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Encapsulation effects of galactomannans combined with xanthan on the survival of two lactic strains under simulated digestive hostilities

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ABSTRACT: Galactomannans are the main component of locust bean gum from the fruit of the carob tree, *Ceratonia siliqua* L. They are a reserve of polysaccharides, found in the translucent endosperm of the seeds. They are designated as the best gels with thickening capacity and are, therefore, widely used as a natural food additive (E410) in many food, pharmaceutical and cosmetic preparations. In this study, we aim to exploit this gelling property of carob galactomannans in the microencapsulation of lactic bacteria in order to protect them from the negative effects of simulated digestive conditions. Two beneficial bacteria are used: *Lactobacillus rhamnosus* LBRE-LSAS and *Bifidobacterium animalis* subsp. *lactis* Bb12. Their survival in the free state or encapsulated in pure carob galactomannan gel combined with xanthan, was determined after residence in simulated *in vitro* digestive conditions (gastric: pH 2, pepsin 3 g/l and intestinal: bile 0.3%: W/V, pH 6.5. The results obtained show that gel encapsulation of carob galactomannans combined with xanthan improves the survival of these two beneficial strains to simulated digestive hostilities. the loss under gastric conditions 36.79% (3.55 log CFU/mL) for the non-encapsulated cells and only 12% (1.2 log CFU/mL) for the encapsulated ones. However, galactomannans alone do not appear to be effective in keeping a minimum of 10^6 bacterial cells viable when confronted with the hostile conditions of the digestive tract where they will be called upon to exert their positive effect on health.

Keywords: Galactomannans; Xanthan; Encapsulation; Survival; Digestive Hostilities.

1. INTRODUCTION

Probiotics are live microorganisms which, when administered in sufficient quantities, confer beneficial health effects on the host [1,2]. To exert health effects, it is recommended to ingest foods containing at least 10^6-10^7 colony forming units (CFU)/g of viable probiotics [3]. One of the major difficulties in the use of probiotics is their low viability during the crossing and stay in the different compartments of the digestive system where a large part of the cells is lost or inhibited by the bile secretions. Indeed, to be effective in their role, these micro-organisms must then colonize the intestine and multiply there.

The low tolerance to acidity of certain bacterial species requires the implementation of means of protection and preservation of their integrity and survival in highly acidic environments such as the gastric

environment [4,5]. One of these means is represented by the encapsulation of these cells in matrices or gels which act as a barrier. The encapsulation must bring a plus in terms of survival of the strains concerned. This means of protection has found an application for the maintenance in life of microorganisms with probiotic status, and thus of digestive interest. [6].

Microencapsulation is a process by which microbial cells are enclosed in a protective layer. Encapsulation reduces the loss of cell viability by separating the bacterial cells from the adverse environment. The protective layer reduces cell loss and injury by blocking aggressive or inhibiting agents such as moisture, oxygen from the air, and acids [7,8]. Starting in the 1990s, new dosage forms capable of immobilizing lactic acid bacteria have emerged; biopolymer-based beads such as alginate, gelatin and xanthan gums, and carrageenan are the most widely used for microencapsulation of lactic acid bacteria [9].

The fruit of the carob tree, carob, has applications in the food industry, and is used mainly in the form of flour and gum (known worldwide as LBG or Locust Bean Gum) [10].

The objective of this study is to test the effect of the biomaterials used (galactomannans extracted from carob seed endosperms, combined with Xanthan, on the survival of bacteria of interest (*Lactobacillus rhamnosus* LBRE-LSAS and *Bifidobacterium animalis* subsp. *lactis* Bb12) under simulated digestive conditions in vitro.

2. MATERIALS AND METHODS

2.1. Bacterial strains used

Lactobacillus rhamnosus: experimental strain LBRE-LSAS from the collection of the Laboratory of Beneficial Microorganisms, Functional Foods and Health (LMBAFS, University of Mostaganem) where it was isolated from the stools of healthy infants not receiving antibiotic therapy, exclusively breastfed and aged 2 to 3 weeks.

Bifidobacterium animalis subsp. *lactis*: probiotic reference strain, commercially known as Bb-12 (Chr. Hansen-Danemark)

2.2. Preparation of the inoculum

72 hours before starting each experiment, cultures are revived by a series of three 200 µl inoculations in 10 ml SRM broth and incubated at 37°C for 24 hours in an anaerobic jar with a CO_2 generator system (Anaerocult).

2.3. Preparation of the galactomannan solution

Galactomannans were extracted from carob following the method described by Dakia et al. [11]. 100 g of carob seeds (~780 seeds) were heated to boiling (100°C) in 800 mL of distilled water for 1 hour. The seed coat and germ of the endosperm were separated manually, subsequently; the endosperms were dried in an oven at 100°C, for 1-2 hours and crushed to powder. The galactomannan solution was prepared with a concentration of 2% (w/v) in sterile distilled water.

2.4. Procedure for encapsulation

The encapsulation of the bacterial cells was carried out according to the method described by Ziar et al. [12]. Individual bacterial cell suspensions were made by centrifuging 80 mL of a 24-hour culture at 5000 g for 20 minutes. The cells were washed twice with saline solution after centrifugation (20 mL). Individually, 10 mL of 18 g/L sodium alginate and 20 g/L resistant starch(RS) were combined with washed bacterial cells.

Following preliminary research, sodium alginate and RS concentrations were chosen. The RS was added to the alginate to increase the stiffness of the beads. A sterile syringe with a 27.5 G needle was used to inject one mL of the microbial suspension into a 5 mL sterile syringe (Terumo, Leuven, Belgium).

Aseptically, the suspension was placed into 100 mL vegetable oil (canola and sunflower oil) containing 1 mL polysorbate 80 (E 433; A and Z Food Additives Co. Ltd, China). After 20 minutes of agitation at 200-400 rpm, 100-200 mL of 0.1 M calcium chloride was swiftly poured down the side of the beaker, causing the oil-water emulsion to phase separate.

Decantation was used to separate Bb12 or LBRE-LSAS beads, which were then washed with a solution containing 0.1 m calcium chloride and 5% glycerol. The distance between the syringe and the $CaCl_2$ solution was regulated to a maximum of 20 cm, yielding capsules with a diameter of 0.2-0.3 mm. Within two days, the beads were aliquoted into separate vials and used.

2.5. Survival tests of encapsulated strains to simulated digestive conditions *in vitro* (simulated gastrointestinal model)

Encapsulated and free strains were confronted with digestive hostilities simulated in vitro by reproducing the pH, enzyme and bile conditions of the human digestive system. Free or encapsulated bacteria (100 µL of a suspension at $1x10^8$ CFU/mL) are exposed to the physico-chemical environment of the stomach (2 h incubation in HCl-KCl buffer (0.1 M) with 0.3% pepsin: W/V, pH 2, shaking 100 rpm) and sterile intestinal (the "100 µL" cells from the first 2h incubation are transferred to phosphate buffer (0.1 M) supplemented with 0.3% bile: W/V, pH 6.5, to be incubated again for 16h under shaking 100 rpm). Incubations are performed at 37°C in an anaerobic jar with the CO generator system Anaerocult. The survival of encapsulated or free bacteria in the hostile conditions of the stomach and intestine is evaluated by counting cells at different incubation time intervals.

2.6. Microbiological analysis

Biomass is determined by surface seeding 100 µL on appropriate medium after making a decimal dilution of the encapsulated cells that survived the physicochemical stress, in sodium phosphate buffer (PBS 0.2 M, pH = 7,) with vigorous shaking (20 min at 4°C) for complete release of the cells from their capsules [13].

2.7. Statistical study of the results

Each test was independently performed in triplicate and each test was repeated three times in a fully randomized design and results obtained were subjected to analysis of variance (ANOVA) using the STATBOX software (version 6.1, France). The comparison of means was performed by the Student-Newman-Keuls test at the 5% threshold for multiple comparisons. At $P<0.05$, the difference is considered significant.

3. RESULTS AND DISCUSSION

3.1. Effects of encapsulation with carob seed galactomannans combined with xanthan on the survival of the two lactic strains LBRE-LSAS and Bb12 under gastric conditions

Carob galactomannans combined with xanthan result in a stiffer gel than the other polymer combinations performed in this study. The evaluation of the effectiveness of this gel in preserving the survival of the *Lactobacillus rhamnosus* LBRE-LSAS strain against gastric conditions, simulated in vitro by a pH equal to 2 and 3 g/L of pepsin, showed that after 30 min of exposure to such conditions, the initial lactobacillus

biomass decreased by 18.37% (i.e., 1.8 log CFU/mL) when unencapsulated (free) and by only 4% (i.e., 0.4 log CFU/mL) when encapsulated (Fig. 1A). If the exposure to these hostile acidic conditions is extended to 60 min and considering the interval 30-60 min, it can be seen that the loss of LBRE-LSAS cells is accentuated regardless of the state in which they are in: 25% (i.e. 2 log CFU/mL) and only 7.29% (i.e. 0.7 log CFU/mL) loss of viability, respectively in the unencapsulated (free) state and encapsulated in the mixed carob and xanthan galactomannan gel (Fig. 1A).

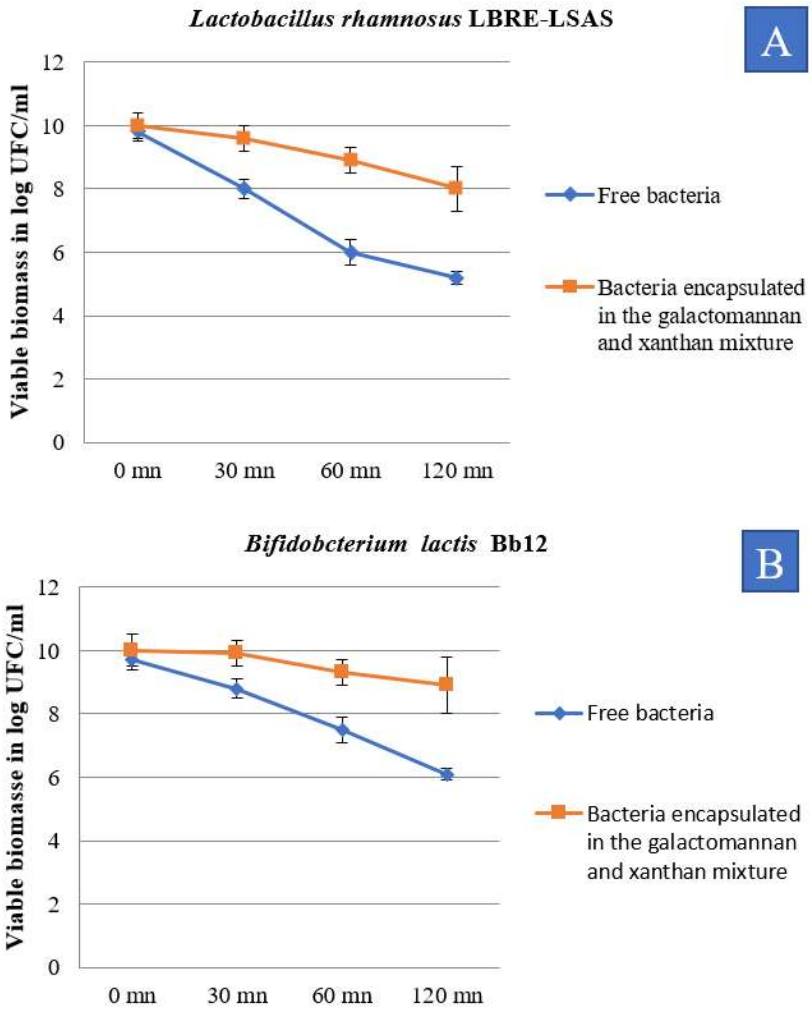

Figure 1. Effects of encapsulation with carob seed galactomannans combined with xanthan on the survival of the two lactic strains: *Lactobacillus rhamnosus* LBRE-LSAS (A) and *Bifidobacterium animalis* subsp. *lactis* Bb12 (B) under simulated gastric conditions *in vitro* (3 g/L pepsin and pH 2).

We noted that in the 0-60 min residence interval, *L. rhamnosus* LBRE-LSAS loses on average 38.77% (i.e. 3.8 log CFU/mL) and only 11% (i.e. 1.1 log CFU/mL) when it is free (non-encapsulated) and encapsulated in this mixed gel. Thus, 3.45 times fewer cells are lost due to encapsulation during the first hour of exposure to gastric conditions. Extending the exposure of the *L. rhamnosus* LBRE-LSAS strain to this acidic environment to 120 min shows that during this second hour, survival is lost (relative to the biomass recorded after 60 min of exposure) by 12.67% (i.e., 0.76 log CFU/mL) and 10.11% (i.e., 0.9 log CFU/mL), respectively, for non-encapsulated (free) and encapsulated cells.

If we consider the survival losses recorded over the entire exposure of the cells to the gastric compartment, i.e. 120 min, we notice that the *L. rhamnosus* LBRE-LSAS strain is lost at an average rate of 46.53% (i.e. 4.56 log CFU/mL) when it is not encapsulated and barely 20% (i.e. 2 log CFU/mL) when it is encapsulated in the gel combining carob galactomannans with xanthan (Fig. 1A).

The survival of this strain is thus more than twice as preserved from gastric hostilities by encapsulation in this type of gel. This level of efficiency is comparable to that obtained with the gel combining carob galactomannans and sodium alginate. The study of the survival of the second probiotic strain, *Bifidobacterium animalis* subsp. *lactis* Bb12, to simulated gastric conditions *in vitro* shows, again, that it is less affected than *L. rhamnosus* LBRE-LSAS when encapsulated or not in the carob galactomannan gel associated with xanthan. However, the combination of these two biomaterials preserves the survival of this strain to acidity (Fig. 1B). *Bifidobacterium* in the control (non-encapsulated cells) are almost 10 times more lost than those in the sample (encapsulated cells) after the first 30 minutes of stay in the simulated gastric environment in vitro. Indeed, there is 9.84% (i.e. 0.95 log CFU/mL) of viability of non-encapsulated cells lost versus 1% (i.e. 0.1 log CFU/mL) for encapsulated cells.

The continuation of this stay of the cells in this hostile environment at 60 min further accentuates this tendency to decrease viability which, in the interval 30-60 min, represents, respectively, 13.79% (or 1.2 log CFU/mL) and 7.07% (or 0.7 log CFU/mL) for the control (non-encapsulated cells) and the sample (encapsulated cells). It appears, thus, that during the first hour of residence (0-60 min), the viability of *B. animalis* subsp. *lactis* Bb12 is lost up to 22.28% (i.e. 2.15 log CFU/mL) in the unencapsulated (free) state and only 8% (i.e. 0.8 log CFU/mL) when encapsulated in the mixed carob and xanthan galactomannan gel (Fig. 1B). From 60 to 120 minutes of exposure of this bifid strain to simulated gastric conditions, its viability losses decrease in intensity compared to those of the 0-60 min interval: 18.67% (or 1.4 log CFU/mL) for unencapsulated (free) cells and 4.34% (or 0.4 log CFU/mL) for encapsulated cells. The effectiveness of the mixed galactomannan and xanthan gel encapsulation in preserving the viability of *B. animalis* subsp. *lactis* Bb12 in the gastric environment is assessed by the cell losses recorded in the 0-120 min exposure interval, which amounted to 36.79% (i.e., 3.55 log CFU/mL) for the unencapsulated cells and only 12% (i.e., 1.2 log CFU/mL) for the encapsulated cells The analysis of variance of the results in the two cases free and encapsulated show a significant difference ($P<0.05$). These results indicate that this bifid strain loses 3.8 times less viability to gastric conditions if encapsulated in the gel combining carob galactomannans with xanthan; whereas *L. rhamnosus* LBRE-LSAS lost only 2.32 times less under the same circumstances.

The results of this experiment regarding the efficacy of xanthan as an encapsulation biomaterial are similar to those reported by Ding and Shah [14]. who, after a comparison of several encapsulation biomaterials, had noted this same efficacy of xanthan in preserving the viability of several tested strains. Furthermore, according to Papiaganni et al. [15], xanthan polymer in emulsion with olive oil improves the viability (which was maintained at 89%) of encapsulated strains exposed for 120 min to simulated gastric conditions in vitro. In a more recent study [16] found that xanthan combined with gellan gum improved the survival of *Lactobacillus rhamonosus* strain under simulated gastric conditions (pH = 2) and whose biomass was preserved at a level of 8 log CFU/mL. Our results are also of this order of magnitude when galactomannan gel (LBG) combined with xanthan was used. Another comparative result [17] on the encapsulation of *Bifidobacterium infantis* ATCC 15697 by xanthan combined with gellan and its exposure to simulated gastric conditions for 120 minutes. These authors recorded a loss of viability of this strain of about 2.7 log CFU/mL.

3.2. Effects of encapsulation with carob seed galactomannans combined with xanthan on the survival of the two lactic strains *Lactobacillus rhamnosus* LBRE-LSAS and *Bifidobacterium animalis* subsp. *lactis* Bb12 under intestinal conditions

Xanthan also appears to be an interesting ingredient when combined with carob seed galactomannans to produce cell protection capsules against digestive hostilities. After 16 h of exposure to simulated in vitro intestinal conditions and based on the biomass recorded after 2 h under gastric conditions, *L. rhamnosus* LBRE-LSAS loses 55.73% (i.e. 2.92 log CFU/mL) in the unencapsulated state and only 12.50% (i.e. 1 log CFU/mL) in the encapsulated form in this mixed gel (Fig. 2A).

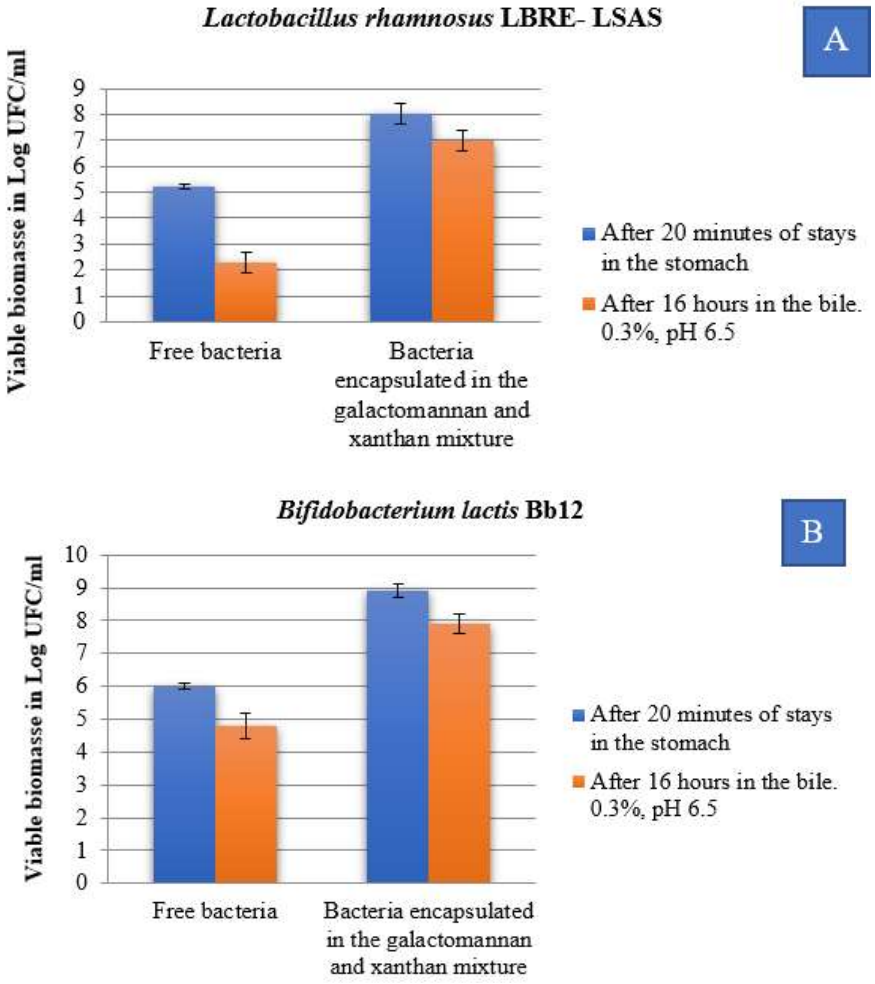

Figure 2. Effects of encapsulation with carob seed galactomannans combined with xanthan on the survival of the two lactic strains: *Lactobacillus rhamnosus* LBRE-LSAS (A) and *Bifidobacterium animalis* subsp. *lactis* Bb12 (B) exposed for 16 hours to simulated intestinal conditions *in vitro* (0.3% bile and pH 6.5).

Under these conditions and considering the intestinal compartment only, the viability of this *Lactobacillus* is multiplied by a factor of 4.85 thanks to the encapsulation of the cells by the combination of carob seed galactomannans and xanthan. The total loss (during all the exposure to digestive conditions: 2 h gastric + 16 h intestinal) of the viability of *L. rhamnosus* LBRE-LSAS is 76.33% (i.e. 7.48 log CFU/mL) without protection (in the non-encapsulated state) and only 30% with protection (in the encapsulated state) in this mixed

gel (Fig. 2A). Over this entire stay in digestive conditions, the viability of *L. rhamnosus* LBRE-LSAS is improved by a factor of 2.54.

The results concerning the second probiotic strain, *B. animalis* subsp. *lactis* Bb12, indicate that the loss of its viability during the 16 hours of residence in intestinal conditions alone amounts to 22.13% (i.e., 1.35 log CFU/mL) in the non-encapsulated state (free = control) and to 10.57% (i.e., 0.93 log CFU/mL) when encapsulated in the mixed gel associating carob seed galactomannans with xanthan (Fig. 2B). Thus, in the simulated intestinal compartment, the viability of bifid cells is multiplied by a factor of more than 2 under the effect of their encapsulation in this mixed gel.

During the entire stay of this strain in the simulated in vitro digestive conditions (2 h in gastric conditions + 16 h in intestinal conditions), the losses of its viability reach levels of 50.78% (i.e. 4.90 log CFU/mL) in the non-encapsulated state (free = control) and 21.30% (i.e. 2.13 log CFU/ML) in the state encapsulated in the gel associating carob seed galactomannans with xanthan (Fig. 2B). Under the digestive conditions (gastric and intestinal) simulated in vitro, the viability of the *B. animalis* subsp. *lactis* Bb12 strain is increased by a factor equal to 2.38 [15], developed xanthan-based capsules to provide stability and increased viability of *Pediococcus* cells in high nutritional value food systems. These authors report that xanthan gum is a safe, high-stability natural material that is tasteless and does not affect the taste of other food ingredients. In such systems, ensuring viability rates of encapsulated cells as high as 85%, and offering levels up to 92% of the initially encapsulated population at the target point are all characteristics attesting to the success of such applications.

4. CONCLUSION

The goal of this study was to use galactomannans from carotenoids combined with other biomaterials such as xanthan in the encapsulation of important lactic bacteria such as *L. rhamnosus* and *B. animalis* subsp. *lactis* in order to protect them from in vitro digestive hostilities. The digestive conditions were simulated using a 3g/L pepsin solution and a pH of 2 with a cell stay time of 2 hours, while the intestinal conditions were simulated using a 0.3 % bile solution and a pH of 6.5 with a cell stay time of 16 hours. Overall, the results clearly demonstrated the positive effect of encapsulation on the survival of the two microorganisms tested.

The strain *B. animalis* subsp. *lactis* Bb12 was found to be more resistant to in vitro digestive hostilities than the strain *L. rhamnosus* LBRE-LSAS. In terms of efficacy, the mixed gel containing carotenoids galactomannans and xanthan is the most effective cell-protective combination. The most significant losses in the viability of the stocks have occurred as a result of the cells' stay in the gastric environment. As a result, the stomach serves as the most significant barrier or factor limiting the survival of digestible nutrients that can be added as a supplement to the host. The viability of *L. rhamnosus* LBRE-LSAS in all of the digestive conditions simulated in this study is multiplied by 2.54 as a result of its encapsulation in galactomannans from carotenoids combined with xanthan gels.

The viability of the strain *B. animalis* subsp. *lactis* Bb12 in all of the digestive conditions simulated in this study is multiplied by a factor of 2.38 as a result of its encapsulation in galactomannans from carotenoids combined with xanthan. These findings clearly show that using only carotenoids galactomannans is not a viable option for effectively protecting the *L. rhamnosus* LBRE-LSAS and *B. animalis* subsp. *lactis* Bb12 strains from digestive hostilities.

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