INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 3, p. 330-337, 2015

RESEARCH PAPER OPEN ACCESS

Survival of free and microencapsulated *Lb. rhamnosus LC705* in simulated gastric and intestinal juice

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Key words: *Lb. Rhamnosus,* microencapsulation, breast milk.

http://dx.doi.org/10.12692/ijb/6.3.330-337 Article published on February 18, 2015

Abstract

Breast milk is an important food for the neonates during their early months of development, primarily educating their immune system and protecting them for pathogens. The aim of this research was to investigate the probiotic properties (resistance to acid and bile salt) of free and microencapsulated *Lb. rhamnosus LC705* strain isolated from mothers' breast milk. *Lb. rhamnosus LC705* was encapsulated into alginate (AL)-whey protein isolate (WPI) microspheres and morphology and particle size of the microcapsules were determined. The Viability of free and encapsulated forms in simulated gastric and intestinal juice was studied. Entrapment efficiency (emulsion method) was ~ 88%. The images of the scanning electron microscope (SEM) illustrated spherical shaped microcapsules with approximate diameters of less than 100 µm. The survival of free and microencapsulated *Lb. rhamnosus LC705* was reduced to 6 and 4 log cycle in simulated gastric juice (pH 2.5, 2 h). Microencapsulated *Lb. rhamnosus LC705* (D-values 27.10 min) survived better than the free cells (D-values 13.38 min) against bile salts (0.5%, 2 h). The survival of microencapsulated cells was significantly ($p < 0.05$) better than the free cells. Therefore microencapsulation of *Lb. rhamnosus LC705* in AL–WPI containing matrix has the potential to increase the strain viability against simulated gastrointestinal conditions.

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Introduction

Lactobacilli are Gram positive (+) bacteria; rod shaped which belong to the group of lactic acid bacteria (LAB). They can be found in plants or material of plant origin, silage, fermented food (yogurt, cheese, olives, pickles, salami, etc.), as well as in the oral cavities, gastrointestinal tracts (GIT), and vaginas of humans and animals (Hammes and Vogel, 1995). The health promoting effects of lactobacilli have been widely explored and include the stabilization of the indigenous microbial population, protection against intestinal infection, alleviation of lactose intolerance, increased nutritional value in foods, reduction of serum cholesterol levels and non-specific enhancement of the immune systems (Suvarna and Boby, 2005).

Lactobacilli are also considered to be probiotic bacteria; they are a living microbial food products/supplement which beneficially affects the human gut by improving the balance of the intestinal microbial flora (Fuller, 1989). Isolation from human hosts, the capability to tolerate acidic pH and bile, antimicrobial activity and good adhesion ability are principle desirable properties in potential probiotics (Dunne *et al.,* 2001). The harsh conditions of the gastrointestinal tract including the acidic environment of the stomach and the bile salts secreted in the duodenum are important obstacle to the viability of probiotic bacteria. However, many probiotic bacteria lack the ability to survive in the harsh acidity and bile concentration commonly encountered in the GIT (Shah and Jelen, 1990; Gardiner *et al.,* 2000). Several methods have been used to enhance the viability of probiotics, including selection of resistant strains, stress adaptation, incorporation of micronutrients, and microencapsulation. Microencapsulation is a method defined as the entrapment of a compound or a system inside a dispersed material for its immobilization, protection, controlled release, structuration and functionalization. The composition of the wall material usually determines the functional properties of the microcapsules. The most common biomaterial used for probiotics encapsulation is alginate. Other supporting biomaterials include carrageenan, gelatin, chitosan, whey proteins, cellulose acetate phthalate, locust bean gum and starches (Anal and Singh, 2007). Alginate is a linear heteropolysaccharide extracted from different types of algae, with two structural units consisting of D-mannuronic and L-guluronic acids. The successful use of alginate in the microencapsulation of probiotics is for the basic protection against the acidity it provides to the cells (Gbassi *et al.,* 2009). Whey proteins are usually used because of their amphoteric character. They can be easily mixed with negatively charged polysaccharides such as alginate, carrageenan or pectin (Doleyres *et al.,* 2004). When the pH is adjusted below their isoelectric point, the net charge of the proteins becomes positive, causing an interaction with the negatively charged polysaccharides (Guerin *et al.,* 2003).

Breast milk is a major factor in the initiation and development of the neonatal gut microbiota because it constitutes a continuous source of microorganisms to the infants gut following birth for the breastfed infant. It is estimated that an infant consumes approximately 800 mL/d will ingest about 1×10^5 – 1×10^7 commensal bacteria while suckling (Martín *et al.,* 2003). In the past, a variety of LAB species with probiotic potential from breast milk, including *Lactobacillus gasseri* and *Lactobacillus fermentum* have been isolated. The results of the study illustrated that breast milk LAB originates from the maternal guts microbiota, following an endogenous route (Heikkilä and Saris, 2003; Martín *et al.,* 2003).

Martin *et al.,* (2004) studied three lactobacilli strains isolated from breast milk to determine whether or not they were potentially probiotic bacteria. Two *Lactobacillus gasseri* and one *Lactobacillus fermentum* strains were evaluated and the results demonstrated the probiotic potential of lactobacilli isolated from the breast milk were similar to strains commonly used in commercial probiotic products. Therefore, the objective of this study was to evaluate the probiotic properties of *Lb. rhamnosus LC705* strain (free and microencapsulated), previously

isolated from breast milk of healthy women (Roozbeh Nasiraii *et al.,* 2011), in the simulated gastric and intestinal juice conditions.

Materials and methods

Preparation of microorganism

Lb. rhamnosus LC705 was originally isolated from breast milk of healthy women during a previous study (Roozbeh Nasiraii *et al.,* 2011). A vial of freeze dried *Lb. rhamnosus LC705* was inoculated into 5 mL de Man, Rogosa and Sharpe (MRS) broth (*Scharlau* Chemie S.A., Spain) and incubated at 37 °C for 16 hours. The cultures were then sub-cultured into 95 mL MRS broth. The bacterial cells were harvested by centrifugation at 5000 g for 10 min at 25 °C and washed twice with 0.1% peptone (Quelab, Canada).

Microencapsulation of microorganism

Whey protein isolate (WPI) powder was rehydrated at 8% w/v in deionized water, agitating the solution for 1 hour at room temperature and then allowed to stand for 2 hours to ensure complete hydration of the proteins. WPI solution was adjusted to pH 8.0 with 1N NaOH and heated at 80 °C for 30 min to denature proteins entirely (Lefe`vre and Subirade, 2000). The denatured WPI solution was then cooled and held at room temperature for 2 hours. Sodium alginate powder (10 g/L, medium viscosity and high mannuronic acid, Sigma-Aldrich, Pool, UK) was dispersed in distilled water and stirred overnight to form a 4.0% (w/v) solution (Chen and Subirade, 2006). Then the alginate solution was added to WPI suspension (a WPI/AL ratio of 2:1). Alginate-whey protein isolate (AL-WPI) microspheres were made using an external gelation technique adapted from Sheu and Marshall (1993) as previously described by Truelstrup Hansen *et al.* (2002) and Allan-Wojtas *et al.* (2008). Briefly, AL-WPI microspheres were produced by mixing 18 g AL-WPI solution with 1.0 g washed bacteria suspension. The AL-WPI bacterial mix was subsequently emulsified in 99 g of vegetable oil containing 10.0 g/L Tween 80 (Sigma-Aldrich, Pool, UK) using a magnetic stirrer set at ca. 900 rpm for 20 min. Gelation was initiated by addition of 32 mL Ca^{2+} containing emulsion (59.5 g vegetable oil, 5 g/L Tween 80 and 62.5 mM $CaCl_2$). The AL-WPI microcapsules were formed during continuous stirring for 20 min. Forty milliliters of peptone saline (10.0 g/L peptone and $CaCl_2$ (0.05 M)) were added and the AL-WPI microcapsules harvested using a separator funnel. The drained slurry was adjusted to approximately a 1:1 ratio (v/v) of AL-WPI microcapsules to liquid.

Encapsulation efficiency

Freshly prepared beads (1 g) were liquefied in 99 mL of 1% (w/v) sterile sodium citrate (Merck, KGaA, Germany) solution at pH 6.0 by gently shaking at room temperature for 10 min and *Lb. rhamnosus LC705* was enumerated in duplicates.

Morphology and particle size

The morphology of the microcapsules was observed with a scanning electron microscope (SEM, VEGA, TESCAN-LMU and Razi Metallurgical Research Center) operating at 15 kV. The surfaces of the microcapsules were splashed with gold particles, observed and photographed. Size analysis was measured by using the particle size analyzer (Analysette 22, Fritsch, Germany). For the particle size analysis, microcapsules were dispersed in ethanol 96% and then were directly placed inside the measuring chamber.

Viability of free and microencapsulated Lb. rhamnosus LC705 in simulated gastric

The method described by Vizoso Pinto *et al.* (2006) was used. The free and encapsulated (1 g) *Lb. rhamnosus LC705* samples were dispersed in 9 mL of sterile simulated gastric juice (6.23 g/L NaCl, 2.29 g/L KCl, 0.229 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$, 0.3% pepsin (Merck, Germany), pH 2.5) and incubated at 37 °C for 30, 60, 90 and 120 min. The survival rates of the microorganisms were determined by the traditional plating method using MRS agar and incubation at 37 °C for 3 days. The results, obtained from two replicates, were expressed as average ± standard deviation log cfu/g.

Survival of free and microencapsulated Lb.

rhamnosus LC705 in Simulated intestinal juice

The free and encapsulated (1 g) *Lb. rhamnosus LC705* samples were placed in 9 mL of described simulated gastric juice and incubated at 37 °C for 60 min. Following incubation, the samples were mixed in 10 mL of sterile simulated intestinal juice (1.28 g/L NaCl, 6.4 g/L NaHCO3, 0.239 g/L KCl, 0.5% bile salt (Sigma-Aldrich GmbH, Germany), pH 7.5) and incubated at 37 °C for 30, 60, 90 and 120 min (Vizoso Pinto *et al.,* 2006). The viability of *Lb. rhamnosus LC705* was determined as described above.

Statistical Analysis

All result means of the two replicates were produced by a completely randomized factorial design used for all analysis the statistical software program (SPSS version 16, SPSS Inc., Chicago, IL, USA) was used to conduct analysis of variance (TUKY) to determine differences between treatments means. Least Significant Difference (LSD) test, at 5% level of significance was used to verify the significance of differences among treatments means.

Results and discussion

Viability of free and microencapsulated cells

Viable cell count of *Lb. rhamnosus LC705* before encapsulation was between 12.54 and 13.17 $\log_{10}$ cfu/g. Capsulation caused a loss in *Lb. rhamnosus LC705* viability of less than 1 $\log_{10}$ cfu/g (11.04 – 11.6 $\log_{10}$ cfu/g).

Table 1. Survival of *Lb. rhamnosus LC705* (log cfu/g) after exposure to a pH 2.5 solution at different time intervals.

Cell type	Time (min)					D-value (min)
	0	30	60	90	120	
Free	$3.5\pm0.1\times10^{12}$	$4\pm0.2\times10^{10}$	$4\pm0.3\times10^{7}$	$4\pm0.1\times10^{5}$	$1.3\pm0.1\times10^{5}$	15.10^{a}
Encapsulated	$1.1\pm0.3\times10^{11}$	$5.5\pm0.1\times10^{10}$	$6\pm0.2\times10^{9}$	$4.75\pm0.2\times10^{8}$	$1.9\pm0.2\times10^{8}$	39.52^{b}

[a, b] Values with the different letters show significant difference ($p < 0.05$).

Values are average ± standard error (n = 2).

The bacterial viability counts during the encapsulation process were high in number, demonstrated by a high efficiency (~ 88%) during the emulsion technique (external gelation). Thus the results during the encapsulation process were insignificant.

The images of the SEM illustrated spherical shaped microcapsules with approximate diameters of less than 100 µm. Also, Figure 1 demonstrated that the bacterial cells were encapsulated at all times with an AL-WPI coating. Thus this method could possibly produce a micron diameter and uniform capsules that could give a smooth texture in live microbial food supplements.

Table 2. Survival (log cfu/g) and D-values of free and microencapsulated *Lb. rhamnosus LC705* in simulated intestinal juice.

Cell type	Time (min)					D-value (min)
	0	30	60	90	120	
Free	$3.5\pm0.1\times10^{12}$	$1\pm0.4\times10^{4}$	$6.1\pm0.2\times10^{3}$	$1.9\pm0.1\times10^{2}$	$1.6\pm0.8\times10^{2}$	13.38^{a}
Encapsulated	$1.1\pm0.3\times10^{11}$	$4.4\pm0.7\times10^{7}$	$3.2\pm0.3\times10^{7}$	$2\pm0.2\times10^{6}$	$1.1\pm0.4\times10^{6}$	27.10^{b}

[a, b] Values with the different letters show significant difference ($p < 0.05$).

Values are average ± standard error (n = 2).

The particle size distribution data graph represents d90 ≤ 115.510 µm (d90 indicates that 90 percent of the particles have a diameter equal or less than 115.510 µm), d50 ≤ 77.039 µm, d10 ≤ 35.546 µm and dpeak ≤ 211.045 µm (Figure 2).

The emulsion technique is easy to scale-up and the size of the beads is small (25 µm – 2 mm) and gives a high survival rate of the bacteria (Chen *et al.*, 2007). The emulsion procedure enables the production of targeted microcapsule size by variation of agitation speed and the water/oil ratio. The gel beads can be introduced into a second polymer solution to create a coating layer that provides additional protection to the cell or may improve organoleptic properties (Kailasapathy, 2009). Mokaram *et al.,* (2009) reported that double layer coated beads (75.339 ± 0.209 µm) provide the best protection of cells in the gastric and intestinal juice.

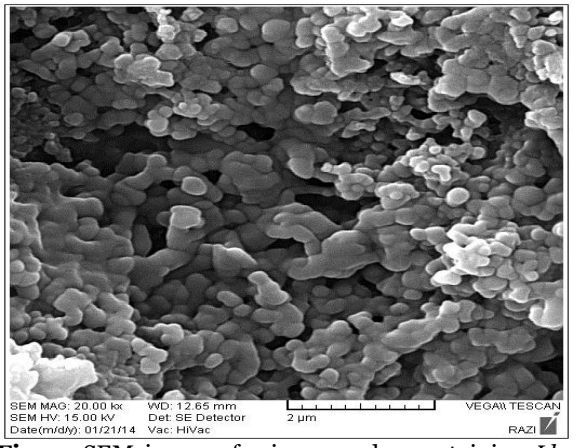

Fig. 1. SEM image of microcapsules containing *Lb. rhamnosus LC705*.

Truelstrup-Hansen *et al.,* (2002) produced Ca-alginate capsules containing four strains of *Bifidobacterium* (*adolescentis 15703*, *breve 15700*, *lactis Bb-12* and *longum Bb-46*) at levels ranging from 6 – 8 $\log_{10}$ cfu/mL. This methodology produced capsule sizes of ~ 70 µm, however during the acid challenge (pH 2.0, 30 min); a 5 $\log_{10}$ cfu/g reduction for all strains was observed, with the exception of *B. lactis* which remained constant.

Viability of free and microencapsulated Lb. rhamnosus LC705 in simulated gastric and intestinal juice

The study of bacteria cells viability during gastric transit (resistance to acid and bile salt) is a major factor in the choice of the probiotic strain. The effect of the simulated gastric environment on the viability of the free and microencapsulated *Lb. rhamnosus LC705* is shown in Table 1.

The initial numbers of free and encapsulated bacterial cells were 3.5×10^{12} and $1.1\pm0.3\times10^{11}$ cfu/g respectively, following a 2 h of exposure to the simulated gastric juice the average viability of free and encapsulated cells was $1.3\pm0.1\times10^{5}$ and $1.9\pm0.2\times10^{8}$ cfu/g. However the survival of free and microencapsulated *Lb. rhamnosus LC705* was reduced to 6 and 4 log cycle. The D-values of cells in capsulated bacteria was higher than the D-values of free cells and these two D-values were significantly different ($p < 0.05$). These results demonstrated that the AL-WPI coating protected microencapsulated cells in the acidic and enzyme conditions of the stomach.

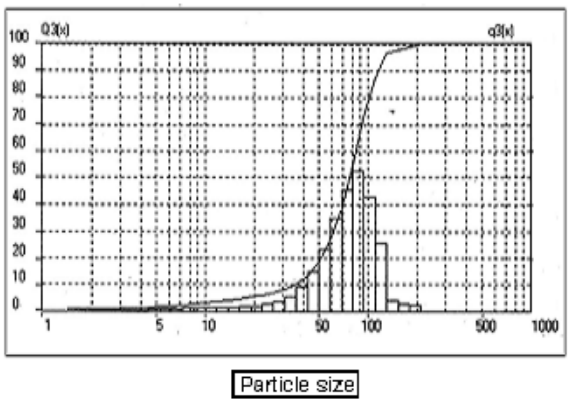

Fig. 2. Particle size analysis of microcapsules.

The effect of simulated intestinal environment on the viability of free and microencapsulated *Lb. rhamnosus LC705* is shown in Table 2. The cultures were put into simulated gastric juice for 60 min, followed by a further incubation in intestinal juice with 0.5% bile salt for 30, 60, 90 and 120 min.

The results suggested that the initial cell population was reduced to $1.6\pm0.8\times10^{2}$ cfu/g after 120 min of exposure to the intestinal condition. Whereas the number of microencapsulated bacteria decreased from $1.1\pm0.8\times10^{11}$ to $1.1\pm0.4\times10^{6}$ cfu/g after 2 h of exposure to the intestinal condition therefore, microencapsulated bacteria (D-values 27.10 min) survived better than the free cells (D-values 13.38 min) against bile salts.

Cellular stress begins in the stomach, which has pH as low as 1.5. After the bacteria pass through the stomach, they enter the upper intestinal tract where

bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given time (Lankaputhra and Shah, 1995).The observation of this study demonstrated the ability of AL-WPI microspheres to enhance the survival rate of the microencapsulated *Lb. rhamnosus LC705* in a gastro-intestinal model which concur with the results reported by Gbassi *et al.,* (2009), who established after a simulated gastric treatment 5 – 7 $\log_{10}$ cfu/mL higher survival rate in alginate-based capsules that were pre-soaked in a whey-protein solution, as compared to capsules solely based on alginate. Ainsley Reid *et al.,* (2005) showed that Microencapsulation of probiotic *Lb. rhamnosus* in a Ca (2+)-induced whey protein gel provided protection against acidic conditions in the stomach after 90 min, as well as against bile after 30, 60 and 90 min in the duodenum. Ying *et al.,* (2013) reported that microencapsulated *Lb. rhamnosus GG* formulations containing WPI alone or WPI in combination with a physically-modified resistant starch (RS) at various ratios (4:1, 1:1 and 1:4) provided better protection to *Lb. rhamnosus GG* in apple juice or citrate buffer (pH 3.5) as compared to the formulation containing RS alone.

Rajam *et al.,* (2012) showed that in simulated gastric environment (pH 2.5) and bile conditions denatured whey protein encapsulated cells showed better stability than undenatured whey protein. This study indicated that the combination of denatured whey protein isolate and sodium alginate wall matrix was able to deliver probiotics with an improved survival rate as well as suitable for controlled core release applications.

Conclusion

The aim of this research was to investigate the probiotic properties of native bacteria (*Lb. rhamnosus LC705*). One of the important characteristics of a probiotic microbe is its human origin. As previously mentioned *Lb. rhamnosus LC705* was isolated from mothers' breast milk (Roozbeh Nasiraii *et al.,* 2011). Breastfeeding is significant source of lactobacilli for the breast fed infant gut, affecting the overall composition of the neonate gut microbiota and exerting biological functions in the immune system and protecting the infant against pathogens. In the past, a variety of LAB species from breast milk have been isolated, and has demonstrated a matching correlation of the microflora to a high degree, between the infant gut and that of the mothers' breast milk, which affect the qualitative and quantitative composition of the neonate gut (Heikkilä and Saris, 2003; Martín *et al.,* 2003). The other properties of a probiotic bacterium is its' tolerance to acid and bile salts gastric tract. The results indicated that microencapsulated bacteria had an enhanced survival rate than the free cells in the simulated gastric and intestinal juice. The observations made in this study suggested that the protection afforded by AL-WPI microspheres is the result of the ability for the whey protein isolates to create a buffered microenvironment within the hydrated colloid particle surrounding the embedded *Lb. rhamnosus LC705*, thus isolating the bacteria against adverse conditions of the gastro-intestinal tract .Further studies and research are required to evaluate the use of *Lb. rhamnosus LC705* as a probiotic such as antimicrobial activity, antibiotic resistance profile, adhesion to intestinal cells, etc.

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