ISOLATION, CHARACTERIZATION AND MICROENCAPSULATION OF PROBIOTIC *Lactobacillus curvatus* G7 FROM CHICKEN CROP

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Abstract: The controlled release of bioactive substances to their site of action in the GIT is essential in modern drug and food industries. The major obstacles that probiotic bacteria should overcome are stomach acidity and bile salts. In this research a *Lactobacillus curvatus* strain was isolated from chicken crop; it was identified based on morphological and biochemical characteristics and tested for its probiotic properties. Furthermore; the survival of free and microencapsulated *Lb curvatus* in 1 % sodium alginate was evaluated in GIT-like conditions. The results showed that 27.87 % of the free cells were found to be resistant to acidic conditions (pH 2) after 1 hour of incubation, while only 2.09 % survived after 2 hours of incubation therefore the bacteria could not be capable of resisting in the stomach. Microencapsulation improved the viability particularly after 2 hours for the reason that 11.36 % of the cells survived after 2 hours. On the other hand, in bile salts, the percentage of survival of the free cells of *Lb curvatus* was 47 after 4 hours of incubation and decreased to 40 after 8 hours. However, the microencapsulated form resists more since 66 % of the cells survived after 4 hours and more than 52 % survived in bile salts after 8 hours. It appears evidently that cell entrapment in sodium alginate protects the bacteria from gastric and intestinal hostile conditions.

Keywords: *Lactobacillus curvatus*, probiotic, microencapsulation, chicken crop

INTRODUCTION

Probiotics are defined by the FAO as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" **(FAO report).**

Currently, probiotics are being used extensively in veterinary to replace the use of antibiotics. In poultry farming, probiotics are essentially used to provide beneficial microorganisms that were basically absent in chicken's digestive tract, thus, the least can profit by favorable effects offered by the introduced microorganisms **(Lutful Kabirm, 2009; Gournier-Château et *al*., 1994)**. The two main commercial preparations are targeting the crop and the anterior small intestine as well as the caecum **(Fuller et Turvey, 1997).** The effects of some probiotic bacteria were reported; they include modification of the microbial composition and metabolic activity of the intestinal flora, inhibition of infective pathogens like *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* by competitive exclusion, and enhancing the growth and development indexes in chicken **(Higgins et al., 2010, Awad et al. 2009, Reque et al. 2000).** However, during GI passage, cultures are required to tolerate the low pH of the stomach, and the antimicrobial activity of bile salts, for that reason, it is important to find methods for enhancing the viability of microbial cells in the digestive tract, one of them is microencapsulation which consists of the technology for packaging active materials in miniature, sealed capsules that can release their contents at controlled rates under specific conditions according to **Shahidi and Han (1993)**. Several studies have shown that microencapsulation of bacteria with alginate at different concentrations or other gels protects them against acid stress, allowing the cells to survive in the stomach and to be delivered in the intestine **(Lee et al. 2004, Crittenden et al. 2006).** Generally, most of the researchers are in agreement that alginate is the most suitable material for encapsulating food ingredients even though the recent studies are providing new improvements in capsule texture and rheology characteristics.

In the present study, a lactic acid bacterium from the crop content of chickens was isolated, identified and assessed for its ability to inhibit the growth of some pathogenic bacteria and to attach to intestinal epithelium. In addition, the tolerance of the bacterium to GIT-like conditions was evaluated before and after microencapsulation in 2 % sodium alginate.

MATERIALS AND METHODS

Isolation of lactic acid bacteria

A 10 g sample of the content of local chicken crop was serially diluted in normal saline, then; the appropriate dilutions were plated on MRS agar and incubated for 24 hours at 37°C. The obtained colonies were cultured in MRS broth and further purified.

Test organisms

The following strains were used as test organisms for antimicrobial activity; *Escherichia coli, Klebsiella* spp. from a local rabbit GIT, *E. coli* ATCC 25929 and *Staphylococcus aureus.*

Identification of LAB

The isolated strains were identified based on their morphological and biochemical properties according to **Bergey (1994).** The tests included gram stain, catalase, arginine dihydrolase, acetoine production, citrate utilization, growth in hypersaline solution, fermentation type and sugars fermentation. The obtained results were analyzed by API-LAB program at the "Laboratoire de Biologie des Microorganismes et Biotechnologie" at Es-Senia University, Oran.

Antibacterial activity

The antibacterial activity of Lb. curvatus culture, the cell-free supernatant and the NaOH neutralized supernatant (pH 6) against the cited bacteria was evaluated according to **Tagg et al. (1976)** based on disc diffusion method.

Assay of the *in vitro* adherence of LAB to epithelial cells

The method described by Lin et al. 2007 was used for the assay of the in vitro adherence of LAB to epithelial cells. Segment of chicken crop were opened and washed with sterilized phosphate-buffer saline (PBS, pH 7.2). It was held in PBS at 4 °C for 30 min to remove the surface mucus and then washed three times with PBS. Epithelial cells were scrapped into sterilized PBS. The cell suspension was examined by microscopy to ensure that contaminated bacteria had been removed and the epithelial cell concentration was adjusted to approximately $5x10^4$ cells/ml. The adherence of LAB strain to the epithelial cells was assayed as follows: the overnight culture of LAB in MRS broth was centrifuged and the cell pellet was resuspended to approximately 1.108 CFU/ml in PBS (pH 7.2). One milliliter of the bacterial suspension was mixed with 1 ml of the suspension of epithelial cells from chicken. The mixture in a tube was rotated at 20 rev/min at 37 °C for 30 min. The adhesion was observed using light microscopy (magnification fold, 100x) after stained with 0.5% crystal violet for 5 min **(Lin et al. 2007)**.

Microencapsulation of LAB in 2% sodium alginate

Alginate (2 % w/v) capsules containing the *Lb. curvatus* cells were prepared by dissolving 2 g of sodium alginate in 80 mL distilled water under constant mechanical stirring, and heating at 80ºC. The solution was autoclaved and cooled to 40ºC to which 20 mL of a freshly prepared cell suspension was added and homogenized. The final solution contained approximately 88.10^{11} UFC/mL. The mixture was injected through a needle into 100 mL of autoclaved and pre-cooled 0.05M CaCl2 crosslinking bath. The resultant capsules were allowed to harden in the cross-linking solution for 30 min, and then washed three times with distilled water **(Boyaval *et al.*, 1985)**.

Survival of LAB in acidic conditions

The viability of free and microencapsulated cells of *Lb. curvatus* in acidic conditions was tested by incubating MRS broth (pH 2) inoculated with approximately 10^{10} UFC/ml (free or encapsulated cells) for 2 hours at 37° C. A viable count on MRS agar was carried out at 1h intervals over the assay period after appropriate serial dilution in normal saline. The plates were incubated at 37ºC for 48 h. For microencapsulated cells, the count was determined after lysis of the capsules in 2M M phosphate buffer (pH7)

Tolerance to bile

The viability of free and microencapsulated cells of *Lb. curvatus* in bile conditions was studied by incubating MRS broth supplemented with 0.3% bile salts with approximately 10^{10} UFC/ml (free or encapsulated cells) for 8 hours at 37° C. A viable count on MRS agar was carried out at 1h intervals over the assay period after appropriate serial dilution in normal saline. The plates were incubated at 37ºC for 48 h. For microencapsulated cells, the count was determined as described before.

RESULTS AND DISCUSSION

Eighteen strains were isolated on MRS medium from chicken crop, after biochemical identification it appeared that most of them belonged to *Lactobacillus curvatus. Lactobacillus curvatus* J7 was chosen for further investigations.

Antimicrobial activity test

The antimicrobial activity of the selected bacterium against some bacteria was evaluated in three ways in order to determine the nature of the inhibitory element. The crude culture, the crude cell-free supernatant as well as the neutralized supernatant were used to analyze the antagonistic effect; the results are shown in **table 1**.

Table 1 Effect of different culture fractions on some test microorganisms.

Test microorganisms / Tested fractions	**Inhibition zone diameter (mm)**			
	Klebsiella	***E. coli***	***E. coli*** ATCC 25929	***Staphylococcus aureus***
Crude culture	**21**	**18**	**19**	**17**
Cell-free supernatant	**19**	**20**	**23**	**22**
Neutralized supernatant	**12**	**14**	**14**	**15**

The culture of *Lb. curvatus* showed a good inhibitory effect against the four tested strains, the inhibition zone diameters are ranging from 17 mm for *S. aureus* to 21 mm for *Klebsiella*, in addition, the cell-free supernatant displayed also an inhibitory effect whereas the neutralized supernatant did not lost the whole inhibitory activity although the diameters of the zones are less important (from 12 for *Klebsiella* to 15 for *S. aureus*). Several mechanisms have been reported to describe antagonistic action of probiotic bacteria such as competitive exclusion, production of antimicrobial compounds, modulation of immune response, alternation of intestinal bacterial metabolic activity, alteration of microecology of the animal intestine, and inhibition of bacterial translocation. The production of antimicrobial agents could be easily demonstrated *in vitro* by the disc diffusion assay; they include fatty acids, organic acids, hydrogen peroxide, and diacetyl, acetoin and the small, heat-stable inhibitory peptides called 'bacteriocins'**(Soomro et al., 2002; Simova et al., 2009)**.

In our experiment, the probiotic *Lb. curvatus* decreased the growth of the tested microorganisms not only by the production of lactic acid but other substances could be involved like bacteriocins or hydrogen peroxide this was confirmed by the residual activity found in the neutralized supernantants.

***In vitro* adhesion test**

The adhesion test of *Lb. curvatus* to epithelial cells was conducted as described before as it is one of the most important criteria to select probiotic bacteria **(Roy et *al.*, 2006)**; the results shown in **figure** 1 indicated that the cells of the lactic acid bacterium are adherent to the selected epithelial tissue.

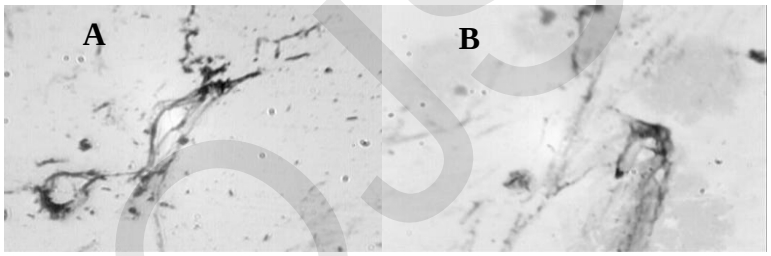

Fig. 1 Adhesion of *Lb. curvatus* to epithelial cells (A: positive result, B: negative control).

As described **by Lin *et al.* 2007**, *Lb. fermentum* cells; isolated from chicken crop highly attach the epithelial cells, which make them; in addition to the other properties; a good candidate to be selected as a probiotic.

The mechanism of adhesion of these cells is not completely understood, although it was suggested that some lactic acid bacteria like *Lb. plantarum* and *Lb. rhamnosus* are capable of colonizing the lower digestive tract for a long period resulting in the inhibition of pathogenic bacteria by competing to specific receptors required for adherence **(Robin and Rouchy, 2001; Roy et *al.*, 2006).**

Survival test

Microencapsulation of *Lb. curvatus* was conducted by using 1% sodium alginate; the microcapsules prepared by extrusion technique were spherical and uniform in size (3 mm), each bead contains approximately 9.10^{12}UFC. The survival of free and microencapsulated cells in acidic pH of the stomach was evaluated by a 2 h *in vitro* SGJ survival assay (Figure 2).

The viability of the free cells decreased intensively after the first hour of incubation in acidic conditions, it reached approximately 28 %; moreover, only 2 % of the cells remained viable after 2 hours, however, the cells in a microencapsulated state are slightly more resistant since after one hour, 40% of cells survived and after 2 hours, the viability attained 11%.

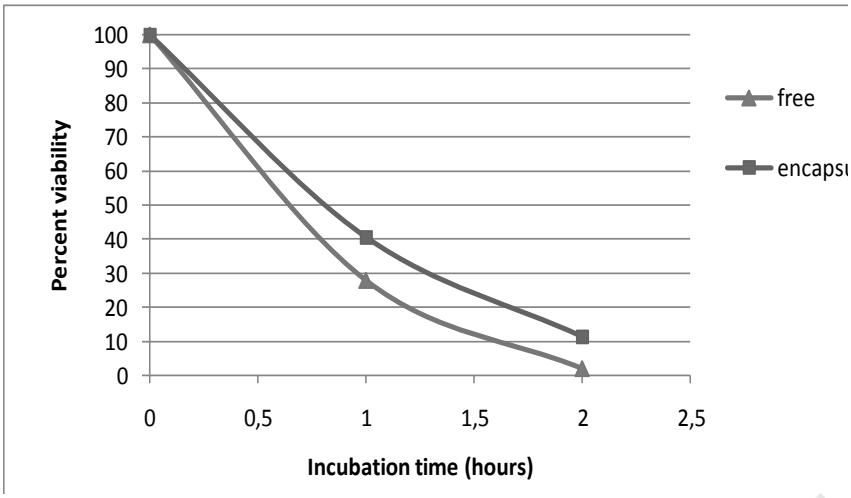

Fig. 2 Effect of acidic pH (2) on the survival of free and microencapsulated *Lb. curvatus* cells.

The chicken GIT contained a complex microbial community distributed unequally in its different compartments; the normal flora consists mainly of lactic acid bacteria particularly lactobacilli, enterobacteria and other groups are also found **(Gabriel et *al*. 2005; Lin et *al*. 2007).** Gizzard's microbial community is less abundant due to the high acidity; the hostile conditions of the duodenum reduced as well the incidence of microbes, although some lactobacilli, enterococci and coliforms were isolated (**Fuller, 1984).** Probiotics must then survive the transit through the gizzard to exert beneficial effects; therefore, resistance to a low pH (2) for at least 2 hours is required for a probiotic cell to be delivered effectively to the intestine. Several microencapsulation materials were used to protect probiotic cells including sodium alginate, carraghenane, pectin, whey proteins… (**Voo *et al.* 2011**; **Kailasapathy, 2002**). However; alginate matrix system is the most widely used and investigated biopolymer for cell bioencapsulation. It is biocompatible, and it can gel at mild condition with the presence of calcium cations. In a related study; *Lb. acidophilus* and *Lb. rhamnosus* were significantly protected from stomach conditions (**Ding and Shah 2009**), similarly; microencapsulated *Lb. acidophilus* and *Bifidobacterium sp*. showed 16 % and 16.7 % increase in viability after incubation at pH 2 for 2 hours when compared to free cells **(Vidhyalakshmien, 2009)**. **Lee and Heo (2000**) showed that *Bifidobacterium longum* encapsulated in calcium alginate containing 2.0, 3.0, and 4.0% sodium alginate tolerated significantly incubation in a simulated gastric juice (pH 1.5) better than free cells. The death rate of the cells in the beads decreased proportionally with an increase in both the alginate gel concentration and bead size.

Figure 2 shows that *Lactobacillus curvatus* was most likely to survive the passage through the stomach, furthermore microencapsulation within alginate capsules resulted in an approximate 5.4-fold increase in the survival of cells in pH 2 after 2 hours of incubation.

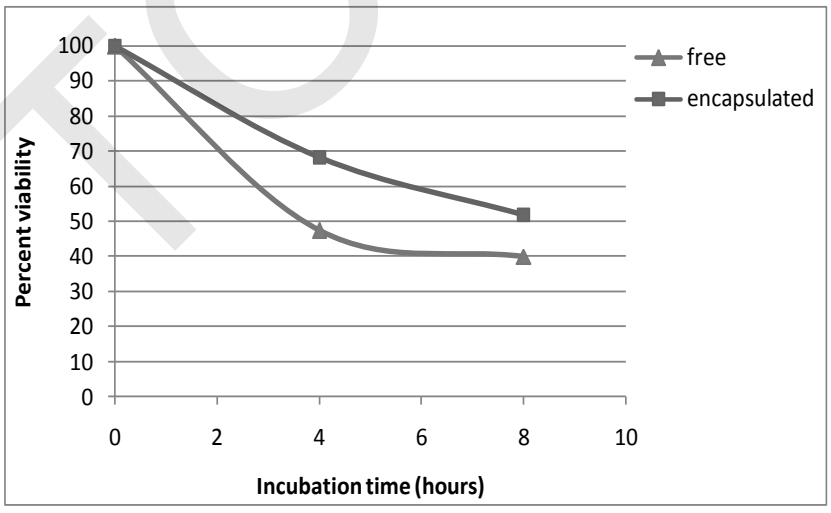

Fig. 3 Effect of bile salts (0.3%) on the survival of free and microencapsulated *Lb. curvatus* cells.

Viability of probiotic cells in the presence of bile salts was conducted as described by incubating the cells in MRS medium supplemented with 0.3 % bile salts. Results showed in **figure 3** indicated that the viable count of free cells decreased by approximately 53 % after 4 hours of incubation, it decreased to reach 40 % after 8 hours with an average cell concentration of about (152.10^{12} UFC/mL), moreover; the gel-enclosed cells resisted more, more than

68 % of the cells were found to survive bile treatment for 4 hours; and more than 50 % tolerated the treatment after 8 hours.

Bile salts are the second barrier that probiotic cells should bypass to attain their site of action. In general, the required concentration of bile salts considered necessary to screen for resistant strains for human and animal use is 0.3% (**Pacheko et al. 2010, Lin et al. 2007**). Several studies reported the improvement of cell viability when exposed to bile salts by microencapsulation, **Ding and Shah** (2009) found that *Lb. plantarum* and *Bifidobacterium lactis type Bi-07* were slightly sensitive to bile toxicity (39 % of the cells survived the treatment); however, microencapsulation in 3% alginate enhanced the viability by 2-fold. In a different study; *L. bulgaricus* KFRI 673, an acid-sensitive strain was found to survive SGI exposure when protected in alginate microparticles coated with a high molecular weight chitosan (**Lee et al. 2004).** Conversely, *Bifidobacterium infantis, Lactobacillus casei* and *L. acidophilus* encapsulated in symbiotic beads composed of Hi-Maize starch (a prebiotic) and sodium alginate did not demonstrate a significant increase in survival when subjected to *in vitro* high acid and bile salt conditions **(Sultana et al .2000**).

In this study; *Lb. curvatus* cells were found to be relatively resistant to bile toxicity, in addition; the use of alginate gel improved their tolerance, as approximately 1.3 –fold increase in viability was observed after 8 hours of treatment.

In conclusion, the isolated *Lb. curvatus* was found to present good probiotic properties, it displayed antimicrobial activity against some selected pathogenic bacteria, and a substantial adhesion capacity. Furthermore, the viability in GIT like conditions was increased by the alginate microencapsulation, which provides additional evidence that alginate-based microparticles are suitable for food ingredient delivery.

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