Association of carob galactomannans with probiotic bacteria in synbiotic fermented milk and colon targeted-release carrier

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Abstract
For the first time, this study presented the use of raw locust bean gum (LBG) as a prebiotic, with one probiotic strain in synbiotic fermented milk or combined with Na-alginate as a biopolymer, for a targeted release of bacteria under colon-like conditions. For this purpose, the fermentative characteristics (biomass, pH), bacterial survival, and developed viscosities of the stored fermented milks were determined. The survival rates of microencapsulated bacteria using the emulsion technique under simulated gastrointestinal conditions (stomach: pH 2 + 0.3% pepsin; colon: pH 6.5 + 1% pancreatin + 0.3% bile) were also evaluated. Results showed that all the tested bacteria maintained better biomass and acidifying activities in the presence of LBG, especially at 2%. During cold storage, the viscosities of the LBG-fermented milks were regulated and better appreciated, especially at 2%. Lactobacillus rhamnosus LbRE-LSAS and Bifidobacterium animalis subsp. lactis Bb12 microencapsulated separately in Ca-alginate-raw carob gum maintained good survival rates (51-66%) as compared to free cells (21-59%) under simulated digestive conditions, and were released under colon-like conditions. Therefore, the formulation of LBG-enriched fermented milks containing probiotic bacteria could represent a very good candidate for industrial application. Ca-alginate-raw LBG beads for the specific release of probiotics in the colon could benefit consumers with celiac disease or other digestive disorders because LBG is naturally gluten-free.

Keywords
raw carob bean gum (LBG), probiotic bacteria, fermentation, microencapsulation, targeted release

Introduction
In recent years, the growing popularity of foods that are supposed to be good for our health, also known as functional foods, has led to an explosion of probiotic fermented milk markets worldwide (Ávila et al., 2020). In fact, several studies have been devoted to probiotics, and their results indicate their capacities to improve intestinal balance and prevent certain intestinal and inflammatory diseases (Srutkova et al., 2015; Kerry et al., 2018). At the same time, the application of probiotics in different food products has been widely studied. This interest is partly due to the growing awareness and concern about food allergies and intolerances. Nowadays, probiotics are introduced in a wide range of food products with probiotic claims, starting with mass-market dairy products such as yogurts, cheeses, or infant milk. However, two issues remain largely unresolved in the functional food industry, namely the viability of the probiotics used, and the organoleptic aspect of the product (Maurya et al., 2014).

Loss in probiotic bacteria viability is a negative point that has been the focus of most scientific analyses (Maurya et al., 2014; Champagne et al., 2018; Neffe-Skocinska et al., 2018). In fact, the integrity of microorganisms is subject to severe conditions, particularly the physicochemical treatments performed during the manufacturing of the functional foods. During consumption, the action of digestive enzymes, gastric acidity, and bile significantly reduce the survival rate of probiotic free cells; whereas to exert their beneficial effects,
probiotics must survive at a minimum number of 10^6 viable cells per gram through the product shelf-life (Neffe-Skocińska et al., 2018).

Several reports have indicated a low survival rate of probiotic bacteria in dairy products containing free cells (De Vos et al., 2010). Therefore, it is essential to consider solutions to address the significant loss of viable cells during and after manufacturing. Microencapsulation of probiotics using biopolymers is one of the most promising and least expensive methods to overcome the problems posed by environmental adversity. This technology can preserve cell viability and metabolism, thus allowing more viable cells to enter the intestine. Recently, various scientific studies have reported using different encapsulation techniques, where emulsification seems to be the best to encapsulate probiotics properly. Alginate is still widely used to produce probiotic microparticles. However, there is increasing interest in its total or partial substitution by other polysaccharides such as gums and prebiotic compounds (Dokoohaki et al., 2019).

Probiotic-fermented milk products may exhibit textural defects and taste alterations such as insufficient gel firmness, changes in viscosity, and whey expulsion (syneresis) (Teggatz and Morris, 1990). Appropriately, in many formulations, hydrocolloids thickening and/or gelling agents compensate for the lack of texture due to reducing fat content. They improve consistency (increase viscosity) and reduce syneresis. The agents frequently used are gelatine, pectins, carrageenans, methylcellulose derivatives, gum Arabic, starches, and galactomannans. The latter are reserve polysaccharides found in the translucent endospem of many legume seeds (Daas et al., 2000) such as guar (Cyamopsis tetragonoloba (L.) Taub.), tara (Caesalpinia spinose Kuntze), and carob (Ceratonia siliqua L.).

The Food and Agriculture Organization (FAO) shows that the global production of carob fruit is about 158.61 t/year, produced from about 66.87 hectares, 13% of which is in Africa (FAOSTAT, 2016). The commercial application of carob bean gum is widely used, and many patents have described its use in foods such as jelly foods, baby foods, and others (EFSA, 2017). In addition, researchers have attempted to formulate carob-based milk drinks using pod syrups (Strout et al., 2016).

However, to the best of our knowledge, there are no published data on the use of carob seed galactomannans as prebiotics. At the same time, a few studies have reported the prebiotic effect of fenugreek seed galactomannans (Majeed et al., 2018) and guar beans (Zartl et al., 2018). As a popular natural polymer, carob bean gum can also be used as a biopolymer to control biologically active agents’ systemic or local delivery (Jana et al., 2015).

Due to the high concentrations of galactomannans, fibres, and polyphenols in carob seeds, and to promote the use of carob tree products and by-products, raw locust or carob bean gum (LBG) was studied in the present work. First, we produced symbiotic fermented milk containing carob LBG as a prebiotic and associated with probiotic bacteria. Then, the aim was to ensure the effectiveness of the alginate-LBG mixture as a biopolymer for the targeted release of probiotic bacteria in the intestines.

Materials and methods

Bacterial strains

*Lactobacillus rhamnosus* LbRE-LSAS was a bacterial strain from the collection of beneficial microorganisms, functional foods, and health laboratory (LMBAFS, University of Mostaganem, Algeria). It was isolated from the faeces of a healthy three-week-old baby who had not received any antibiotic therapy, and was exclusively breastfed. *Bifidobacterium animalis* subsp. *lactis* was the probiotic reference strain, commercially known as Bb-12 (Chr. Hansen-Denmark). The yogurt starters used, *Lactobacillus delbrueckii* subsp. *bulgaricus* LB 340 and *Streptococcus thermophilus* TA 040 were purchased from Danisco (France).

All strain cultures were maintained at -70°C using Microbank cryovials (Pro-Lab Diagnostics, UK). Bacteria were serially propagated three times in the appropriate broth (Biomérieux, France: MRS with cysteine-HCl for LbRE-LSAS and Bb12, MRS for LB340, and M17 for TA040) before experimental use. A 1% inoculum was used, and incubation was performed at 37°C for 24 h under anaerobic conditions. In all experiments, cultures of each strain were taken at the exponential growth phase.

**Carob seeds**

Source of galactomannans, carob seeds, were obtained from pods harvested in the Zenata Forest (Tlemcen City, Algeria). They were stored in opaque, hermetically sealed bags in the dark and away from humidity.
Preparation of locust bean gum (LBG)

Three trials were conducted for the preparation of LBG. Raw and purified gums (in ethanol or isopropanol + acetone) were tested for their efficiency and synergy as prebiotic substrates for probiotic bacteria.

Raw carob bean gum

First, the carob pods were crushed, and the seeds were collected and sorted (Dakia et al., 2008). Next, 100 g of carob seeds (780 seeds) were immersed in 800 mL of distilled water, and heated to 100°C for 1 h in a water bath. The seeds absorbed the water and swelled without destroying the integument envelopes. The seeds were then collected from the water and washed, the envelopes were separated manually, and the germ was removed after the division of the endosperms. The endosperms were dried at 100°C for 1 - 2 h to a constant weight, ground into fine particles, and sieved to obtain the raw gum flour (locust bean gum, LBG).

Purified carob bean gum

Two methods were used to obtain the purified LBG. In the first method, the mixed endosperm in the blender was filtered through a nylon mesh followed by centrifugation at 3,800 g (20 min, 20°C). Galactomannan precipitation was performed by adding the supernatant to absolute ethanol (purity 99.7%) in a ratio of 1:2. The ethanol was decanted, and the precipitated galactomannans were dried and stored in a desiccator until use. In the second method (Bouzouita et al., 2007), 1.3 g of crude endosperm was added to 100 mL of distilled water, stirred gently for 2 h at room temperature, and stored overnight at 4°C. Then, the solutions were heated to 80°C in a water bath (30 min, continuous stirring).

The resulting solution was allowed to cool and centrifuged for 1 h at 7,500 g (20°C). The supernatant containing solubilised galactomannan was purified by pouring two volumes of isopropanol, and letting the mixture stand for 30 min. The white fibrous material was collected and filtered through a sieve (53 µm), and washed twice with isopropanol and acetone. After 48 h of freeze-drying, the resulting solid was ground into a fine powder. The galactomannan content was 84.7% in raw LBG (water extraction method) and 95% in purified LBG (isopropanol and acetone method). Their respective molecular weights were 523 and 862 KDa, as determined by the intrinsic viscosity method.

Fermentation of LBG by bacteria

For the milk fermentation assays, pasteurised (30 min, 80°C) solutions of raw LBG at final concentrations of 0, 0.1, 0.5, 1, 1.5, or 2% (w/v) were added to skim milk sterilised by tyndallisation. Then, 100 µL of a single 16-h bacterial culture (TA040, LB340, LbRE-LSAS, or Bb12) was added at a final load of 1×10⁷ to 5×10⁷ cells/mL. The inoculated milks were incubated at 37°C under anaerobic conditions (anaerobic jar with CO₂ generator system; Anaerocult). The fermentative activities of the bacteria were assessed after 24 h of incubation. The number of viable cells, determined three times, was calculated from the colonies obtained after incubation on the appropriate media, and was expressed as log CFU/mL. The acidifying activity developed by each strain in milk was estimated by measuring the pH with a digital pH meter (WTW pH meter 330; Weilheim, Germany).

Determination of bacterial survival in fermented milk stored at 4°C

Bacterial strain survival was determined weekly on a 1 g sample of fermented milk diluted at 1:10 with a sterile diluent solution (0.1% peptone, w/v), incubated on appropriate medium, and the colonies enumerated thereafter.

Measurement and monitoring of fermented skimmed milk viscosity

The viscosity developed in the fermented milk was measured after 1, 7, 14, 21, and 28 days of storage using a viscometer (SNB-1 digital viscometer; Brookfield Engineering Labs. Inc., USA) equipped with four rotating mobiles. The viscosity measurement was performed after the samples were initially stirred by hand for 1 min to obtain homogeneous samples for viscosity measurements. The viscosity was recorded every 60 s for 30 min, and the average of the last 10 measurements was given as the viscosity value of the sample.

Bacterial cell microencapsulation method

Only one concentration of raw LBG gum (among 0.1, 0.5, 1, 1.5, or 2%) based on the optimal micro efficiency value, and the two strains giving the best results with LBG as a prebiotic were tested for specifically targeted release under colon-like conditions. The emulsification technique described by Ziar et al. (2012) was followed. Briefly, sterile solutions of 1.8% sodium alginate and 0.1 M CaCl₂...
were prepared. In a sterile beaker containing 100 mL of vegetable oil (sunflower and soybean), 1 mL of polysorbate 80 was added, and a mixture of bacterial culture (pellet washed once at 10 log CFU/g), sterile LBG solution (by boiling at 80°C/30 min), and sodium alginate solution were prepared. Microdroplets (using a 27.5G needle) were deposited from this mixture on the oil (stirred between 200 and 400 rpm for 20 min). Then, a CaCl$_2$ solution (100 to 200 mL) was added by pouring it on the walls until the emulsion broke. After 15 min of stirring, the formed calcium alginate-LBG microcapsules were separated from the aqueous phase, rinsed twice with sterile saline solution (0.9% NaCl + 5% glycerol), and stored in hermetically sealed sterile tubes at 4°C.

The concentration of sodium alginate was set at 1.8% (w/v) to optimise the concentration of carob bean gum. The microencapsulation process was performed using different concentrations of LBG solution, namely 0.1, 0.5, 1, 1.5, and 2% (w/v). The microencapsulation efficiency was used to determine the optimum concentration of carob bean gum and was calculated using Eq. 1:

\[
\text{Microencapsulation efficiency (\%) = } \left( \frac{\log N}{\log N_0} \right) \times 100
\]  

(Eq. 1)

where, $N$ = number of microencapsulated probiotics released from the beads, and $N_0$ = number of probiotics in the initial microbial solution.

**Survival under simulated digestive conditions (simulated gastrointestinal model)**

The same protocol described by Ziar et al. (2012) was performed with the following modifications: survival of microencapsulated or free bacteria was evaluated in a simulated continuous digestive model at 37°C under anaerobic conditions, conditions similar to those in the stomach and colon. Samples of 0.5 g of free and microencapsulated cells were added to 4.5 mL of simulated gastric juice (SGJ, 37°C). SGJ consisted of a buffered solution of 0.02 M KCl-HCl with 0.3% pepsin, and the pH was adjusted to 2 using 1 M HCl. Next, 0.5 g of washed bacterial pellets of free and microencapsulated cells that had completed their gastric residence were added to 4.5 mL of simulated intestinal juice (SIJ, 37°C). The SIJ consisted of 0.02 M phosphate buffer (PBS) with 1% porcine pancreatin, 0.3% bile salt, and the pH was adjusted to 6.5 using 1 M NaOH.

For viable biomass studies, aliquots at different gastric exposure times were taken from the vessel’s bottom using a selected laboratory tool (diameter = 3.5 mm). Counting was performed on MRS-cys agar after lysis in sodium phosphate buffer (PBS, pH = 7, 0.2 M, vigorous stirring for 20 min at 4°C).

Another count was performed on MRS-cys agar without lysis in PBS to estimate the integrity of the beads. For SIJ samples, the bacterial pellet was collected. A count was performed on MRS-cys agar without lysis in PBS. The survival rates were determined using Eq. 2:

\[
\text{Survival rate (\%) = } \left( \frac{\text{Initial load of entrapped bacteria CFU/g} - \text{load of entrapped bacteria at T Time level of exposure}}{\text{Initial load of entrapped bacteria CFU/g}} \right) \times 100
\]  

(Eq. 2)

**Statistical analysis**

Statistical analysis was performed by analysis of variance (ANOVA) using STATISTICA software (Data Analysis Software System, v. 8.0; StatSoft Inc., Tulsa, OK, USA). Difference was considered significant at $p < 0.05$. The main sources of variation, which were considered statistically significant, were compared using the LSD (least significant difference) test. All results represent the means of three independent experiments ($n = 9$).

**Results and discussion**

**Fermentative activities of bacteria in the presence of LBG**

Both purified LBG failed to fulfill the prebiotic property since other bacteria could use them as the sole carbon source. With LBG extracted from water, our results (unpublished results) revealed an inhibitory effect against pathogens. However, this inhibitory effect was moderate for lactic acid bacteria of dairy origin, and absent for probiotic bacteria isolated from the intestines. The objective of the present work was achieved with raw LBG and not with purified LBG.

Results for the fermentative activities of *Lactobacillus rhamnosus* LbRE-LSAS, *Bifidobacterium animalis* subsp. *lactis* Bb12, *Lactobacillus delbrueckii* subsp. *bulgaricus* LB340, and *Streptococcus thermophilus* TA040 were expressed in terms of biomass (log CFU/mL) (Figure 1) and pH (Figure 2) recorded after 24 h at 37°C under
anaerobic conditions. After 24 h in the presence of LBG, all strains showed an increase in biomass (Figure 1) where +0.25, +0.64, +1.26, and +1.24 log units were monitored in control fermented milks with LbRE-LSAS, Bb12, TA040, or LB340 strain, respectively.

With 0.1% LBG (Figure 1), LbRE-LSAS reached a high biomass (p < 0.05) of 8.35 log CFU/mL (an increase of +0.57 log unit) as compared to Bb12, TA 040, and LB340; where 8.19, 7.94, and 8.05 log CFU/mL were recorded, respectively. By increasing the concentration of added LBG to 0.5% (Figure 1), LbRE-LSAS and Bb12 strains showed better biomasses (p < 0.05) of 8.06 and 8.14 log CFU/mL, respectively.

However, TA040 and LB340 were attenuated (-0.22 and -1.05 log cycle, respectively). The moderate biomasses accumulated with S. thermophilus and L. bulgaricus monocultures enriched with 0.5% LBG could be related to the prolonged lag phase (as compared to their respective control cultures) observed for both yogurt starters. Milk coagulation occurred after up to 11 h of fermentation. Since the probiotic used in the present work was raw gum, we suggest that yogurt bacteria’s lactic acid production might be related to their preferred protein assimilation from both origins, milk and raw LBG (4.8%), rather than sugar fermentation.

With 1% LBG (Figure 1), Bb12 and TA040 reached the highest biomasses (p > 0.05), 8.29 and 8.15 log CFU/mL, respectively, while LbRE-LSAS showed high biomass similar to that recorded with 0.1% LBG. The Lactobacillus LB340 starter appeared to be slightly (p > 0.05) attenuated (-0.03 log unit). One reason for this behaviour could be the composition of the raw carob flour which is rich in total polyphenols (19 mg/g fresh matter), condensed (2.75 mg/g), and water-soluble tannins (0.5 mg/g). These compounds are known for their antimicrobial activity (Avallone et al., 1997).

With the 1.5% LBG concentration (Figure 1), strains LbRE-LSAS, Bb12, TA040, and LB340 showed an increase (p < 0.05) of +0.18, +1.12, +0.74, and +0.65 log units, respectively (from 0 to 24 h). With 2% LBG (Figure 1), the increase in biomass was approximately +0.5 log units regardless of strain (p < 0.05), despite a slight decrease of -0.13 log cycles in the biomass of TA040. This result reflected the consumption of LBG used as a prebiotic.

The best-known prebiotics on the market are inulin and fructooligosaccharides, but legumes are interesting sources of prebiotics, which can be used by bifidobacteria and lactobacilli. Carob seeds are composed mainly of galactomannans, highly polydisperse linear polysaccharides consisting of a β-(1→6)-mannosyl backbone with a single D-galactopyranosyl unit attached via α-(1→6) linkages as a side branch. Galactomannann polysaccharides can be obtained from carob beans by aqueous or aqueous alkaline extraction. Their content in the seeds can reach 85% (Dakia et al., 2008). In the present work, the ratio of protein, crude fibre, fat, and galactomannan in carob bean gum powder was 4.8%:1.17%:0.43%:84.7%, respectively.

Galactomannans are complex polysaccharides not easily fermented by common bacteria. However, some probiotic bacteria possess the enzymatic ability to break down galactomannans. In a recent study by Zarl et al. (2018), seven out of 20 selected probiotic strains could metabolise guar galactomannans. In fact, S. salivarius subsp. thermophilus metabolised all tested oligosaccharides well, including guar oligosaccharides. In the same study, L. rhamnosus GG and B. animalis subsp. lactis Bb12 showed the highest fermentation rate of guar oligosaccharides. Similar observations were obtained in the present work, while in a previous study (Ziar et al., 2014), we demonstrated that our strains could ferment many different prebiotic carbohydrates.

The pH results recorded after 24 h of fermentation of LBG-enriched milks are shown in Figure 2. The recorded values showed a decrease (p < 0.05) in pH proportional to the increase in LBG concentration in all the formulated fermented milks. The milk acidification indicated that all the strains retained their acidifying activities in the presence of LBG as compared to their optimal pH reference values of 4.2 to 5.8, recorded after 24 h in MRS-glucose (0.5%) as the sole carbon source (Ziar et al., 2014).

Despite this, the most acidic values were recorded with the probiotic strain Bb 12, which showed very low pH values (p < 0.05) below 5 (from 4.88 to 4.76), when LBG was added at a concentration greater than or equal to 1% (Figure 2). As compared to the rest of the strains tested, Bb12 appeared to retain (p > 0.05) the same acidifying activity when the LBG concentration was increased from 1 to 1.5% (Figure 2).
Figure 1. Biomass (log CFU/mL, 24 h/37°C) registered in milks fermented with *L. rhamnosus* LbRE-LSAS, *B. lactis* Bb12, *S. thermophilus* TA040, or *L. bulgaricus* LB340, containing raw carob bean gum (LBG) at final concentrations (w/v) of 0 ( ), 0.1 ( ), 0.5 ( ), 1 ( ), 1.5 ( ), or 2% ( ). *a*-Statistically different according to LSD test.

Figure 2. pH recorded after 24 h fermentation of milks with *L. rhamnosus* LbRE-LSAS, *B. lactis* Bb12, *S. thermophilus* TA040, or *L. bulgaricus* LB340, containing raw carob bean gum (LBG) at final concentrations (w/v) of 0 ( ), 0.1 ( ), 0.5 ( ), 1 ( ), 1.5 ( ), or 2% ( ). *a*-Statistically different according to LSD test.
The acidifying activity of all strains reached its maximum \((p < 0.05)\) in the presence of 2% LBG, but remained less pronounced as compared to the controls where the lowest pH value was recorded with the bifidobacterial strain Bb12 (4.76; 2% LBG). The recorded pH values could be due to the buffering action caused by the carob proteins, and the increase in solids from the carob gum supplementation (Al-Dabbas et al., 2010). Jrad et al. (2020) reported that camel yogurt with 2% carob powder had the highest pH value as compared to plain yogurt. These results corroborate our results, where control fermented milks with Bb12, LbRE-LSAS, LB340, or TA040 showed better acidifying activities \((p < 0.05)\) than LBG-enriched fermented milks.

**Viability**

The viability of bacteria assessed in stored fermented milks containing LBG is presented in Figure 3 as viable log CFU/g. A gradual decrease in the number of viable cells was recorded week by week throughout the cold storage for all strains. However, it is recognised that for probiotic bacteria, a minimum number of 10\(^7\) viable cells per gram of product is required to exert their health benefits (Knorr, 1998).

On the first day of storage, and in the absence of LBG, the beneficial strain Bb12 recorded a loss of viability of -0.66 log cycles (7.12 log CFU/g viable cells). This loss gradually increased during the storage period, and the level of viable cells remained satisfactory until the 7th day. After that, it decreased significantly to 4.26 log CFU/g on the 28th day of storage (Figure 3).

In the present work, carob galactomannans significantly improved \((p < 0.05)\) the viability of Bb12 in cold-stored fermented milks. Indeed, the loss of viable cells was slightly increased in the presence of LBG. At the end of storage, we recorded 4.66, 5.57, 6.49, 6.81, and 7.21 log CFU/g of viable cells in the presence of 0.1, 0.5, 1, 1.5, and 2% LBG, respectively (Figure 3).

In general, the viability of Bb12 at the end of storage was 57, 69.2, 78.3, 83.4, and 87.5% recorded in the presence of 0.1, 0.5, 1, 1.5, and 2% LBG, respectively, while it was only 54.76% in the absence of this polymer. The same observations were also highlighted in the fermented milks containing LbRE-LSAS. On the other hand, it maintained higher viability rates \((p < 0.05)\) as compared to the bifidobacterial strain. By the last week of refrigerated storage (28th day), the viability of the LbRE-LSAS strain still exceeded 90% in the presence of 2% LBG.

We also calculated improvement rates of 14 - 28% (depending on the concentration of added LBG) in the remaining enriched fermented milks as compared to the control. Similar trends were also reported in a recent study by Pereira et al. (2020), where probiotic bacteria *L. acidophilus* LA-5 and *B. lactis* Bb12 showed high viability during 28 days of storage, with counts above 6 log CFU/g in 5% inulin hydrolysed fermented milks.

For yogurt starters, the number of viable cells at the end of storage was 5.13 and 7.17 log CFU/g for *L. bulgaricus*, 5.23 and 7.19 CFU/g for *S. thermophilus*, recorded in the presence of 0.5 and 2% LBG, respectively (Figure 3), as compared to only 5.28 and 5.46 log CFU/g in their respective control fermented milks without LBG addition. Similar results were observed in a recent study (Jrad et al., 2020), where *S. thermophilus* maintained appropriate levels (8 log CFU/g) in ultrafiltered camel yogurt with 2% carob pod powder for three weeks at 4°C.

From our results, we noted that the viability of bacteria improved \((p < 0.05)\) by the addition of 1 - 2% galactomannans from carob seeds, maintaining a number of viable cells exceeding the FAO/WHO required level of 6 log CFU/g after four weeks of refrigerated storage (Neffe-Skocinska et al., 2018).

**Effect of carob galactomannans on viscosity of fermented milks**

The changes in viscosity of the fermented milks during 28 days of storage at 4°C are listed in Table 1. The initial viscosity of the fermented milk (1 day) depended on the LBG content added to the skim milk. It ranged from 1014 to 1080 mPa.s in the absence of LBG, and reached 1014 to 1075, 1088 to 1195 \((p < 0.05)\), 1322 to 1520 \((p < 0.05)\), and 1399 to 1582 \((p < 0.05)\) mPa.s, followed in fermented milk containing 0.1, 0.5, 1, 1.5, and 2% LBG, respectively (Table 1).

The variation in viscosity during the storage period can be attributed to syneresis, protein hydration, and exopolysaccharide production (Aliakbarian et al., 2016). In the present work, viscosity values increased \((p < 0.05)\) in fermented milks containing strain TA040 and 0, 0.1, or 0.5% LBG from the second week. However, fermented milks containing the same strain and 1, 1.5, or 2% LBG had higher viscosity values until the 14th day, and decreased thereafter (Table 1).
Figure 3. Viability (log CFU/g, 4°C) of *L. rhamnosus* LbRE-LSAS, *B. lactis* Bb12, *S. thermophilus* TA040, or *L. bulgaricus* LB340 in stored fermented milks containing raw carob bean gum (LBG) at final concentrations (w/v) of 0 ( ), 0.1 ( ), 0.5 ( ), 1 ( ), 1.5 ( ), or 2% ( ). *a-f* Statistically different according to LSD test.
Table 1. Evolution of the viscosity values (mPa.s) in LBG-enriched fermented milks stored at 4°C.

<table>
<thead>
<tr>
<th>LBG (w/v, %)</th>
<th>Fermented skim milk containing one strain</th>
<th>1 day</th>
<th>7 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>S. thermophilus TA040</td>
<td>1080 ± 0.1^{Aa}</td>
<td>1100 ± 0.91^{Ab}</td>
<td>1300 ± 0.1^{Bb}</td>
<td>1400 ± 0.3^{Cc}</td>
<td>1500 ± 0.4^{Dd}</td>
</tr>
<tr>
<td></td>
<td>L. bulgaricus LB340</td>
<td>1060 ± 0.01^{Ba}</td>
<td>1060 ± 0.21^{Aa}</td>
<td>1080 ± 0.01^{Ab}</td>
<td>1067 ± 0.22^{Ba}</td>
<td>1068 ± 0.03^{Aa}</td>
</tr>
<tr>
<td></td>
<td>B. lactis Bb12</td>
<td>1014 ± 0.2^{Aa}</td>
<td>1057 ± 0.3^{Aa}</td>
<td>1066 ± 0.02^{Aa}</td>
<td>1047 ± 0.02^{Aa}</td>
<td>1028 ± 0.01^{Aa}</td>
</tr>
<tr>
<td></td>
<td>L. rhamnosus LbRE-LSAS</td>
<td>1041 ± 0.14^{Aa}</td>
<td>1071 ± 0.4^{Aa}</td>
<td>1078 ± 0.02^{Aa}</td>
<td>1043 ± 0.11^{Aa}</td>
<td>1019 ± 0.02^{Aa}</td>
</tr>
<tr>
<td>0.1</td>
<td>S. thermophilus TA040</td>
<td>1047 ± 0.21^{Ba}</td>
<td>1119 ± 0.7^{Bb}</td>
<td>1170 ± 0.1^{Cc}</td>
<td>1337 ± 0.3^{Cd}</td>
<td>1432 ± 0.02^{Ee}</td>
</tr>
<tr>
<td></td>
<td>L. bulgaricus LB340</td>
<td>1014 ± 0.7^{Aa}</td>
<td>1032 ± 0.4^{Aa}</td>
<td>1160 ± 0.11^{Aa}</td>
<td>1320 ± 0.1^{Aa}</td>
<td>1360 ± 0.06^{Nd}</td>
</tr>
<tr>
<td></td>
<td>B. lactis Bb12</td>
<td>1020 ± 0.4^{Aa}</td>
<td>1051 ± 0.1^{Aa}</td>
<td>1070 ± 0.02^{Aa}</td>
<td>1039 ± 0.24^{Aa}</td>
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</table>

* Comparisons were made between strains in the same column for a particular LBG concentration at T time of storage. ** Comparisons were made for each strain in the same row for a particular LBG concentration at different T times of storage.

On the 28th day of cold storage, we recorded a decrease (p < 0.05) in viscosity values in all LBG-enriched fermented milks containing LbRE-LSAS or Bb12 (except for a non-significant increase (p > 0.05) observed with 1% LBG) as compared to the viscosity values recorded on the 21st day. Fermented milks containing Bb12 or LbRE-LSAS and 0, 0.1, or 0.5% LBG had the lowest viscosity values at the end of storage (p < 0.05) (Table 1). Ünal et al. (2003) reported that cold storage could significantly influence the viscosity and syneresis of LBG-enriched fermented dairy products. In fact, in the present work, viscosity decreased when LBG concentration was higher than 2%, while syneresis increased.

In the present work, the 2% LBG-enriched fermented milks containing S. thermophilus TA040 showed the highest (p < 0.05) viscosity value (> 1500 mPa.s) after 28 days of storage. A similar value (p > 0.05) was also recorded in the 2% LBG-enriched fermented milks containing the LbRE-LSAS strain (Table 1). Jrad et al. (2020) pointed out that 2% carob pod powder provided lower syneresis values in ultrafiltered dromedary yogurt throughout 21 days of storage.

In general, the presence of carob galactomannans had a very positive effect on the viscosity of fermented milk. The firmness remained stable during storage (unpublished results). This could be attributed to protein aggregates formed by the interaction via intermolecular disulphide bonds of casein micelles with denatured whey proteins. Park et al. (2019) demonstrated that the water absorption capacity increased with the addition of carob bean gum. The authors stated that the xanthan and carob supplemented yogurt for the experimental goat yogurt
showed significantly ($p < 0.05$) better consistency than the control and other gum added yogurts. Interestingly, according to Koksoy and Kilic (2004), locust bean gum has synergistic effects with other stabilisers in decreasing serum separation and increasing viscosity.

In the present work, adding this polysaccharide did not contribute to any undesirable flavour or taste to the finished product (unpublished results). This result is in good agreement with the work of Teles and Flores (2007). The authors reported that, unlike locust bean gum, a high concentration of xanthan or guar gum reduces the organoleptic acceptability of the product.

**Optimisation of carob bean gum concentration and choice of strains for microencapsulation**

In summary, with regard to fermentation abilities and viscosity test, LbRE-LSAS and Bb12 strains gave the best results ($p < 0.05$) with 2% LBG, which are interesting as compared to yogurt starters (LB340 and TA040). Moreover, the results suggested that fermented milks containing carob gum could be sufficiently complete and rich in nutrients to allow the strains’ good growth and metabolic activity. All these combined parameters favoured the selection of *L. rhamnosus* LbRE-LSAS and *B. animalis* subsp. *lactis* Bb12 as excellent candidates for microencapsulation with LBG.

It was found that when the concentration of carob bean gum increased from 0.5 to 1.5% (w/v), the microencapsulation efficiency of the beads decreased from 94.98 to 90.37%. However, from 1.5 to 2% (w/v) LBG, the microencapsulation efficiency of beads increased from 90.37 to 97.22% ($p < 0.05$).

Thus, the 2% carob bean gum was chosen to prepare the Ca-alginate-LBG bio-carrier of probiotic bacteria (bead size = 329 µm on average). Gul and Dervisoglu (2017) reported that the microencapsulation efficiency ranged from 86.71 to 95.25%, and increased with the alginate concentration in the emulsification method. The authors also concluded that 2% alginate could effectively encapsulate the probiotic *Lactobacillus casei* Shirota (LCS).

**Survival rates of microencapsulated probiotic bacteria under continuous simulated gastrointestinal conditions**

**Under gastric like-conditions**

The effects of simulated gastric conditions on bacterial survival rates were evaluated at 0, 30, 60, and 120 min residence time intervals. LbRE-LSAS and Bb12 strains (microencapsulated or free) were subjected to pepsin (3 g/L) combined with pH 2 acidity simulating gastric conditions (Table 2). At 0 min of exposure, the viable load of LbRE-LSAS was more affected in the free state than in the microencapsulated state, with respective survival rates of 79.5 and 96% (Table 2). After 30 min, the viability of microencapsulated LbRE-LSAS further decreased by one log unit, and the survival rates remained almost the same until 60 min of exposure. For free cells, however, biomass was virtually stable ($p > 0.05$) until 30 min of exposure (77.8%), after which we noticed a sharp ($p < 0.05$) drop in cell viability (51.1%) when the stay was extended to 60 min. Subsequently, 67 and 49.1% survival rates were recorded in microencapsulated and free LbRE-LSAS cells, respectively, at the end of 120 min of residence in simulated gastric conditions (Table 2).

<table>
<thead>
<tr>
<th>Time of residence</th>
<th>B. lactis Bb12</th>
<th>L. rhamnosus LbRE-LSAS</th>
</tr>
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<tbody>
<tr>
<td>Free Microencapsulated</td>
<td>Free Microencapsulated</td>
<td></td>
</tr>
<tr>
<td><strong>Gastric simulated condition (pH 2 + pepsin)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>90.68&lt;sup&gt;BD&lt;/sup&gt; 94.66&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>79.5&lt;sup&gt;C&lt;/sup&gt; 96.06&lt;sup&gt;CD&lt;/sup&gt;</td>
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<td>30 min</td>
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<td>60 min</td>
<td>80.15&lt;sup&gt;BC&lt;/sup&gt; 82.54&lt;sup&gt;BB&lt;/sup&gt;</td>
<td>51.1&lt;sup&gt;B&lt;/sup&gt; 83.23&lt;sup&gt;BC&lt;/sup&gt;</td>
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<td>120 min</td>
<td>74.53&lt;sup&gt;BB&lt;/sup&gt; 82.44&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>49.14&lt;sup&gt;B&lt;/sup&gt; 67&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Intestinal simulated condition (0.3% bile + pancreatin)</strong></td>
<td></td>
<td></td>
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<tr>
<td>16 h</td>
<td>59.4&lt;sup&gt;A&lt;/sup&gt; 66.13&lt;sup&gt;DA&lt;/sup&gt;</td>
<td>21.28&lt;sup&gt;AA&lt;/sup&gt; 50.68&lt;sup&gt;BA&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means followed by different superscripts differ statistically at $p < 0.05$; <sup>ab</sup> statistically different in the same row; <sup>ABC</sup> statistically different in the same column.
With strain Bb12 (Table 2) and from pepsin and gastric acid exposure (0 min), 94.6 and 90.6% ($p > 0.05$) viable cells were recorded for microencapsulated and free Bb12, respectively. At 30 min, microencapsulated biomass remained stable (93.7%), while that of free cells dropped ($p < 0.05$) (82.3%). At 120 min, the survival rate of microencapsulated cells was about 82.4%, while that in the free state did not exceed 75%. Therefore, the mixed Ca-alginate-galactomannan gel seems to effectively mitigate the harmful effects of our similar gastric conditions, managing to maintain an acceptable and high level of viable cells.

Ding and Shah (2009) performed microencapsulation of several probiotic bacteria with carob gum as a matrix. The probiotic strain Howaru _rhamnosus_ showed a biomass loss in the simulated gastric medium (120 min at pH 2), corresponding to 5.91 log units as compared to the initial biomass. Using a mixed Ca-alginate-galactomannan gel, our microencapsulation technique allowed a loss of only 2.9 log units in _L. rhamnosus_ LbRE-LSAS. These results reflected the effectiveness of the protective aspect of galactomannans and the integrity of the microencapsulated cells.

**Under intestinal like-conditions**

After the gastric conditions, the bacteria were transferred to simulated intestinal conditions under the action of bile (0.3%, w/v) and 1% pancreatin (w/v) for 16 h. At this level, LbRE-LSAS free cells drastically lost viable biomass ($p < 0.05$). The survival rate was about 21.3%, calculated against the viable biomass persisting at the end of the gastric stay. On the other hand, the viable biomass of microencapsulated LbRE-LSAS cells remained higher, with a survival rate of 50.7% (Table 2). The Bb12 strain showed a similar decrease in viability ($p > 0.05$) for free and microencapsulated cells. The survival rates were 59.4 and 66.1%, respectively, calculated against the viable biomass recorded after 120 min of gastric stay (Table 2).

In general, the microencapsulated cells were more resistant ($p < 0.05$) to digestive hostility than the free state. Furthermore, the probiotic strain Bb12 appeared to be more resistant ($p < 0.05$) to these hostile conditions than the isolated strain LbRE-LSAS. The biomass required for a probiotic strain to produce a beneficial effect must be greater than 6 log CFU/mL. In the present work, only strain Bb12 met this requirement when microencapsulated in raw LBG-Ca-alginate beads, and was therefore considered capable of producing a beneficial effect. In our previous work, both Bb12 and LbRE-LSAS strains displayed more than 7.5 log CFU/mL at the end of the simulated gastrointestinal conditions. However, this could be due to the buffering effect generated by the simulated food bolus on the microcapsules used (Ziar et al., 2012).

In the present work, all microcapsules containing bacteria dissolved after 16 h under intestinal conditions. Moreover, β-mannanase and other enzymes are present in the human colon which could lead to _in vivo_ carob bean gum degradation (Jain et al., 2007).

**Conclusion**

As a natural product, carob is not only beneficial to human health, but also of great economic and environmental importance, and more attention should be paid to it. The synergistic combination of probiotic bacteria and LBG gum as a prebiotic is still little explored. The present work investigated the prebiotic quality of raw carob gum with two yogurt starters and two probiotic strains with approved cholesterol assimilation activities. All fermented carob milks containing one strain showed improved growth abilities ($p < 0.05$). Probiotic counts in 1 or 2% raw LBG stored fermented milks were above the recommended level (6 log CFU/g), and significantly ($p < 0.05$) higher than controls. Similarly, viscosity measurements are related to survival counts in LBG-enriched yogurt, and thus can be developed as an effective functional food by improving physicochemical characteristics such as apparent viscosity.

We also used raw LBG in combination with sodium alginate to generate a protective vehicle for the colon. Under simulated digestive conditions, our results indicated that 67 - 82% of viable probiotic cells were recorded after gastric transit, and 51 - 66% in the colon when protected in raw LBG-Ca-alginate beads. The LBG constituents of carob seeds will be further characterised in our future research. Clinical studies on _in vivo_ physiological benefits are also needed to confirm a prebiotic status. The present work is considered a preliminary evaluation, and may also provide evidence for formulating synbiotic fermented milk containing carob seed.
galactomannans and the appropriate probiotic strain. Due to the low cost and simplicity of the process, our suggested emulsion technique could be suitable for large-scale production of microcapsules in the food and medical industries, and potentially for delivery of probiotics to the gut.

Acknowledgement

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