CHAPTER 6

Encapsulation of Folate Producing *Lactococcus lactis* Strains for Enhanced Gastrointestinal Survival

6.1. Introduction

The growing awareness among consumers for what they eat lead to the development of probiotic functional foods enriched with health promoting ingredients including B group vitamins, low calorie sugars and exopolysaccharides (Divya et al., 2012). An adequate level of viable bacteria in the food product at an appropriate daily dose ($\geq 10^7$ CFU/g) is essential to exert its beneficial effect on host (Charalampopoulos et al., 2002). When probiotics are administered orally they must be protected from various factors like the acidic pH of the stomach, digestive enzymes, and bile salts which limit the survival of probiotics and prevent them from exerting their positive effects.

Microencapsulation provides a physical barrier to the probiotics against the harsh environmental conditions and gastrointestinal passage and thus increases the number of viable cells reaching the small intestine (Kailasapathy, 2002). It is the entrapment of probiotic bacteria into food grade matrices like alginate, starch and milk proteins. Encapsulation also aids in the passage of metabolites and controlled release of the probiotics. Calcium alginate is the most widely used encapsulation matrix for probiotics due to the relatively simple and cheap procedure and non toxic nature. But, the alginate beads are susceptible to acidic environment which cause crackling and loss of mechanical strength of the beads (Eikmeier & Rehm, 1987; Mortazavian et al., 2007). Also, alginate is unstable in presence of chelating agents like lactate which have affinity towards calcium and this could result in the disintegration of beads during lactic acid fermentation resulting in the release of the probiotics before reaching the lower intestinal tract or colon (Smidsrød & Skja, 1990). However, blending of alginate with certain encapsulating additives such as prebiotics, milk proteins and starch can increase its mechanical and chemical stability.

The aim of the present study was to increase the gastrointestinal survival of the two folate producing *Lactococcus lactis* strains by extrusion method employing different encapsulation matrices.

6.2. Materials and Methods

6.2.1. Bacterial Strains and Culture Conditions

L. lactis CM22 and *L. lactis* CM28 were used for the encapsulation studies. Culture propagation and maintenance were described in 2.2.1.f.

6.2.2. Encapsulation of the LAB strains

The encapsulation was carried out by extrusion technique using two different encapsulation matrices.

a. ***Co-encapsulation***

The co-encapsulation was done by a modified method of Dianawati and Shah (2011). Overnight culture (25 mL, 18 h) was pelleted and washed with saline. The pellet was resuspended in 25 mL of alginate (2.5% w/v) - mannitol (2.5% w/v) solution and kept at room temperature for 45 min. This was then added drop by drop into 0.1M $CaCl_2$ solution using a pipette. After 1 h the beads were transferred to saline to enforce the gel structure. Hardened gel beads were transferred to sterile distilled water and gently mixed to wash the residual $CaCl_2$.The prepared beads containing individual culture were then lyophilized and stored in fridge.

b. Hybrid Entrapment

Overnight culture (30 mL, 18 h) was pelleted and washed with saline. To the pellet 20 mL encapsulation medium (10% (w/v) skim milk, 5% (v/v) glycerol, 0.1% (w/v) $CaCO_3$) was added. This was followed by the addition of 20 mL of sodium alginate (2.5% w/v) solution and kept at room temperature for 45 min (Reyed, 2007). Beads were prepared by extrusion method as described above.

c. SEM analysis

The free cells/beads were fixed with 4% (v/v) glutaraldehyde and adequately dried using increasing concentration of ethanol (20, 50, 70 and 90% (v/v)). The samples were then mounted on a brass stud and spur coated with gold and viewed under scanning electron microscope (Zeiss EVO 17SE, Germany).

6.2.3. ***Survival of Encapsulated LAB in Simulated Gastric Juice (SGJ)***

SGJ was prepared by dissolving NaCl (9 g/L) and pepsin (3 g/L) and the pH was adjusted to 2.5 and 3.0 using 0.1 N HCl (Chavarri et al., 2010). Encapsulated bacteria (1 g) were added to 10 mL SGJ (pH 2.5 and 3.0). Samples were collected at 0, 1 and 2 h, homogenized in phosphate buffer (pH 7.0), serially diluted with saline and the appropriate dilutions were plated onto M17 plates supplemented with 0.5% (w/v) glucose.

6.2.4. Survival of Encapsulated LAB in Simulated Intestinal Juice (SIJ)

SIJ was prepared by the method describe by Ivanovska et al. (2012) with certain modifications. 1.5% (w/v) bile salt (bile salts mixture, Himedia) was filter sterilized and added to pre-sterilized KH_2PO_4 (0.05 M) solution and the pH was adjusted to 7. Encapsulated bacteria (1.0 g) were transferred to SGJ (10 mL, pH 3.0) and incubated at 37 ºC for 1 h. The beads were then transferred to SIJ (10 mL, pH 7.0) and incubated at 37 ºC for 2 h. Samples were collected at 1 h interval for 3 h. Beads were homogenized in phosphate buffer (pH 7.0) and then serially diluted and plated onto M17 agar with 0.5% (w/v) glucose. Plates were incubated at 37 ºC. Results were expressed as log CFU/mL. Survival of free cells in SGJ and SIJ was also determined as control.

All the experiments were carried out in triplicates and the results were expressed as mean ± SD.

6.3. Results and Discussion

6.3.1. Encapsulation of Lactococcus strains

The two probiotic LAB strains were encapsulated by co-encapsulation and hybrid entrapment techniques. **Fig. 6.1A** shows the freshly prepared probiotic beads of *L. lactis* CM22 by co-encapsulation and the lyophilized probiotic beads are presented in **Fig. 6.1B**. Freeze drying is preferred for storing the encapsulated probiotics due to the fact that low temperature results in low thermal stress. But, the formation of extracellular ice crystals and subsequent increase in the extracellular osmolality are the detrimental effects of freeze drying. Addition of cryoprotectants such as milk solids, glycerol, sugar alcohols etc can prevent the damage to the cells (Morgan et al., 2006). **Fig 6.1C** represents the SEM image of free *L. lactis* CM22 cells while **Fig 6.1D** is the SEM image of encapsulated *L. lactis* CM22. Similarly, the encapsulation of *L. lactis* CM28 was confirmed by scanning electron microscopy.

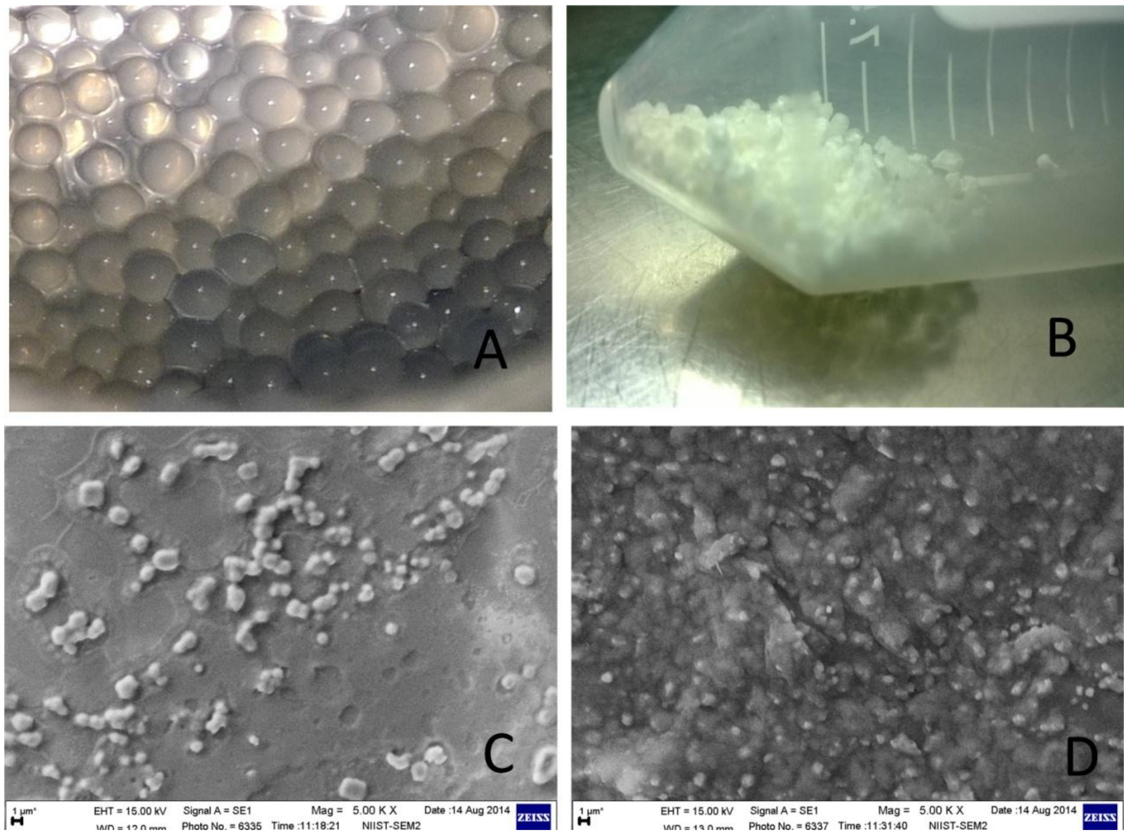

Fig. 6.1. Encapsulation of *L. lactis* CM22

(A) Freshly prepared probiotic beads by co-encapsulation method; (B) Lyophilized probiotic beads; (C) SEM image of free *L. lactis* CM22; (D) SEM image of encapsulated *L. lactis* CM22

6.3.2. Survival of Free and Encapsulated LAB in Simulated Gastric Conditions

In order to find out the survival rate of LAB strains on oral administration, the free as well as encapsulated bacteria were tested *in vitro* for stability in SGJ (pH 2.5 and 3) and SIJ (pH 7). **Fig. 6.2A** and **6.2B** show the survival of free and encapsulated *L. lactis* CM22 in SGJ at pH 2.5 and 3.0 respectively.

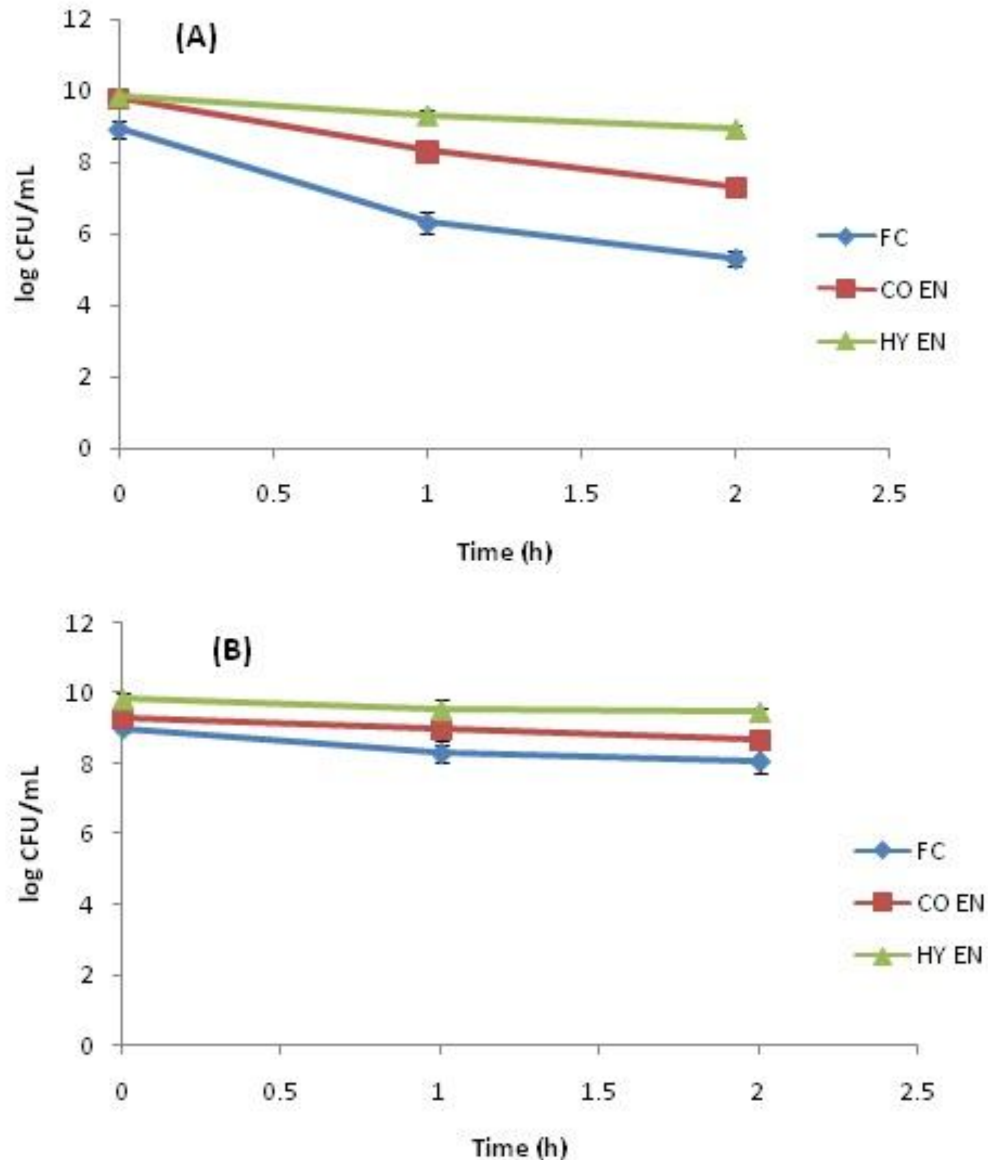

Fig 6.2. Survival of free and encapsulated *L. lactis* CM22 in SGJ

(A) Survival at pH 2.5; (B) Survival at pH 3.0

FC – Free cells, CO EN – Co-encapsulation, HY EN – Hybrid entrapment

The survival of encapsulated *L. lactis* CM22 was higher than that of the free cells. There was less than a log unit reduction (9.85 ± 0.07 log CFU/mL to 8.92 ± 0.11 log CFU/mL) in the viability of cells encapsulated by hybrid entrapment method where as the viability of the free cells was reduced to more than three log units (8.93 ± 0.24 log CFU/mL to 5.3 ± 0.22 log CFU/mL) in SGJ of pH 2.5. At pH 3.0 also the encapsulated cells survived better than the free cells.

Similarly, encapsulation improved the survival of *L. lactis* CM28 at pH 2.5. There was more than 3 log unit reduction for the free cells at pH 2.5 while only a two log unit reduction was observed in encapsulated cells prepared by hybrid entrapment method. The co-encapsulated cells also exhibited better survival than free cells at pH 2.5 (**Fig. 6.3**).

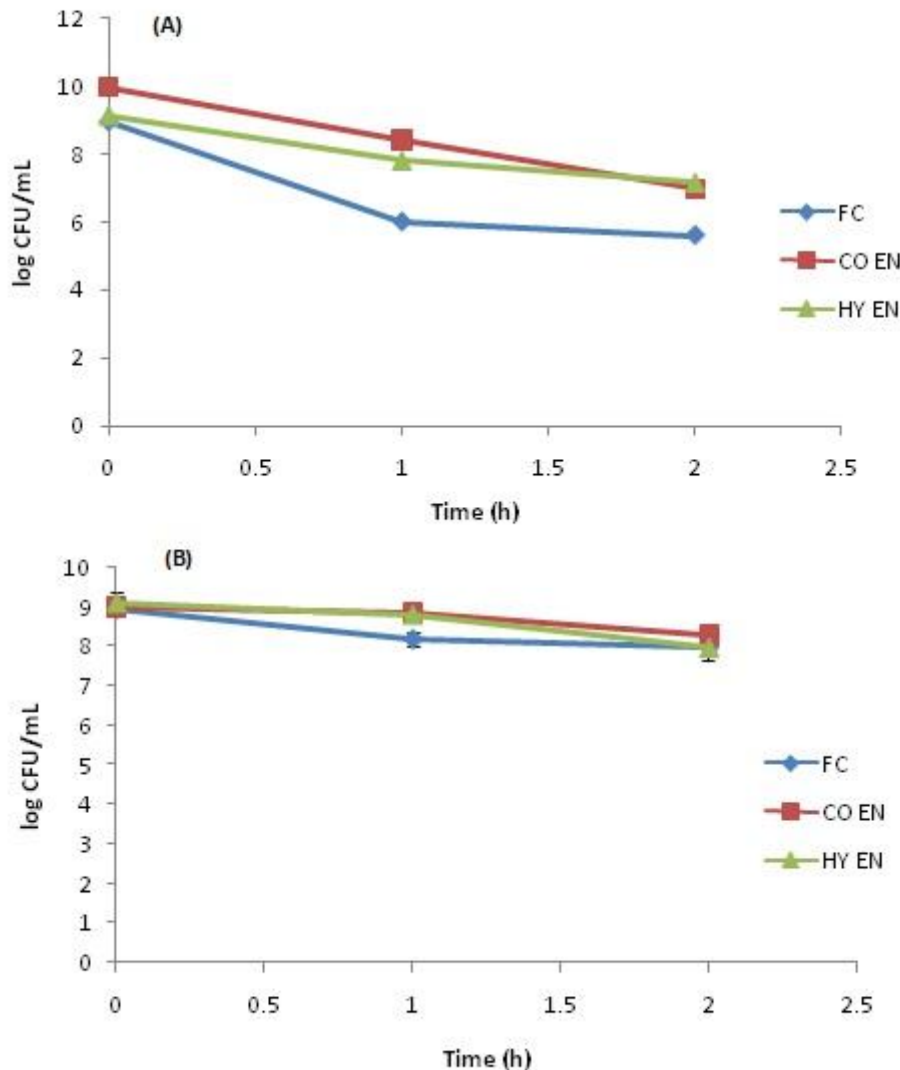

Fig 6.3. Survival of free and encapsulated *L. lactis* CM28 in SGJ

(A) Survival at pH 2.5; (B) Survival at pH 3.0

FC – Free cells, CO EN – Co-encapsulation, HY EN – Hybrid entrapment

As it was difficult to maintain constant CFU/mL at 0 h for all the three conditions the corresponding percentage survival of the isolates in SGJ was calculated. The results are shown in **Fig 6.4**.

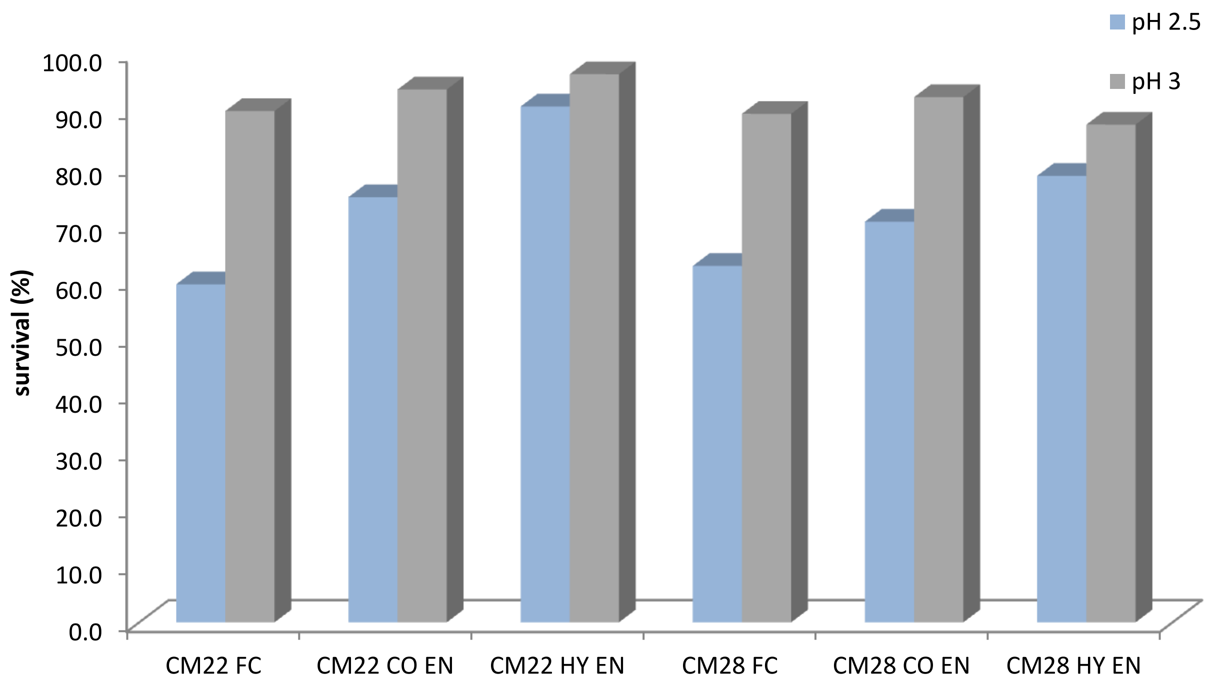

Fig 6.4. Percentage survival of the free and encapsulated *L. lactis* strains in simulated gastric conditions after incubation period (2 h)

CM22 FC – Free cells of *L. lactis* CM22, CM22 CO EN – Co-encapsulated *L. lactis* CM22, CM22 HY EN – Hybrid entrapped *L. lactis* CM22; CM28 FC – Free cells of *L. lactis* CM28, CM28 CO EN – Co-encapsulated *L. lactis* CM28, CM28 HY EN – Hybrid entrapped *L. lactis* CM28

The percentage survival of free cells of *L. lactis* CM22 was only 59.4% after 2 h incubation in SGJ of pH 2.5 but 74.6 and 90.6% cells retained viability in the case of co-encapsulation and hybrid entrapment respectively. A similar trend was followed by *L. lactis* CM28. Only 62.8% free cells survived at pH 2.5 whereas 70.4% co-encapsulated cells and 78.4% hybrid entrapped cells remained viable at the end of incubation period. For both the strains, the percentage survival of free cells at pH 3.0 was comparable to that of the encapsulated cells while considerable increase in survival was observed for encapsulated cells at pH 2.5.

The acid tolerance of LAB depends on the pH profile of H^+-ATPase and on the composition of the cytoplasmic membrane which in turn depends on the type of bacterium, growth medium and the incubation conditions (Mokarram et al., 2009). Kim et al. (2008) reported that non encapsulated cells of *Lactobacillus acidophilus* were

completely destroyed when exposed to artificial gastric juice of pH 1.2 and 1.5, while the treatment declined the viable count of encapsulated samples only by 3 log units. Increased acid and bile tolerance of spray-dried *Lb. acidophilus* La-05 and *Bifidobacterium lactis* Bb-12 was demonstrated with the use of a complex formulation consisting of cellulose, maltodextrin, prebiotic, glycerol and reconstituted milk (Favaro-Trindade & Grosso, 2002). Higher survival of *Lb. rhamnosus* in double layer coated alginate microspheres at low pH environment was reported by Mokarram et al. (2009). They have stated that the reduced pore size of the membrane in coated beads might have reduced the diffusion of gastric juice into the beads thereby protecting the cells.

6.3.3. Survival of Free and Encapsulated LAB in Simulated Intestinal Conditions

The viable count of free and encapsulated bacteria was determined after sequential incubation in SGJ and SIJ. The results obtained revealed that co-encapsulation and hybrid entrapment method effectively increased the survival of *L. lactis* CM22 and *L. lactis* CM28 (**Fig. 6.5**).

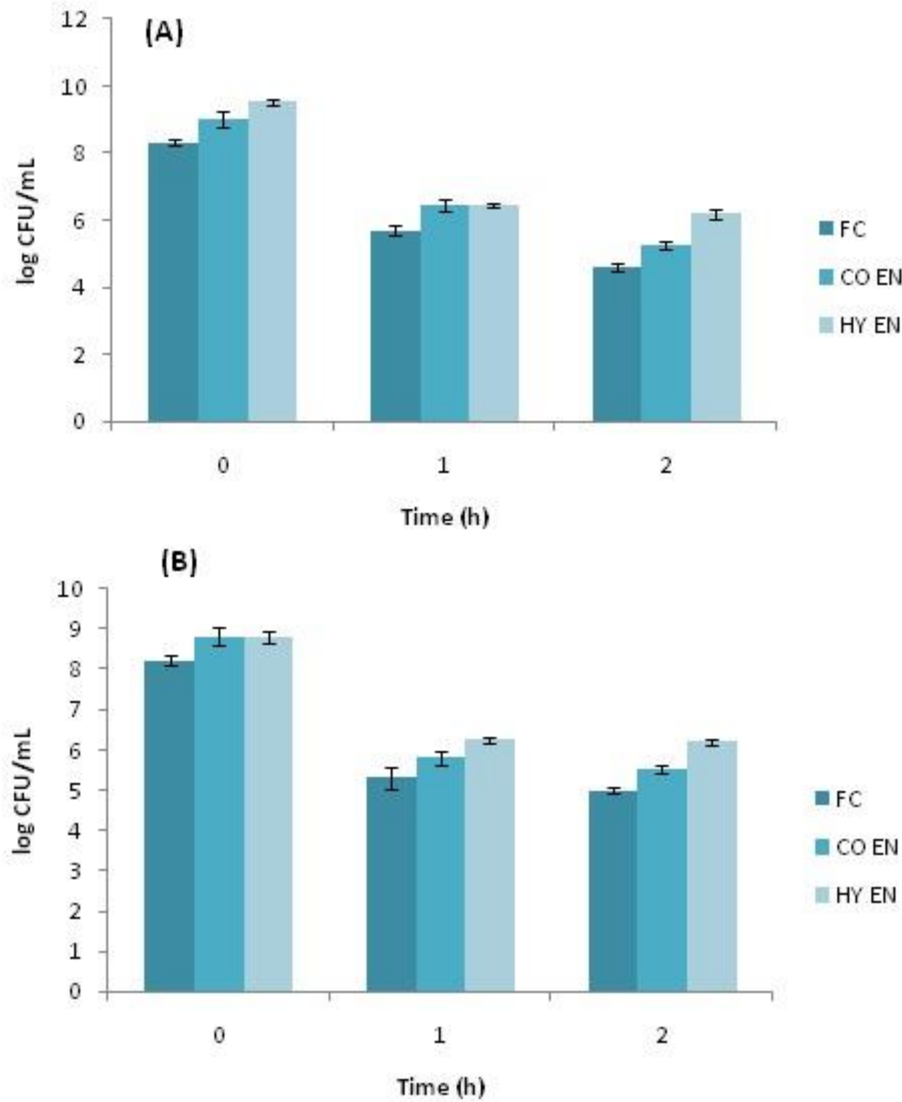

Fig. 6.5. Survival of free and encapsulated *L. lactis* strains in SIJ

(A) Survival of free and encapsulated *L. lactis* CM22; (B) Survival of free and encapsulated *L. lactis* CM28

FC – Free cells, CO EN – Co-encapsulation, HY EN – Hybrid entrapment

Based on these results the corresponding percentage survival of the free and encapsulated probiotics was calculated and the results are presented in **Fig. 6.6**.

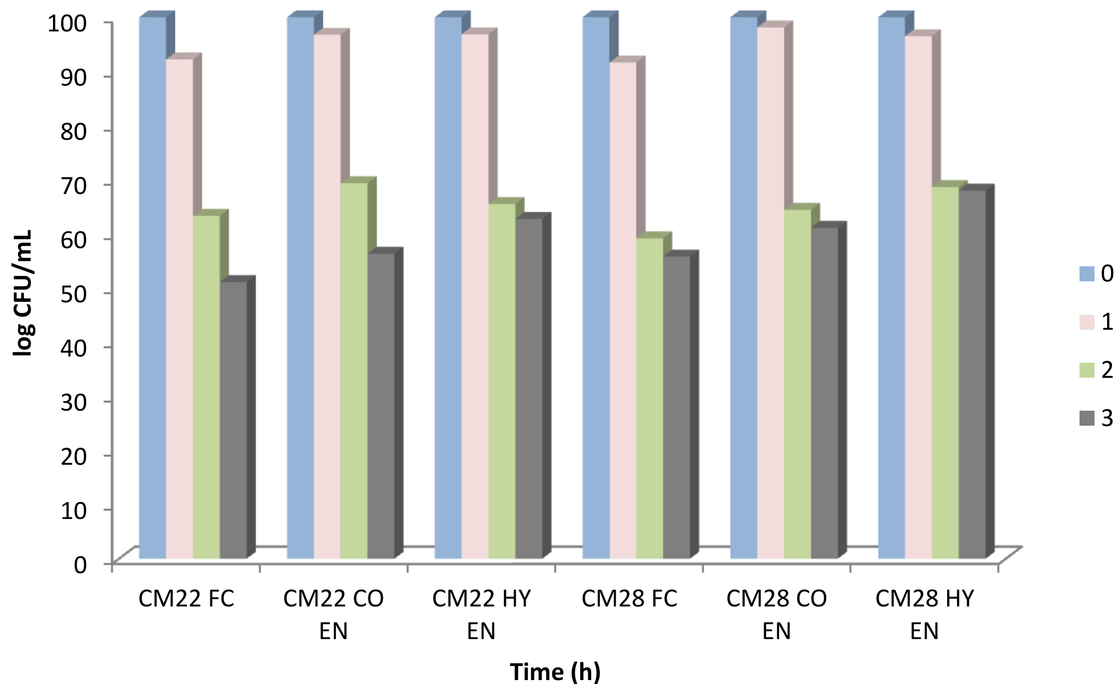

Fig 6.6. Percentage survival of the free and encapsulated LAB strains in simulated gastrointestinal conditions

CM22 FC – Free cells of *L. lactis* CM22, CM22 CO EN – Co-encapsulated *L. lactis* CM22, CM22 HY EN – Hybrid entrapped *L. lactis* CM22; CM28 FC – Free cells of *L. lactis* CM28, CM28 CO EN – Co-encapsulated *L. lactis* CM28, CM28 HY EN – Hybrid entrapped *L. lactis* CM28

After incubation in simulated gastrointestinal conditions, 51.1% free *L. lactis* CM22 cells were viable whereas for co-encapsulated and hybrid entrapped cells the survival was 56.34% and 62.74% cells respectively. For *L. lactis* CM28, hybrid entrapment retained 68% viability while co-encapsulation resulted in 61.1% survival and for free cells the survival was 55.8%. Enhanced survival of probiotic bacteria in gastrointestinal conditions when encapsulated with alginate-chitosan or poly-L-lysine was reported by Krasaekoopt et al. (2004). In another study by Guerin et al. (2003) considerable improvement in the survival of *Bifidobacterium* cells in SGJ and SIJ was observed when a combination of alginate, pectin and whey protein was used as the encapsulation matrix.

The survival of encapsulated probiotics is dependent on a number of factors such as concentration of the polymer used, capsule size, composition. Dianawati et al. (2012) reported that sugar alcohols like sorbitol and mannitol interact with the polar site of phospholipid bilayer hence provide protection to the probiotics. However, higher

concentration of mannitol could adversely affect the probiotic survival due to the formation of mannitol crystals (Berner & Viernstein, 2006). When milk proteins were used as encapsulating medium along with alginate the highly dense gel formed offered a favourable milieu for the probiotics. The pH inside the gel matrix will be higher than outside thus protecting the probiotics (Heidebach et al., 2009). Several factors come into play *in vivo* conditions like inconsistency in the level of bile acids, presence of food that could affect the survival of bacteria. The microenvironments formed by certain food matrix may even bind bile acids and prevent them from exerting toxicity (Mokarram et al., 2009). Nevertheless, the probiotic bacteria must survive the passage through the acidic gastric juice before reaching the intestine. As it is difficult to simulate the exact *in vivo* conditions in a laboratory setting further studies need to be carried out to prove the efficiency of encapsulation.

6.4. Conclusion

The study demonstrated the efficacy of two encapsulation techniques in improving the survival of *L. lactis* strains in simulated gastrointestinal condition. Among the two encapsulation methods employed, hybrid entrapment method offered better protection to the cells. This strategy could be effectively used for delivering the viable probiotics to the intestine without any significant adverse effect on their functionalities including folate production. However, *in vivo* studies are necessary to fully validate the *in vitro* studies to ascertain the functionality of the encapsulated bacteria.