RESEARCH ARTICLE

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Effect of permeability of 6-carboxy fluorescein dye and the survival of probiotic bacteria *Lactobacillus casei* in acidic and gastric conditions

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ABSTRACT
Microencapsulation of probiotic bacteria has been investigated to improve probiotic bacteria survival in foods and the gastrointestinal environment. In this study, *Lactobacillus casei* Shirota was isolated from the probiotic drink "Yakult" which was encapsulated with various materials and exposed to varied pH and bile concentrations. The results suggested better survival of encapsulated probiotic bacteria than free cells when exposed to acidic conditions (at pH 2.0 and pH 3.0) and high bile salt concentrations (3% taurocholic acid). Also, 4% sodium alginate, 3% alginate + starch, and 3% xanthan gum capsules retained much more of the water-soluble fluorescent dye, and the alginate + starch complex proved to be the best encapsulating material. The results revealed that 2% of sodium alginate retention of the dye up to 2 weeks storage. The size of all microcapsules ranged from 15μm – 20 μm.

Key words: *L. casei;* Probiotic; Microencapsulation; Acid bile tolerance; 6-Carboxyfluorescein

Introduction

Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Lactic acid bacteria such as *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Enterococcus*, *Pediococcus*, and bifidobacteria are the foremost commonly used microbes as probiotics (Shah, 2000; Bhagwat et al., 2019; Bhagwat and Annapure, 2019;). Probiotics term is used to describe the "friendly" bacteria that normally live in the intestinal tract and which contribute to good health. Health benefits that have been attributed to probiotics include anti-mutagenic, anti-carcinogenic, anti-infective, and immune-stimulating properties (Sah et al., 2014). Also, one can benefit from serum cholesterol reduction, alleviation of lactose intolerance as well as a nutritional enhancement (Oak & Jha, 2019). Presently probiotics are available as ingredients in a variety of dairy products like milk, yogurt, cheese, butter, and supplements in the form of capsules, tablets, and powder. The key factors for effective use of probiotics in food are the maintenance of culture viability and activity of the bacteria that have to survive in the food during its shelf life, during transit through acidic conditions of the stomach, and the enzyme and bile salts in the small intestine. Its therefore necessary that probiotic foods meet the criterion of a minimum of 106 cells/mL of the probiotic bacteria at the expiry date, as the minimum therapeutic dose/ day is suggested to be 108-109 cells/mL (Kailasapathy, 2002).

To obtain the desired therapeutic effects, the probiotic bacteria must be available in sufficient numbers. Probiotic bacteria should have the ability to resist the digestion process in the stomach and the intestinal tract. Hence, there is a need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine which contain bile. Thus, strains selected to be used as probiotic bacteria should be able to tolerate stomach acid and bile salts, adhere to the epithelium, and grow in the lower intestinal tract, to provide real health benefits. Shelf life should be controlled to ensure the product has adequate bacterial counts to obtain the health-promoting effects of the probiotic cultures (Fenster et al., 2019).

Encapsulation has been studied for protecting bacteria and improving their survival in adverse conditions. Micro-encapsulation is a process wherein tiny particles or droplets are surrounded by a coating forming small capsules. It is a technology widely used in the packaging of solids, liquids, or gases, in the form of sealed capsules that can release their contents at controlled rates under specific conditions. In other words, a microcapsule is a small sphere with a uniform wall around it. The microcapsule consists of a core and the wall sometimes is known as a shell, coating, or membrane. Most microcapsules have diameters between a few micrometers and

a few millimeters (Shahidi & Han, 1993; Gharsallaoui et al., 2007).

Microencapsulation of the probiotic cells is one of the widely used and efficient methods and is now targeted and developed by many researchers. Encapsulation methods have been applied to increase the survival and delivery of bacterial cultures. Several methods have been developed for the encapsulation of bacteria for use in fermentation, as well as for incorporating into products. Encapsulation helps in segregating the bacterial cells from the adverse environment thus potentially reducing cell loss. The encapsulation process and the capsule material influence the viability of bacteria, under different conditions as compared to when bacteria were in the non-encapsulated state. Regarding the utility of microencapsulation, a few publications are dealing with the effect of different capsule materials especially polysaccharides e.g., alginate (Sultana et al., 2000).

The present study used several different encapsulating materials and their effects on protecting and stabilizing the survival of *Lactobacillus casei* in some adverse environments and conditions were evaluated. Microcapsules made of alginate polymers are reported to increase the survival and viability of probiotic bacteria in acidic food products during cold storage. To date, alginate has been widely used for encapsulation (Sultana et al., 2000); however, only a few studies have explored other potential encapsulating materials. The main aim of this study was to examine the effect of various encapsulating materials on the survival of probiotic bacteria under acidic conditions and exposure to bile salts.

Materials and Methods

Bacterial growth and conditions

Lactobacillus casei Shirota strain isolated from commercial probiotic drink "Yakult" was used in the study. About 5 mL of probiotic drink Yakult with *L. casei* Shirota was enriched in 50 mL of sterile MRS broth and incubated for 24 h at RT under microaerophilic conditions. The culture obtained was then isolated on MRS agar plates and incubated under microaerophilic conditions at RT for 48 h and colony characteristics were observed. Isolated colonies on the sterile MRS agar plates were identified based on their colony characteristics, Catalase test, Gram-nature, morphology, and biochemical tests. Saline suspension of isolates was inoculated into sterile 1% peptone water base with Andrade's indicator containing 1% of any one of the sugars like arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, rhamnose, ribose, sorbitol, sucrose, and xylose. The isolate was confirmed by referring to Bergey's Manual of Bacteriology (Garrity & Holt, 2001). The strain was grown on MRS agar slants and maintained at 4ºC and sub-cultured weekly.

Encapsulation procedure

L. casei Shirota strain was inoculated in 100 mL of MRS broth and incubated under microaerophilic conditions for 24 h. Cells were harvested by centrifugation and adjusted to the density of10^9cfu/mL. Culture (OD=0.4) adjusted were added to different encapsulating materials like 4% sodium alginate, 3% alginate+starch, 4% gelatin, and 3% xanthan gum. Encapsulation was carried out by two methods: extrusion and emulsion technique. In the extrusion technique, the capsule material was mixed with the probiotic bacteria using a syringe and the beads were prepared in a chilled 6% $CaCl_2$ solution. In the emulsion method, the capsule material was mixed with probiotic bacteria that were slightly dispensed into vegetable oil and 1% tween 80. Then, the $CaCl_2$ solution was gently added to the size of the beaker until emulsification was broken. After phase separation, beads were dropped into the $CaCl_2$ layer. The excess oil was drained off. After 15 minutes, the beads were removed from the aqueous phase and were refrigerated at 4ºCto allow the beads to fully harden. The bead size was evaluated by using a stage micrometer slide and oculomotor (Mandal et al., 2006).

Encapsulation efficiency

One gram of beads was dissolved in 0.4 M phosphate buffer (pH 7.0) to make serial dilutions and spread plate on sterile MRS agar plates. The plates were incubated in microaerophilic conditions for 48 h. The viability can be evaluated by the plate count method.

Viability of free and microencapsulated L. casei under acidic conditions

Sterile MRS broth was adjusted to pH 2.0and 3.0. Approximately 10^9 cfu/mL of encapsulated beads and free probiotic bacteria were inoculated into the acidified sterile MRS broth and incubated at 37 ºC for 2 h. Samples were taken for plate counts at 0, 30, 60, 90, 120-minute intervals. For the enumeration of encapsulated probiotic organisms, the bacteria were released from the capsules by sequestering calcium ions with 0.4 M phosphate buffer (pH 7). Acid tolerance was determined by comparing the final plate count after 2 h with the initial plate count at 0 h. Plates were incubated at 37 ºC. All acid tolerance tests were repeated twice to estimate an average (Rao et al., 1989).

Viability of free and microencapsulated L. casei in bile

Sterile MRS broth was mixed with 3.0% sodium taurocholate – a bile salt. Approximately 10^9 cfu/mL of encapsulated beads and free probiotic bacteria were inoculated into the acidified sterile MRS broth and incubated at 37 ºC for 2h. Samples were taken for plate counts at 0, 30, 60, 90, 120-minute intervals. For the enumeration of encapsulated probiotic organisms, the bacteria were released from the capsules by sequestering calcium ions with 0.4 M PO_4 buffer

at pH 7.0. Bile tolerance was determined by comparing the final plate count after 2 h with the initial plate count. Plates were incubated at 37 ºC. All bile tolerance tests were repeated 2 times to estimate an average (Lee et al., 2004).

To check the permeability of encapsulating materials by fluorescent dye

The permeability of the microcapsules and their ability to hold small molecules when stored in an aqueous environment was measured by monitoring the release of a water-soluble fluorescent dye, namely 6-carboxyfluorescein (6-CF). The dye was chosen for its small molecular weight of 376.28 g/mole, which is similar to that of many disaccharides. About 1% solution (v/v) of fluoroscein was encapsulated by incorporating the fluorescent dye into the various encapsulating materials and followed the same procedure of encapsulation. Beads were washed twice in distilled water and stored at 4ºC overnight to harden the beads. One gram of microcapsules containing water-soluble fluorescent dye was stored in beakers containing 10 mL of water for 2 weeks. Readings were observed on day zero, after 1 week and 2 weeks. Initial reading (0 day) was taken by suspending beads in 3 mL distilled water and kept at RT for 3 h. The amount of dye oozed out was estimated by UV-visible spectrophotometer at 487 nm. 3 mL aliquots were taken from the storage medium and centrifuged at 5000 rpm for 25 minutes and the supernatant was used to analyze the amount of dye released during storage. A UV-visible spectrophotometer set to 487 nm was used to detect the dye release. The quantity of dye released by the capsules was detected by comparing the quantity of dye found in the final storage period at 2 weeks to that of the initial storage period (Ding & Shah, 2007).

Results

Isolation of probiotic bacteria

The probiotic bacteria were isolated from probiotic drink milk "Yakult" which contains *L. casei* strain Shirota. The results indicated that all the colonies isolated on MRS agar plates were of LAB as they were Gram-positive and catalase-negative. Biochemical tests were performed by referring to Bergey's Manual of Bacteriology (Garrity & Holt, 2001). The organism isolated from probiotic drink milk was confirmed as *Lactobacillus casei* Shirota (Table 1).

Acid tolerance

This research evaluated the *in vitro* tolerance of both free and microencapsulated *Lactobacillus casei* to pH and bile levels similar to those encountered in the human stomach and intestine, respectively. Cells were inoculated into 0.1 N HCl solutions of pH 2.0 and pH 3.0. The effect of acidic conditions on the viability of free probiotic organisms and encapsulated organisms is shown in Figure 1. The free probiotic organism tested showed a gradual loss in viability when exposed to acidic conditions. At pH 2.0 initially, there was an average of 6.55 log cfu/mL of viable probiotic bacteria, but after 1 h of exposure the average viability of cells was reduced to 4.49 log cfu/mL and the viability was further reduced to an average of 2.64 log cfu/mL after 2 h of exposure. The effect of low pH on the viability of probiotic bacteria encapsulated in various encapsulating materials, namely alginate, alginate + starch, gelatin, and xanthan gum in comparison with free cells are shown in Figure 1. Results suggested that 1 h of exposure of these encapsulated probiotic organisms to acidic conditions would still confer health benefits, but 2 h of exposure would

Table 1. *Biochemical characteristics of the isolate*.

Sugars	*L. casei* subsp. *casei*	*L. casei* subsp. *pseudoplantarum*	*L. casei* subsp. *rhamnosus*	*L. casei* subsp.*tolerans*	Isolate
Arabinose	-	-	D	-	-
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Glucose	+	+	+	+	+
Lactose	D	+	+	+	+
Maltose	+	+	+	-	+
Mannitol	+	+	+	-	+
Mannose	+	+	+	-	+
Raffinose	-	-	-	-	-
Rhamnose	-	-	+	-	-
Ribose	+	+	+	-	+
Sorbitol	+	+	+	-	+
Sucrose	+	+	+	-	+
Trehalose	+	+	+	-	+
Xylose	-	-	-	-	-
Cellobiose	+	+	+	-	+
Gluconate	+	+	+	-	+

not. At 2 h, probiotic organisms encapsulated in gelatin showed poor survival with an average survival of 3.35 log cfu/mL, respectively. The composition of 3% alginate + starch was found to be the best encapsulating material compare to all other materials.

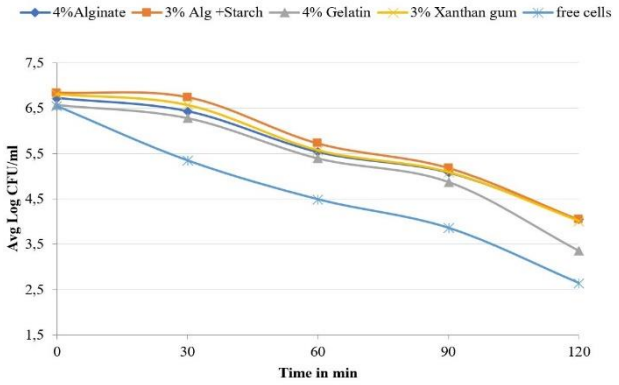

Figure 1. *Tolerance to pH 2.0.*

Similarly, at pH 3.0, initially, there was an average of 6.61 log cfu/mL of viable probiotic bacteria, but after 1 h of exposure the average viability of cells was reduced to 4.69 log cfu/mL and the viability was further reduced to an average of 2.75 log cfu/mL after 2 h of exposure. The effect of low pH on the viability of probiotic bacteria encapsulated in various encapsulating materials, namely alginate, alginate + starch, gelatin, and xanthan gum in comparison with free cells are shown in Figure 2. Results suggest that 1 h of exposure of these encapsulated probiotic organisms to acidic conditions would still confer health benefits, but 2 h of exposure would not. At 2 h, probiotic organisms encapsulated in gelatin showed poor survival with an average survival of 3.85log cfu/mL, respectively. The composition of 3% alginate starch was found to be the best encapsulating material compare to all other materials.

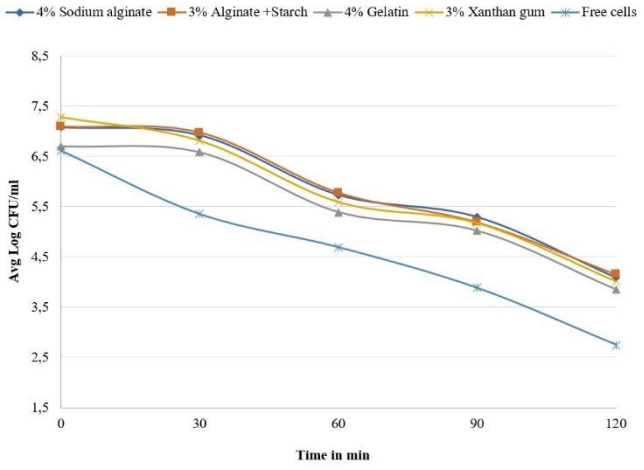

Figure 2. *Tolerance to pH 3.0.*

Bile tolerance with 3% Sodium Taurocholate

The bile concentrations tested were 3% taurocholic acid as it is the maximum concentration found in the human small intestine. The effect of 3% (w/v) sodium taurocholate on the viability of free and encapsulated probiotic bacteria with different encapsulating materials is presented in Figure 3. The free probiotic organism tested showed a gradual loss in viability when exposed to bile conditions. Initially, there was an average of 5.4 log cfu/mL of the free viable probiotic bacteria, but after 3 h of exposure the average viability of cells was reduced to 3.39 log cfu/mL and the viability was further reduced to an average of 2.39 log cfu/mL after 6 h of exposure. Results suggest that 3 h of exposure of these encapsulated probiotic organisms to bile conditions would still confer health benefits, but 6 h of exposure would not. At 6 h, probiotic organisms encapsulated in gelatin showed poor survival of 2.41 log cfu/mL, respectively. The composition of 3% alginate + starch was found to be the best encapsulating material compare to all other materials.

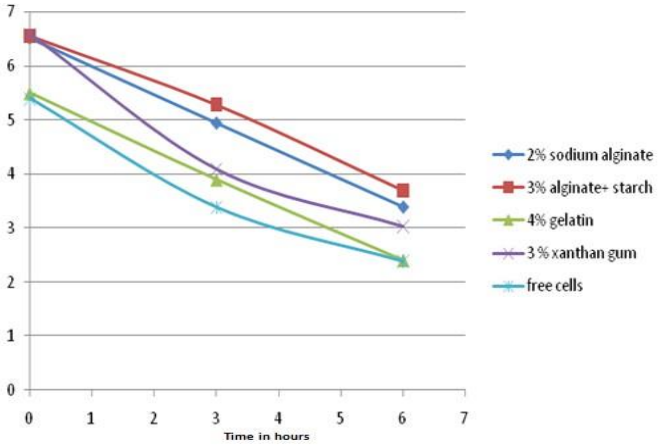

Figure 3. *Taurocholic acid tolerance.*

Permeability of encapsulated material by fluorescent dye

This research also evaluated the permeability of encapsulated material by fluorescent dye**.** All capsules released small amounts of fluorescent dye. Figure 4 shows that 4% sodium alginate, 3% alginate+ starch, and 3% xanthan gum capsules retained much more water-soluble fluorescent dye than 3% gelatin. The alginate starch complex was proved as the best encapsulating material. Results indicate that 2% of sodium alginate retained more sodium fluorescein dye than other encapsulating materials when stored for 2 weeks. Results also indicate that small water-soluble dyes with a molecular weight of 376.28 g/mole could permeate in or out of the capsule. The capsules of different materials were measured using an ocular micrometer under the 100X eyepiece of a compound microscope. The average size of all encapsulated materials ranged from 15µm – 20 µm.

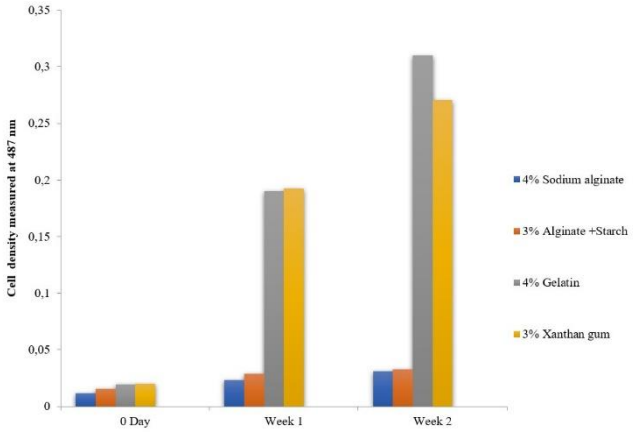

Figure 4. *Permeability of encapsulated materials by fluorescent dye.*

Discussion

Recently, increasing research has been conducted involving the usage of live bacterial cells for therapeutic purposes. The ability of these cells in being genetically engineered to synthesize products with therapeutic potential has resulted in considerable interest and curiosity among healthcare professionals. However, a major issue or hindrance is in the complexity of delivering these live cells to the target tissues. Hence, several ways like oral delivery, lyophilized forms, and immobilized cells have been attempted with a limited success rate. The major issue lies with the incapability of survival of bacterial cells in the gastrointestinal tract. In several cases, delivering live cells orally has provoked undesirable immunogenic responses (Prakash & Jones, 2005).

The present study evaluated the survival of free and microencapsulated *L. casei* under acidic conditions in HCl solution of pH 2.0 and pH 3.0 with MRS broth at 30-minute intervals. Results suggest that 1 h of exposure of these encapsulated probiotic organisms to acidic conditions would still confer health benefits, but 2 h of exposure would not. At 2 h, probiotic organisms encapsulated in gelatin showed poor survival with an average survival of 3.35 and 3.85 log cfu/mL, respectively. 3% alginate+starch composition was found to be the best encapsulating material compare to all other materials.

Acid and bile resistance are important characteristics to be considered when selecting a culture, which should be used as a dietary adjunct. Cellular stress begins in the stomach, which has a very low pH. After the bacteria have passed through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. Thus, strains selected to be used as probiotic bacteria should be able to tolerate stomach acid and bile salts, adhere to the epithelium, and grow in the lower intestinal tract, to provide health benefits (Chou & Weimer, 1999; Dos Santos Leandro et al., 2013). The probiotic strains must tolerate and survive in the stomach for at least 90 minutes to provide health benefits. Different regions of the gastrointestinal tract have varying acid levels. The stomach and the regions immediately following have the highest acidity and the pH of these areas may fall to as low as 1.5. To use as dietary adjuncts, *L. casei* must be able to survive these harsh conditions and colonize in the gut (Shah et al., 1995).

Chou & Weimer (1999) have reported that lactic acid bacteria are useful health adjunct when supplied with food delivery mechanism. The present study showed that 3 h of exposure of these encapsulated probiotic organisms to bile conditions would still confer health benefits, but 6 h of exposure would not. At 6 h, probiotic organisms encapsulated in gelatin showed poor survival with the survival of 2.41 log cfu/mL, while 3% alginate + starch was found to be the best encapsulating material with 3.75 log cfu/mL when compared to all other materials.

In a similar study, Ding & Shah (2007) encapsulated ten probiotic bacteria, belonging to *Lactobacillus* and *Bifidobacterium* species in alginate and carrageenan, guar, xanthan, and locust bean gums. The encapsulated probiotic bacteria were studied for their acid and bile tolerance. The permeability of the capsules was also examined using a water-soluble dye, 6-carboxyfluorescein (6-CF). Results revealed that probiotic bacteria encapsulated in alginate, xanthan gum, and carrageenan gum survived significantly better under acidic conditions than free bacteria. The bile tolerance test revealed that the viability of free probiotic bacteria was reduced by 6.36 log cfu/mL, while probiotic organisms encapsulated in alginate, xanthan gum, and carrageenan gum gave better protection by reducing the viability by 3.63, 3.27, and 4.12 log cfu/mL respectively. All the microcapsules released small amounts of 6-CF, with alginate and xanthan gum retaining 22.1% and 18.6% more fluorescent dye as compared to guar gum. So, the probiotic bacteria microcapsules embedded in with alginate, xanthan gum, and carrageenan gum greatly improved the survival of probiotic bacteria when exposed to acidic conditions and bile salts (Ding & Shah, 2007). In contrast to the above results, in the present study, the 6-CF dye was used to check the remaining capacity of the capsules and 2% sodium alginate and 3% sodium alginate retained the maximum amount of dye as compared to 2% xanthan gum, 3% xanthan gum, 2% guar gum, and 3% guar gum.

Adhikari et al. (2003) studied microencapsulated probiotic cells of *Bifidobacterium longum* in κ-carrageenan and stored at 4.4°C for 30 days. Encapsulated cells indicated no decline in viability but a significant reduction in the non-encapsulated cell population. The results suggested the protection of bifidobacteria due to microencapsulation in acidic environments (Adhikari et al., 2003). P. Capela et al. (2006) studied the survival of probiotic microorganisms including *L. acidophilus*, *L. casei*, *L. rhamnosus*, and *Bifidobacterium*

species in yogurt and freeze-dried yogurt after processing and storage. Alginate microencapsulation improved the viability of selected multi-strain probiotic organisms by 0.31 log in freeze-dried yogurt stored at 21°C.

Lee & Heo (2000) studied *Bifidobacterium longum* KCTC 3128 and HLC 3742 by immobilizing calcium alginate beads containing 2, 3, and 4% sodium alginate. The results revealed the protection property of calcium alginate and sodium alginate after exposing the bifidobacteria to simulated gastric juices and a bile salt solution. The death rate of the cells in the beads decreased proportionally with an increase in both the alginate gel concentration and bead size.

In another study by Kailasapathy (2006), increased survival of 2 and 1 log cell numbers of *L. acidophilus* and *B. lactis* was observed in calcium-induced alginate–starch encapsulated probiotic bacteria in acidic stress. The study observed that microencapsulation of probiotic bacteria helped in enhancing the survival of probiotic bacteria in yogurts during storage. In our study different encapsulating materials were used to increase the viability of probiotic bacteria in acidic and bile conditions found in the gastrointestinal tract and amongst the different encapsulating materials used i.e., 4% alginate, 3% alginate+ starch, 4% gelatin, and 3% xanthan gum, 3% alginate + starch was found to be best encapsulating material.

Mirlohi et al. (2009) studied the growth and survival of three native strains of lactobacilli with two commercial probiotic strains in their tolerance to acid and bile. The results suggested that *L. plantarum* is a potential probiotic with resistance to acid and bile. It also showed that the growth-rate designed studies and survival studies evaluating transit tolerance might give varied results with regards to different species of lactobacilli having different growth and metabolic activities).

Encapsulation may prove to be an important method of improving the viability of probiotic bacteria in acidic food products to help deliver viable bacteria to the host's gastrointestinal tract.

Sodium alginate, alginate starch mixture, gelatin, and xanthan gum appeared to protect probiotic cells from harsh environmental conditions and also retained small probiotic bacteria by slow release of the dye which leads to a higher survival rate for the retention period in the digestive tract.

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