of corn oil containing 0.2 % Tween 80 and was stirred vigorously (at 19×*g* for 20 min) until full emulsification. Then the emulsion was broken by adding 500 mL of 0.1 M calcium chloride while stirring. The mixture was allowed to stand for 30 min to separate the prepared calcium alginate beads in the calcium chloride layer at the bottom of beaker. The oil layer was drained and beads in the calcium chloride solution were collected by low speed centrifuge at 350×*g* for 10 min and then washed with 0.9 % saline solution containing 5 % glycerol and stored at 4 °C (*3,9,10, 14*). Low-molecular-mass chitosan (0.4 g; Sigma-Aldrich) was dissolved in 90 mL of distilled water acidified with 0.4 mL of glacial acetic acid to achieve a final concentration of 4 g/L. The pH was then adjusted to between 5.7 and 6.0 by adding 1 M NaOH. The mixture was filtered through Whatman filter paper no. 4 and the volume adjusted to 100 mL before autoclaving at 121 °C for 15 min. Then a mass of 15 g of washed beads was immersed in 100 mL of chitosan solution for coating with gentle shaking at 1×*g* for 40 min on an orbital shaker (two-step method). The chitosan-coated beads were washed and kept in 0.1 g per 100 g of peptone solution at 4 °C for not more than 1 h (*15*), and then used on the same day.

Preparation of bread with encapsulated bacteria

Hamburger buns contained the following ingredients: sugar 3, salt 1, fresh yeast 5 and fat 3 g per 100 g of wheat flour (extraction rate 72 %). Ingredients in white pan bread were: sugar 2, salt 1, fresh yeast 4 and fat 2 g per 100 g of wheat flour (extraction rate 72 %). A mass of 1 g of microencapsulated bacteria was added per 100 g of final product. Even distribution of the bacteria in the dough was obtained by mixing. Inulin was added so that 100 g of wheat flour contained 5 g of HPX inulin (Beneo, Mannheim, Germany) to obtain prebiotic effect per slice of bread equivalent to 0.7–1.2 g of inulin. Studies have shown that the addition of inulin to bread generally results in smaller loaves with a harder crumb, darker colour and decreased overall acceptability. However, a fortification with 5 % inulin appears to be acceptable (*4*). After initial proofing, dough was divided into 60- and 450-gram pieces for hamburger buns and white pan bread loaves, respectively. Dough pieces were rounded and shaped, then transferred to proofing cabinet in trays and pans for 45 min at 37 °C with relative humidity of 85 %.

Baking conditions

Hamburger buns were baked for 15 min at 180 °C and white pan bread loaves were baked for 25 min also at 180 °C. Rotary oven was used for heating and the temperature of crumb centre was measured by thermocouple.

Enumeration of encapsulated probiotics

Bacterial counts were determined before and immediately after microencapsulation, less than 24 h after baking and during 4 days of storage at room temperature. Enumeration of probiotic bacteria was achieved as described by Haynes and Playne (*16*). All enumerating plates were incubated at 37 °C for 72 h under aerobic conditions. The average values of all results were expressed as colony-forming units per gram of sample (CFU/g) (*3*). To count the encapsulated bacteria, the entrapped bacteria were released from the beads according to the method of Sheu and Marshall (*10*). A mass of 10 g of bread was resuspended in 100 mL of phosphate buffer (0.1 M, pH=7.0), followed by blending in a stomacher for 10 min. Since chitosan-coated beads did not dissolve in phosphate buffer, they were suspended in citrate buffer (0.1 M,

pH=6.2), blended in a stomacher for 1 min and then allowed to stand for 10 min to dissolve. The counts (CFU/g) were determined by plating on MRS agar (Merck, Darmstadt, Germany) as discussed above (*3*,*17*).

Size and morphology of microcapsules

In this study, the size of microcapsules was determined by particle size analyser (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK) with the standard deviation calculated from the cumulative distribution curve. Scanning electron microscopy (SEM) (LEO 440i; Oxford Instruments, Oxford, UK) was used to observe the surface and morphology of microcapsules.

Sensory analysis

Triangle test was performed on the first and fourth day of storage at room temperature. Evaluation was carried out by ten expert panellists recruited among the employees of Sahar bread factory (Tehran, Iran) and Bread Research Centre (Tehran, Iran). The samples were assessed in a standardised tasting room equipped with individual booths along a wall that divided the room from the preparation area (*18*,*19*). The reference samples did not contain encapsulated bacteria and inulin. All samples were served in dishes with three-digit codes: five judges tested two treatment samples and one reference sample, and the other five judges received one treatment sample and two reference samples. The judges were asked to indicate and identify the odd sample regarding flavour and texture. They were also asked to indicate the degree of difference between the duplicate samples and the odd sample and finally the sample they preferred. The degree of difference indicated by the ten judges, who correctly identified the odd samples, was scored on a four-point scale labelled 1 for slight, 2 for moderate, 3 for much and 4 for extreme (*18*,*20*–*24*).

Statistical analysis

A complete randomised factorial design was used for all analyses and all results were expressed as mean values of triplicate trials. Factors selected were bread type, bacterial strain, coating type and storage time. Data analysis was carried out using Statistical Package for Social Sciences (SPSS) software v. 20 (SPSS Inc., Chicago, IL, USA). Significant differences between the treatments were detected using least significant differences at $p<0.05$.

Results and Discussion

Shape and size of calcium alginate microcapsules

Scanning electron microscopy (SEM) showed that the beads were generally globular in shape and also showed that the starch granules were present in the alginate matrix and the cavities (Fig. 1). Previous studies indicated that microencapsulation of water-in-oil emulsions helps to avoid abnormal bead shape and that the selection of appropriate coating material determines the physical and chemical properties of the resulting microcapsules (*12*,*25*). Hi-maize resistant starch, which is a prebiotic, also acts as a synergist with alginate in gelling and may help in providing additional protection to the entrapped bacterial cells. Studies have shown good compatibility between alginate and starch. The combination of calcium alginate with resistant starch produces beads with a good integrated structure that swells and absorbs water but does not gelatinize fully during heating (*3*,*12*,*13*,*26*,*27*). Since alginate gels have a porous structure, a filling material such as starch and a chitosan membrane coating can improve stability, maintaining the spherical shape, decreasing the shrinkage of the microcapsules and reducing bead permeability (*28*).

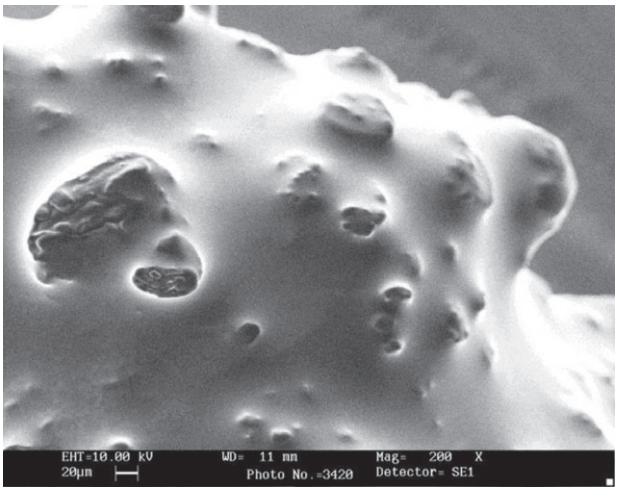

Fig. 1. Scanning electron photomicrograph showing calcium alginate and starch beads at magnification of 200×

Addition of chitosan layer formed a smooth surface (Fig. 2), with Hi-maize resistant starch granules still visible on the surface (Fig. 3). SEM did not show any significant differences in capsule shapes between the calcium alginate-encapsulated and starch-encapsulated probiotic strains produced with and without chitosan coating.

The size distribution of calcium alginate and starch microcapsules was analysed with particle size analyser. Microcapsule diameter of monolayer alginate and starch beads containing *L. acidophilus* LA-5 ranged from 31.0 to 382.9 μm with mean diameter of 216.6 μm, while of those containing *L. casei* 431 ranged from 44.0 to 717.7 μm with mean diameter of 352.8 μm. These results indicated that beads containing *L. casei* 431 were bigger in size than beads containing *L. acidophilus* LA-5, while from a morphologic point of view, no difference was observed. Therefore, our results showed that capsule size depends on the probiotic strain, which is in agreement with Chavárri *et al.* (*2*).

The size distribution of calcium alginate and starch microcapsules coated with chitosan was also analysed. Microcapsule diameter of chitosan-coated beads containing *L. acidophilus* LA-5 ranged from 78.0 to 574.2 μm with mean diameter of 347.4 μm, while of those containing *L. casei* 431 ranged from 93.04 to 895.7 μm with mean diameter of 512.6 μm. Therefore, the mean diameter of double layer chitosan-coated beads was significantly ($p<0.05$) higher than of monolayer alginate and starch beads, which is in agreement with Mokarram *et al.* (*29*). Emulsion technique used in this experiment produces micrometer-sized beads rather than millimeter-sized ones produced by many

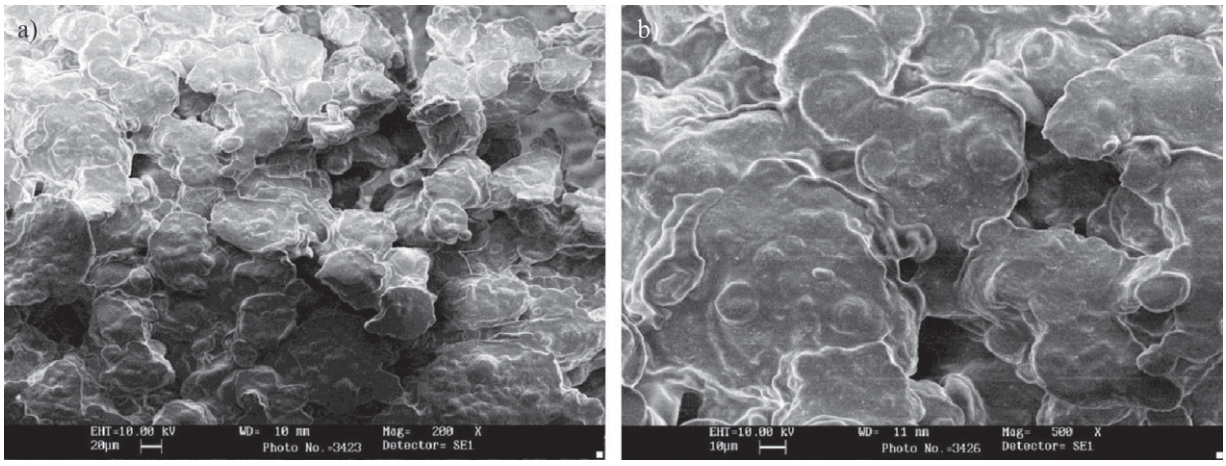

Fig. 2. Scanning electron photomicrograph showing calcium alginate and starch beads coated with chitosan layer at magnification of: a) 200× and b) 500×

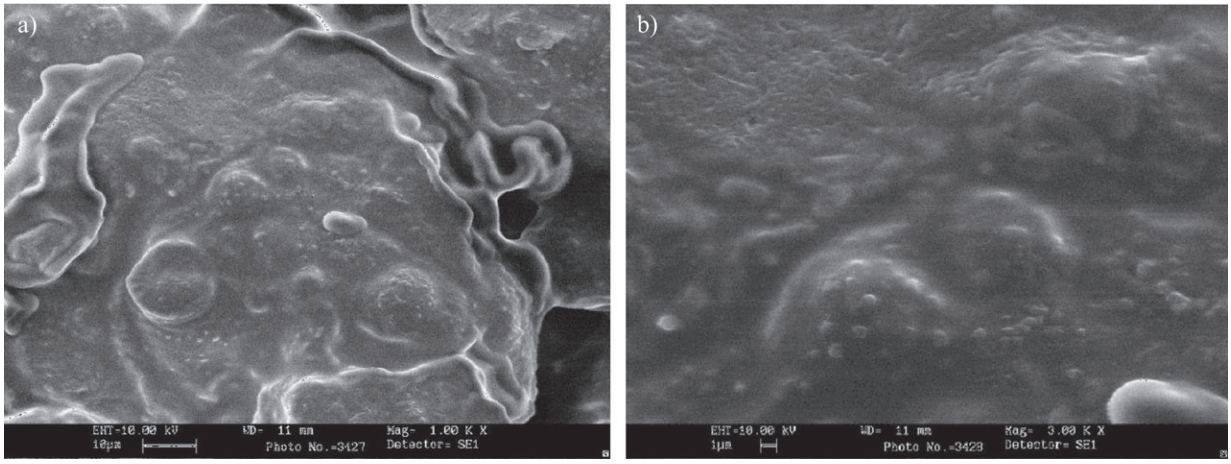

Fig. 3. Scanning electron photomicrograph showing Hi-maize resistant starch granules on the surface of calcium alginate and starch beads coated with chitosan layer at magnification of: a) 1000× and b) 3000×

researchers (*30,31*), as they give a smooth texture when incorporated into products and cause less alteration in the composition of food product and further inhibit the formation of sandy texture. This is in agreement with Mokarram *et al.* (*29*) and Truelstrup Hansen *et al.* (*32*), who reported that very large calcium alginate beads (>1 mm) cause coarseness of texture in live microbial feed supplements.

Survival of encapsulated bacteria in the bread

The initial cell count before and after encapsulation was approx. 10^{11} CFU/g. The results show that there was no significant loss of viability of both strains during encapsulation and coating due to the gentle methods used, and 99.8 % of cells were successfully entrapped. This result implied that the encapsulation and coating methods had no effect on cell viability, which is in agreement with Krasaekoopt *et al.* (*17*) and Mokarram *et al.* (*29*).

The survival of encapsulated probiotics was determined less than 24 h after baking (Fig. 4a) and on day four of storage at room temperature (Fig. 4b). Longer storage time was avoided due to staling. Using alginate and starch beads with and without chitosan coating, viable microorganisms survived after the baking process and both bread types met the standard criteria for probiotic products. Type of bread significantly affected the probiotic survival, which was significantly higher in hamburger buns ($p<0.05$), probably due to shorter baking time than of white pan bread. The temperature of crumb centre was approx. 93–94 °C. Temperatures above 45 °C are known to be critical for the survival of probiotics in free form. Elevated temperatures higher than 45–55 °C for longer time lead to a decrease in probiotic survival. It has been shown that temperature higher than 65 °C is fatal for all free probiotic bacteria. Ding and Shah (*11*) showed that time plays an important role in high temperatures. They observed that exposing encapsulated probiotics to the temperature mentioned above for an hour results in complete bacterial death and concluded that alginate started to disintegrate during this time and as a result, the protective layer surrounding the bacteria was destroyed. Our results indicated that survival of encapsulated probiotics was lower in white pan bread, which could be due to

longer baking time. It is worth mentioning that other factors, *e.g.* heat transfer, dough mass and mould could probably affect the rate of probiotic survival in the bread.

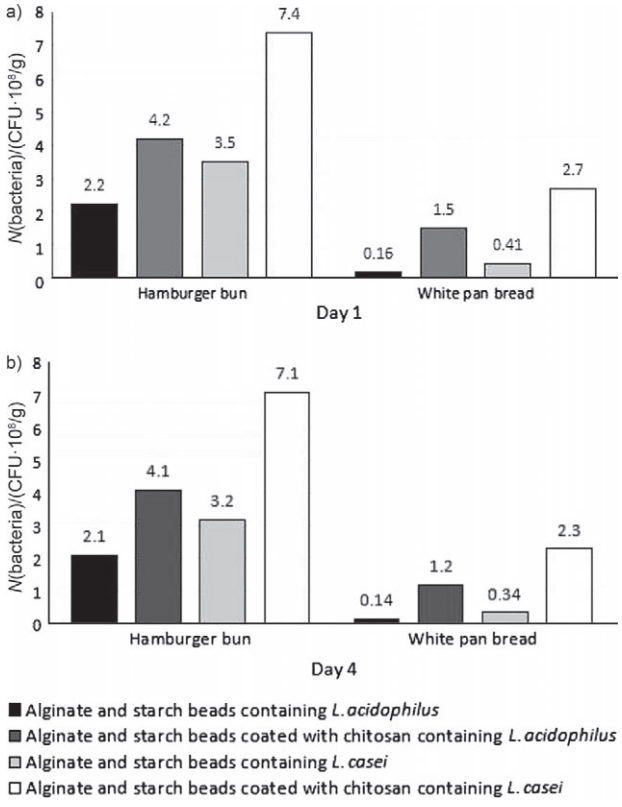

Fig. 4. The survival of encapsulated probiotics determined: a) less than 24 h after baking, and b) on day 4 of storage at ambient temperature

It was noted that four days of storage had no effect on the viability of encapsulated bacteria ($p>0.05$). The goal of encapsulation is to create a microenvironment in which the bacteria will survive during processing and storage and be released at appropriate sites (*e.g.* small intestine) in the digestive tract (*33*). Ravula and Shah (*34,35*) reported that microencapsulation improved the counts of *L. acidophilus* compared to free cells in frozen fermented dairy desserts stored for 12 weeks. In frozen iced milk, 40 % more lactobacilli survived when they were entrapped in calcium alginate beads (*10*). In addition, it was demonstrated by Homayouni *et al*. (*3*) that encapsulated cells required longer time to decrease the viability for one log cycle. Therefore, microencapsulation of probiotic bacteria in beads can increase the viability of probiotics during storage.

A significant increase ($p<0.05$) in probiotic survival was observed when the protective outer layer of chitosan was used in addition to the first layer of calcium alginate and Hi-maize resistant starch. According to Anal and Singh (*36*), the formation of a hydrogel barrier by the compacted sodium alginate layer retards the permeation of the gastric fluid into the cells. Chandramouli *et al.* (*37*) and Iyer and Kailasapathy (*38*) have shown that only the microencapsulated probiotics were able to maintain viability in gastrointestinal conditions. Microencapsulation of probiotics in alginate beads had previously been tested to improve the viability of probiotic bacteria in simulated gastric conditions (*2,8,10–13,27,29*). Studies have shown that the survival of bacteria under different conditions is increased in calcium alginate-immobilized cell cultures, confirming that they are better protected than the non-encapsulated ones (*33*).

Probiotic bacteria encapsulated with Hi-maize resistant starch also survived better than the encapsulated bacteria without the prebiotic (*2*), and further coating with chitosan significantly enhanced their survival (*38*). Capsule membrane in microcapsules with alginate and starch allows sufficient diffusion of nutrients and metabolites to maintain the growth of encapsulated cells and their fermentation ability (*8*). Resistant starch is the starch that is not digested by pancreatic amylases in the small intestine and reaches the colon, where it can be fermented by human and animal gut microflora. The fermentation of carbohydrates by anaerobic bacteria produces short-chain fatty acids and lowers the pH in the lumen. Resistant starch can be used to ensure the viability of probiotic populations from the food in the large intestine. It also provides an ideal surface for adherence of the probiotics to the starch granule during processing, storage and transit through the upper gastrointestinal tract (*33*). Studies have shown that the incorporation of Hi-maize starch improved the encapsulation of viable bacteria compared with the bacteria encapsulated without starch (*12,13,33, 38*). It seems that specific interactions occur during mixing of alginate and starch. Therefore, the precise ratio of the used materials is essential (*39*). Previous investigations have demonstrated that intermolecular interactions and good molecular compatibility take place between starch and alginate (*26,28*). This can be explained by strong interactions like hydrogen bonds and ionic interactions (*26*).

The survival rate of probiotic bacteria entrapped in alginate beads containing chitosan was higher than that of alginate beads without chitosan (*40,41*). Chitosan is a positively charged polyamine that forms a semipermeable membrane around a negatively charged polymer such as alginate. This membrane is not soluble in the presence of Ca^{2+}-chelating or antigelling agents, and thus increases the stability of the gel, providing a barrier to cell release. Studies have reported that the probiotic organisms with chitosan coating had better protection than the uncoated microcapsules and that their encapsulation in chitosan microspheres improved the survival in comparison with free cells (*2,17,42,43*).

L. casei 431 was more resistant to high temperatures than *L. acidophilus* LA-5 ($p<0.05$). Lactic acid bacteria (LAB) are the most important probiotic microorganisms typically associated with the human gastrointestinal tract (*36*). Their probiotic benefits strongly depend on their ability to survive and multiply in the host. Therefore, in order to have beneficial effects in the intestine of the host, the bacteria should be metabolically stable and active during and after processing, and survive the passage through the upper digestive tract in large numbers (*44*). Overall, viability is essential for organisms targeted to proliferate within the human gut (*36*). The results of our

Table 1. Sensory evaluation scores of white pan bread with encapsulated probiotics

t/day				*w*(inulin)/%	Capsule type	Bacteria
1		4				
Texture	Flavour	Texture	Flavour			
7	4	7	6	5	Calcium alginate and Hi-maize resistant starch	*Lactobacillus acidophilus* LA-5
8	4	7	6	5	Calcium alginate and Hi-maize resistant starch coated with chitosan	
8	5	7	5	5	Calcium alginate and Hi-maize resistant starch	*Lactobacillus casei* 431
8	5	8	6	5	Calcium alginate and Hi-maize resistant starch coated with chitosan	

Table 2. Sensory evaluation scores of hamburger buns with encapsulated probiotics

t/day				*w*(inulin)/%	Capsule type	Bacteria
1		4				
Texture	Flavour	Texture	Flavour			
7	6	7	6	5	Calcium alginate and Hi-maize resistant starch	*Lactobacillus acidophilus* LA-5
8	4	7	5	5	Calcium alginate and Hi-maize resistant starch coated with chitosan	
7	5	8	5	5	Calcium alginate and Hi-maize resistant starch	*Lactobacillus casei* 431
8	4	7	4	5	Calcium alginate and Hi-maize resistant starch coated with chitosan	

study showed that the survival of bacteria in unfavourable conditions is species-dependent. This finding was in agreement with those of Homayouni *et al.* (*3*), Haynes and Playne (*16*), and Kailasapathy and Sultana (*45*).

Sensory evaluation

Data from triangle test with replicates were analysed using the corresponding table for repeated triangle tests (*20,22–24,46*). The results of sensory evaluation are shown in Tables 1 and 2. Sensory scores indicated that there were not any significant differences in flavour among the samples of bread and buns containing inulin. These results are in agreement with those of Morris and Morris (*4*), who concluded that bread containing 5 % inulin seems acceptable. In another study, Brasil *et al.* (*47*) evaluated the effect of the addition of inulin on sensory, nutritional and physical parameters of white bread and, according to their results, a level of 6 % of inulin added to bread was considered to give good sensory quality. Our results showed that microcapsules had no significant effect on the flavour and texture of bread. Previous studies had shown that alginate and starch capsules are white and this could have been the reason why they did not impart a significant difference in crumb colour (*27*). Alginate and starch beads in our study were also micrometer-sized, hence did not have an adverse effect on bread texture (*29–32*).

Conclusions

This study indicated that the production of synbiotic bread by using microencapsulation is possible and it can enhance the viability and thermal resistance of probiotic bacteria, and therefore significantly improve their survival in bread and other bakery products. Using alginate and starch beads with and without chitosan coating, viable microorganisms survived after baking and both types of bread met the standard criteria for probiotic products. A significant increase ($p<0.05$) in probiotic survival was observed when the protective outer layer of chitosan was used in addition to the first layer of calcium alginate and Hi-maize resistant starch. *L. casei* 431 was more resistant to high temperature than *L. acidophilus* LA-5 ($p<0.05$), and our study showed that the survival of bacteria in unfavourable conditions was species-dependent. Type of bread significantly affected the probiotic survival, which was significantly higher in hamburger bun ($p<0.05$), probably due to shorter baking time, than in white pan bread. Results showed that microcapsules had no significant effect on flavour and texture of bread and adding 5 % of inulin as prebiotic was acceptable, leading to production of bread with similar characteristics to the common bread, but with additional health benefits. Therefore, this work contributes to this area and its findings can be applied by bakery industry to develop probiotic bread and cereal-based products. Further studies are needed to evaluate the survival of other probiotic strains using different microencapsulation techniques and other coating materials in cereal-based products. More investigations should be considered using consumer- and product-oriented tests to cover the influence of sensorial factors on consumers' attitude towards the product.

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