CHAPTER 7

Folate Fortification with Indigenous *Lactococcus lactis* Isolates

7.1. Introduction

Folate is gaining wide scientific and public health interest in recent years owing to the vital roles it plays in metabolism. Sufficient intake of the vitamin through diet is necessary to prevent its deficiency especially in pregnant women and women of child bearing age. Despite its benefits, the main concern with mandatory folic acid fortification is that it is untargeted thereby exposing a proportion of the general population to an undesirably high levels of the nutrient potentially risking them to the side effects of synthetic folic acid (Hannon-Fletcher et al., 2004). The increased consumption of foods that are naturally rich in folate could offer a possibility to eliminate the health concerns associated with synthetic folic acid (Koebnick et al., 2001). The bioavailability of folate from various foods depends on various factors including the presence of certain food components that can inhibit folate deconjugation and its transport and also the content of mono- and poly glutamyl folates (Hannon-Fletcher et al., 2004). The limited bioavailability and losses of folate while food processing, storage and cooking makes it more difficult to reach recommended targets for folate intake (Jägerstad et al., 2005). Fermentative folate fortification of foods by probiotics is a better option to enhance the folate intake among various populations.

The selection of food matrix for fermentative folate fortification is another important criterion (do Espirito Santo et al., 2011). Milk, although a complete food is only a moderate source of folate but serves as an ideal matrix for the growth and survival of probiotics (Divya & Nampoothiri, 2014). In addition to milk other dairy based matrices like ice cream and milk powder may also offer as good vehicles for probiotic delivery (Araújo et al., 2012). Also, in recent years consumer demand for non-dairy based probiotics has increased. Fruit juices may serve as an alternative matrix for probiotics as they are rich in nutrients, sugars and antioxidants. Even though the low pH of the fruit juice could be detrimental to the viability of probiotics but proteins and dietary fibres could protect the cells from acid stress (Perricone et al., 2015). Incorporation of LAB into fruit juices with low pH may enhance the resistance of bacteria to further acid stress, such as those found in the gastrointestinal tract (Ranadheera et al., 2010). The fermentability of

the substrate by the starter cultures as well as their viability are important and should be complemented by an assessment of the functionality while developing a new probiotic functional foods (Vinderola et al., 2011).

The chapter deals with the folate fortification of apple juice using the co-culture of *Lactococcus lactis* CM22 and CM28. Subsequent studies on folate stability in the fermented beverage on storage were also carried out. Investigations were also undertaken to check the feasibility of using encapsulated *L. lactis* strains prepared by hybrid entrapment method (described in 6.2.2) for fermentative folate fortification of skim milk and ice cream.

7.2. Materials and Methods

7.2.1. Bacterial Strains and Culture Conditions

The LAB strains *L. lactis* CM22 and CM28 (free or encapsulated) were used for the fortification study. *Lb. casei* NCIM 2464 was used for quantification of folate by microbiological assay. The sub culturing and maintenance were discussed in 2.2.1.f.

7.2.2. Folate Fortification of Apple Juice

a. Preparation of Apple Juice

Apples (red delicious variety) purchased from a local market were washed, sliced with skin, removed the seeds and then blended in a juicer. The juice was strained through a cheese cloth and later clarified by centrifugation (8000 x g, 10 min, 4 ºC). Pasteurization of the juice was done by heating at 63 ºC for 30 min. Two different formulations of apple juice AJ1 and AJ2 were made. AJ1 is pasteurized apple juice without any supplements while AJ2 was supplemented with 0.5% (w/v) glucose and 0.2% (w/v) sodium ascorbate.

b. Inoculum Preparation

L. lactis CM22 and *L. lactis* CM28 were grown individually in FAA medium supplemented with nutrient solution. The composition was as described in 3.2.3. These cultures (18 h) were used as inoculum for folate fortification studies.

c. Folate Fortification

Folate fortification studies were carried out in AJ1 and AJ2 with different inoculum ratios (CM22: CM28 in 1:1, 1:2 and 2:1) to get a total inoculum size of 2% (v/v) to get adequate growth as per the requirement of probiotics. Fortification studies were also

performed with individual strains as well with the same inoculum concentration of 2% (v/v). All fermentation steps were performed at 37 ºC for 8 h under static conditions. Analyses of intracellular and extracellular folate in apple juices were performed by microbiological assay by the protocol described before (2.2.2.a). The combinations that gave highest folate production were used for further studies on storage stability.

d. Storage Stability of the Fermented Apple Juice

After fermentation, the selected fermented apple juices were stored under refrigerated conditions at 5 °C for 21 days. Samples were collected at intervals and were analysed for folate, pH, titratable acidity (TA) and viable count of bacteria. Estimation of TA was done as described in 2.2.2.c. The viable count of bacteria was determined as per the method described in 4.2.6. The enumerated CFU were expressed in log CFU/mL.

7.2.3. Folate Fortification of Skim milk and Ice Cream using Encapsulated Bacteria

For the fortification studies, skim milk medium optimized for folate production by *L. lactis* CM22 (SM2 medium) and *L. lactis* CM28 (SM1 medium) were used. The composition of SM1 and SM2 was mentioned in 5.2.3. Encapsulated cells (1.0 g, freshly prepared) of respective cultures were added to corresponding skim milk medium (10 mL) and incubated at 37 ºC for 10 and 15 h under static condition. Free cells of *L. lactis* CM22 and CM28 (1% (v/v) inoculum i.e., ~10^9 CFU/mL) were also inoculated into SM2 and SM1 medium (10 mL) respectively and incubated at 37 ºC for 8 h.

Probiotic ice cream was made by fermenting a standard ice cream mix by the free and encapsulated *L. lactis* strains. The beads prepared by hybrid entrapment method with improved tolerance to simulated gastrointestinal conditions were selected for the study (6.2.2). The mix was pasteurized at 68.3 °C for 30 min, cooled and freshly prepared beads (1.0 g) of each culture were added to 10 mL ice cream mix and allowed to ferment at 37 ºC for 10 and 15 h without shaking. Free cells (1% v/v) of the cultures were also inoculated into ice cream mix and incubated at 37 ºC for 8 h at static condition. The ice cream is then kept at 5 ºC for 4 h and then frozen. Fermented skim milk and ice cream were centrifuged and the pellet containing the cell biomass and supernatant were collected for the estimation of both intra- and extracellular folate. The entrapped bacteria were released from the beads by homogenization in 0.1M phosphate buffer (pH 7.0). Folate extraction and folate analysis were carried out as per the protocol given in 2.2.2.a. The viable count of bacteria in the fermented samples was also enumerated.

Results were expressed as mean ± standard deviation values which were the average of triplicate experiments.

7.3. Results and Discussion

7.3.1. Fermentative Folate Fortification of Apple Juice

The *L. lactis* strains were capable of fermenting the apple juice and produce folate. The monostrain cultures of *L. lactis* CM22 produced 73.8 ± 5.1 µg/L and CM28 produced 68.4 ± 1.5 µg/L folate. However, folate production by the co-culture was higher compared to the individual strains. Among the different inoculum ratios tried the two strains in 1:1 ratio resulted in highest folate titre in both apple juice formulations (AJ1 and AJ2). After fermentation by the probiotic co-culture the folate content in apple juice (AJ1) increased from 10.28 ± 0.7µg/L to 114.15 ± 1.2 µg/L of which 57.41 ± 2.7µg/L remained as bioavailable extracellular folate. In apple juice supplemented with 0.5% (w/v) glucose and 0.2% (w/v) sodium ascorbate (AJ2), irrespective of glucose, the folate yield was lesser. The maximum folate produced in AJ2 was 106.86 ± 1.4 µg/L and most of the folate was retained intracellularly (60.61 ± 1.7 µg/L). The results are shown in **Fig. 7.1**.

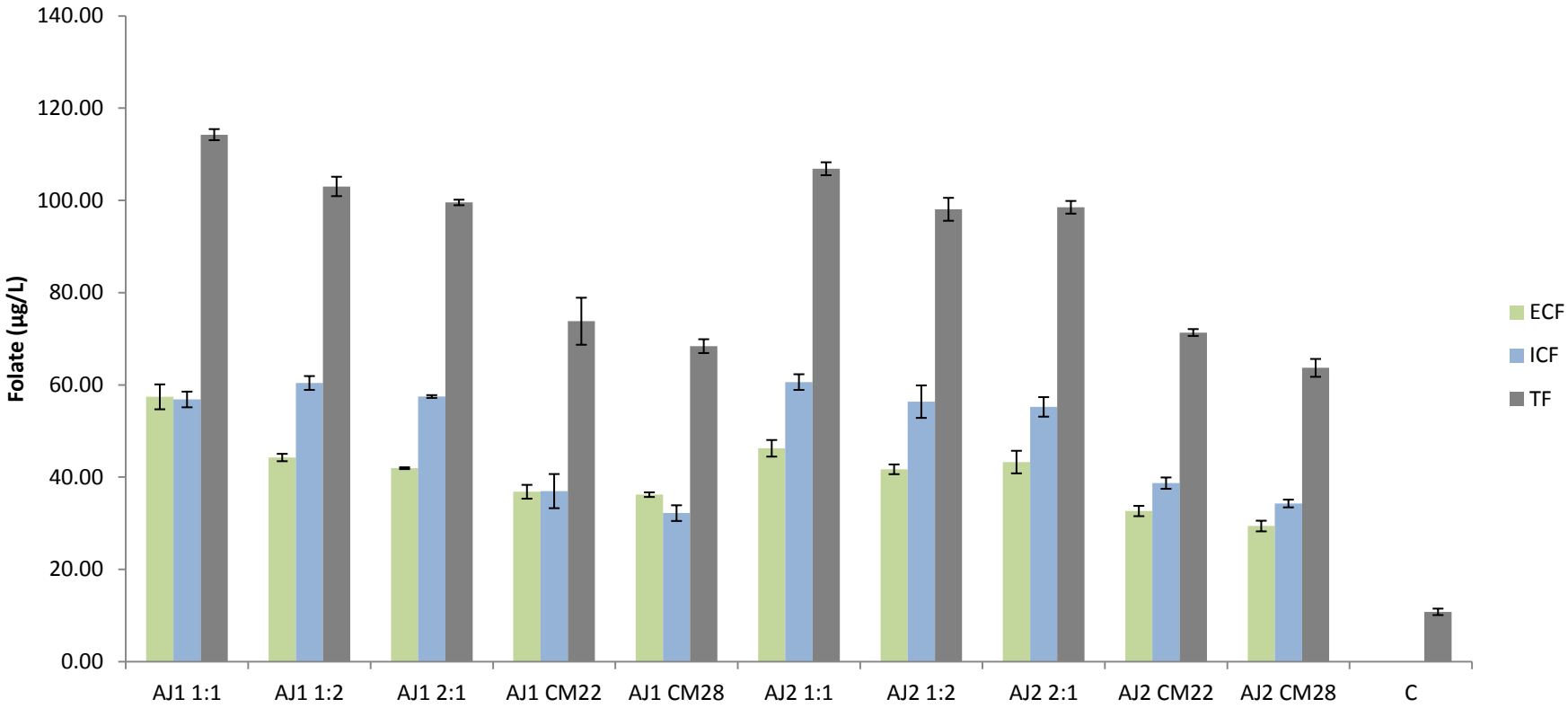

Fig. 7.1 Folate fortification of apple juice by *L. lactis* strains

AJ1 1:1 – AJ1 with 1:1 inoculum ratio, AJ1 1:2 -- AJ1 with 1:2 inoculum ratio, AJ1 2:1 - AJ1 with 2:1 inoculum ratio, AJ1 CM22 – AJ1 with 2% (v/v) CM22, AJ1 CM28 – AJ1 with 2% (v/v) CM28, AJ2 1:1 – AJ2 with 1:1 inoculum ratio, AJ2 1:2 – AJ2 with 1:2 inoculum ratio, AJ2 2:1 – AJ2 with 2:1 inoculum ratio, AJ2 CM22 – AJ2 with 2% (v/v) CM22, AJ2 CM28 – AJ2 with 2% (v/v) CM28, C – control (pasteurized apple juice)

ECF – Extracellular folate, ICF – Intracellular folate, TF – Total folate

Probiotics are traditionally been used to develop yoghurts, fermented milks and other dairy based products. Cholesterol content and lactose intolerance are the two drawbacks associated with such products. This lead to the increased demand for non-dairy based probiotic products by the consumers (Shah, 2001; Yoon et al., 2006). Fruits and vegetables are rich in vitamins, minerals, dietary fibres, antioxidants and are also devoid of any dairy allergens that might prevent their usage by certain segments of population (Luckow & Delahunty, 2004). Apples are good source of vitamin C, dietary fibres and many other essential nutrients but have very low folate content and that was the rationale behind selecting apple juice as the matrix for fermentative fortification.

In a previous study, fermentative fortification of cucumber juice with *L. lactis* ssp *cremoris* increased its folate content from 10 ± 0.2 ng/mL to 60 ± 1.9 ng/mL (Gangadharan & Nampoothiri, 2011). In another study, an increase in folate levels in melon juice fermented by *Lb. reuteri* JCM1112 was demonstrated by Santos et al. (2008).

a. Storage Stability of the Fermented Apple Juice

The fermented apple juices (AJ1 and AJ2) with 1:1 inoculum ratio were selected for storage stability studies due to the higher folate content when compared to other ratios. The stability of folate, changes in viable count of bacteria, TA and pH during the storage period were determined. About 82% of the produced folate in AJ1 and 89.5% in AJ2 were retained in an active form during the storage period. The increased stability of folate in AJ2 could be due to the presence of sodium ascorbate. The results are presented in **Fig 7.2**.

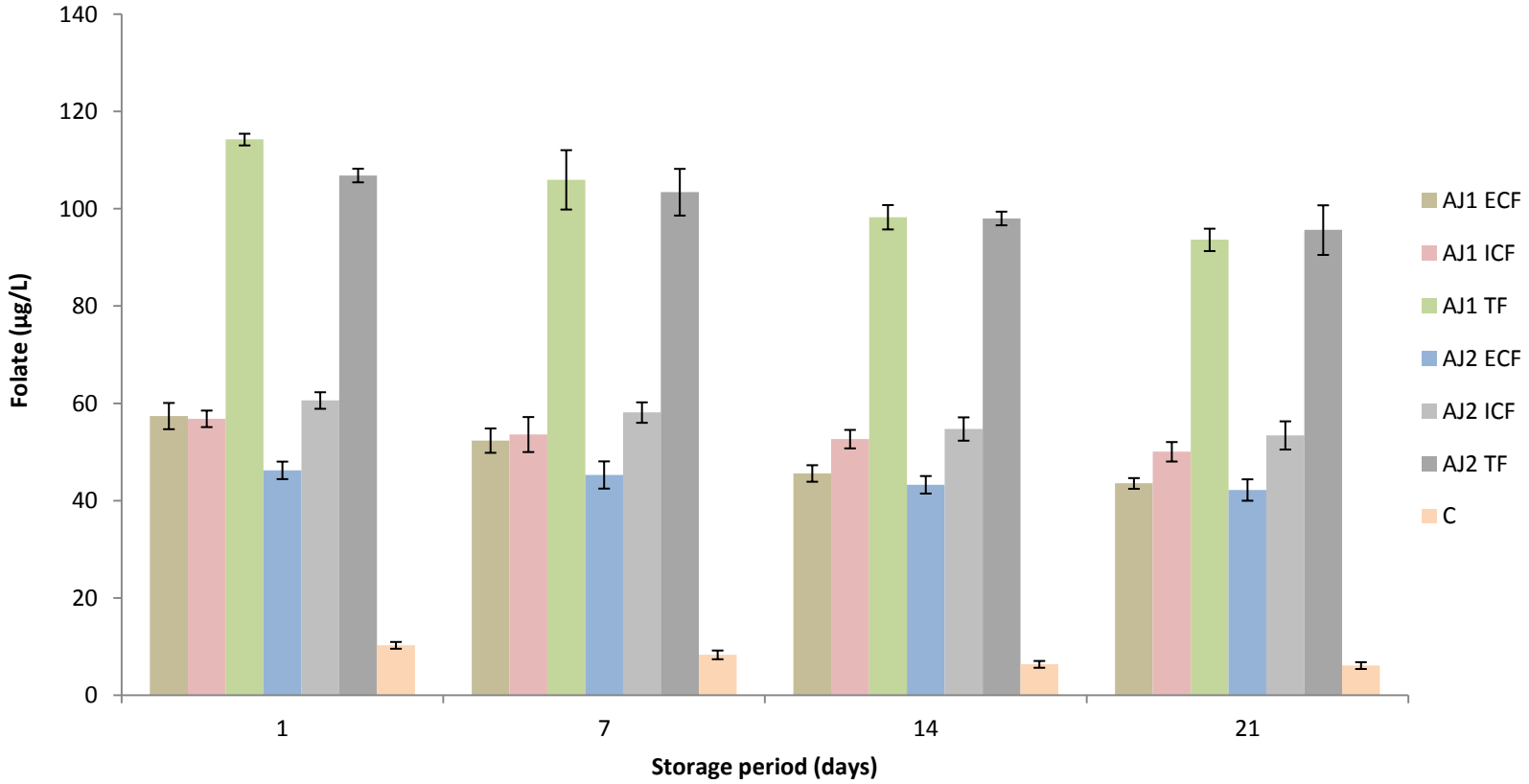

Fig. 7.2. Storage stability of folate in fermented apple juice

AJ1 – pasteurized apple juice without any supplements fermented by the co-culture (1:1), AJ2 - pasteurized apple juice supplemented with 0.2% (w/v) sodium ascorbate and 0.5% (w/v) glucose fermented by the co-culture (1:1), C – control (pasteurized apple juice)

ECF – Extracellular folate, ICF – Intracellular folate, TF – Total folate

The centrifuged juice used for the study was slightly clearer but quite cloudy. Recently, the cloudy apple juice has an increased demand in market due to its sensory and nutritional qualities. The cloudy juice is expected to have a yellowish colour which is the characteristic of the fresh product. Oxidation of phenols to quinones by the enzyme polyphenol oxidase results in browning of juice (Komthong et al., 2007; Ozoglu & Bayindirli, 2002). Enzymatic browning also impairs the nutritional and other sensory qualities thereby decreasing the commercial value of the product. Ascorbic acid is reported to convert *o*-quinones back into *o*-diphenolic compounds and thus prevents the unwanted discolouration of the product (Iyidogan & Bayindirli, 2004). In the present study also the addition of sodium ascorbate retained the colour of the fermented juice as in AJ2. Visible difference in colour was present in fermented AJ2 when compared to the control and fermented AJ1. AJ1 and C showed enzymatic browning whereas AJ2 retained the yellowish colour (**Fig 7.3**).

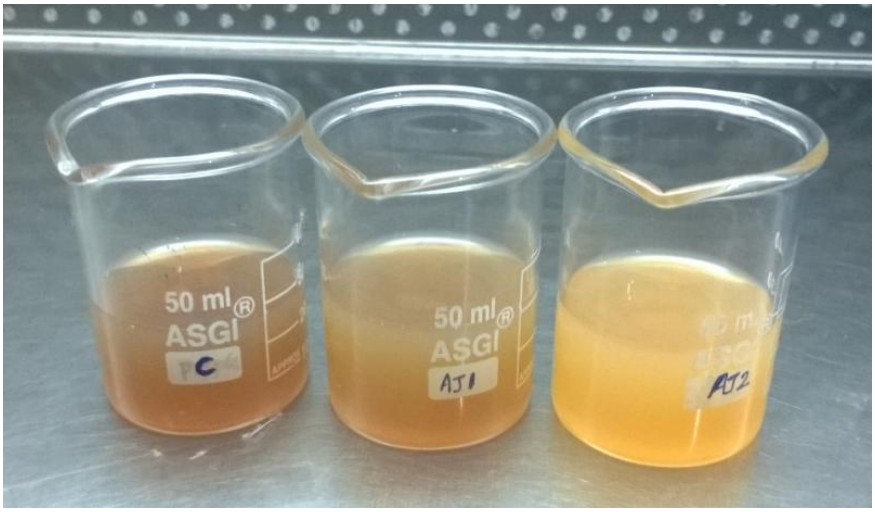

Fig 7.3. Folate fortified apple juice

AJ1 – pasteurized apple juice without any supplements fermented by the co-culture (1:1), AJ2 - pasteurized apple juice supplemented with 0.2% (w/v) sodium ascorbate and 0.5% (w/v) glucose fermented by the co-culture (1:1), C – control (pasteurized apple juice)

Similar to the results with fermented skim milk, in fermented apple juice also there was a decrease in pH and an increase in TA during cold storage (**Fig 7.4 and 7.5**).

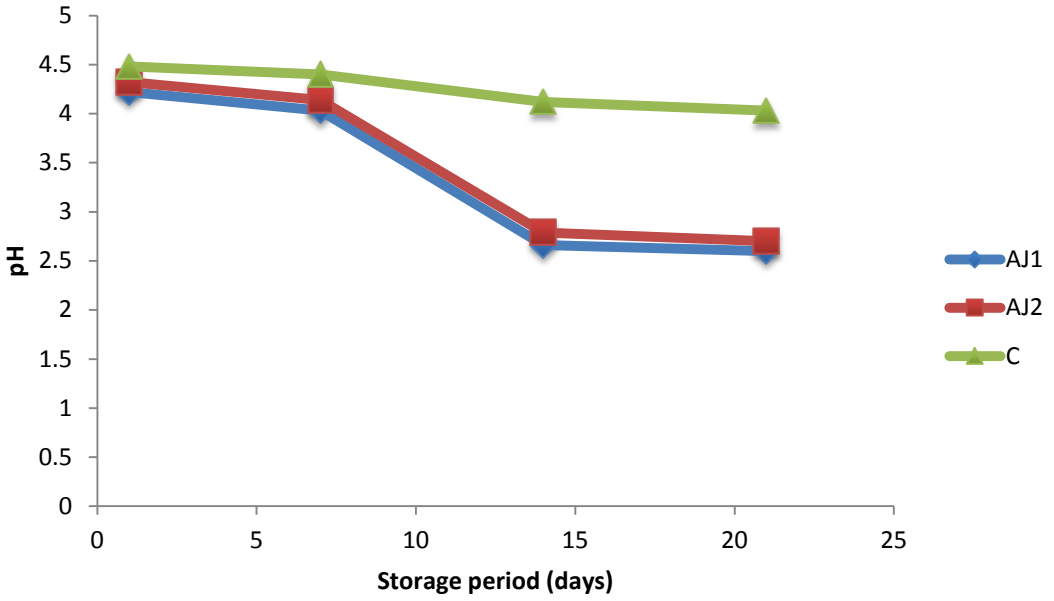

Fig. 7.4. Changes in pH of fermented apple juice during cold storage

AJ1 – pasteurized apple juice without any supplements fermented by the co-culture (1:1), AJ2 - pasteurized apple juice supplemented with 0.2% (w/v) sodium ascorbate and 0.5% (w/v) glucose fermented by the co-culture (1:1), C – control (pasteurized apple juice)

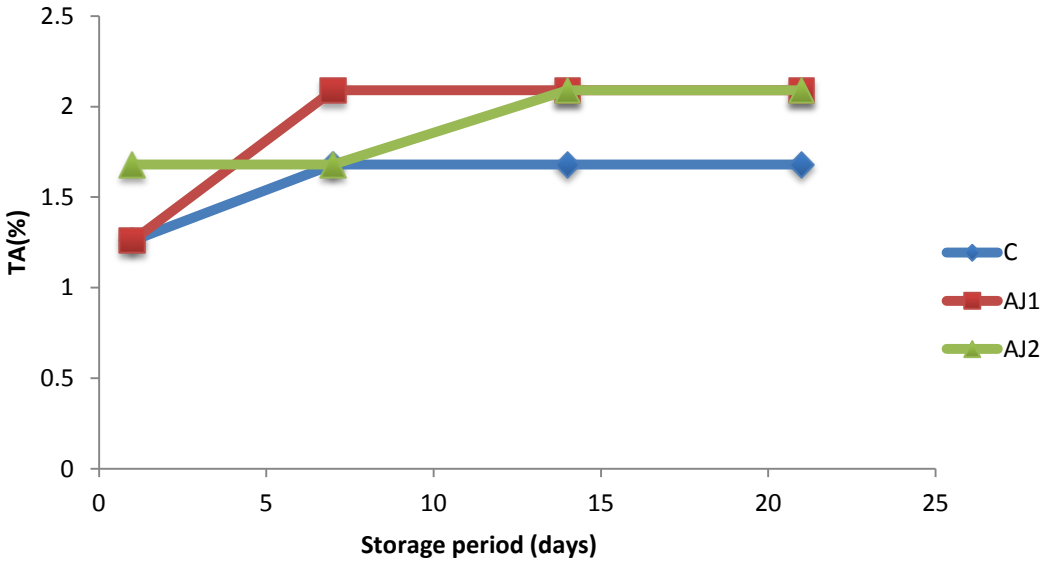

Fig. 7.5. Variation in titratable acidity of fermented apple juice during cold storage

AJ1 – pasteurized apple juice without any supplements fermented by the co-culture (1:1), AJ2 - pasteurized apple juice supplemented with 0.2% (w/v) sodium ascorbate and 0.5% (w/v) glucose fermented by the co-culture (1:1), C – control (pasteurized apple juice)

A similar trend in decline in pH of apple juice fermented by probiotic bacteria over storage was observed by Ding & Shah (2008). The utilization of carbohydrates by the probiotic bacteria and production of small amounts of organic acids during the storage period could be the possible reason for these changes in pH and TA (Ding & Shah, 2008). The viable count of bacteria was above the minimum of biovalue index (MBV) during the storage period of 21 days. Less than 2 log unit reductions in the viable cells was observed in AJ2 after 21 days of refrigerated storage while in AJ1 the viability of cells decreased to 2.32 log units (**Fig.7.6**).

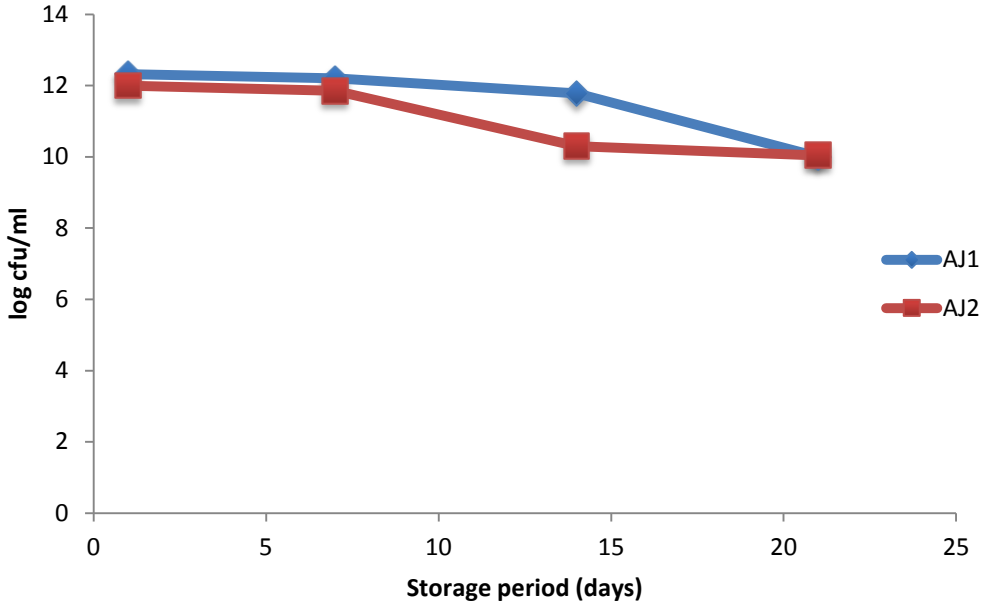

Fig. 7.6. Changes in viability of bacteria in fermented apple juice during cold storage

AJ1 – pasteurized apple juice without any supplements fermented by the co-culture (1:1), AJ2 - pasteurized apple juice supplemented with 0.2% (w/v) sodium ascorbate and 0.5% (w/v) glucose fermented by the co-culture (1:1), C – control (pasteurized apple juice)

7.3.2. Folate Fortification of Ice Cream and Skim Milk by Free and Encapsulated Bacteria

The study was conducted to access the feasibility of encapsulated bacteria with enhanced tolerance to simulated gastrointestinal juice in fermentative folate fortification of ice cream and skim milk. The folate production by the encapsulated probiotics was then compared to that of free cells. Though milk is not a rich source of folate it provides ideal conditions for the two LAB strains to grow and survive. For the folate production, skim milk media (SM1 and SM2) that have already been optimized for the LAB strains were

used. **Fig. 7.7** shows the results of folate fortification by free and encapsulated *L. lactis* CM22 and *L. lactis* CM28 in skim milk.

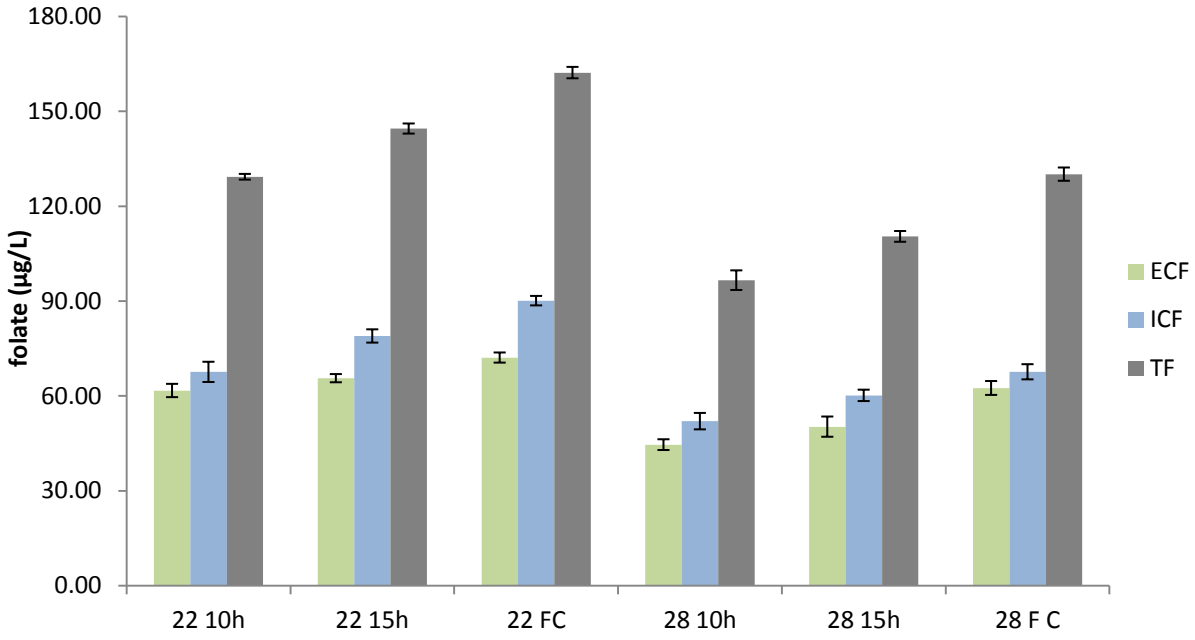

Fig. 7.7. Fermentative folate fortification skim milk by free and encapsulated *L. lactis* strains

22 10h – Encapsulated *L. lactis* CM22, 10 h fermentation; 22 15h – Encapsulated *L. lactis* CM22, 15 h fermentation; 22 FC - *L. lactis* CM22 (free cells), 8 h fermentation; 28 10h – Encapsulated *L. lactis* CM28, 10 h fermentation; 28 15h – Encapsulated *L. lactis* CM28, 15 h fermentation; 28 FC – *L. lactis* CM28 (free cells), 8 h fermentation; ECF – Extracellular folate, ICF – Intracellular folate, TF – Total folate

The fermentation time was extended from 8 h to10 and 15 h for encapsulated probiotics by considering the low metabolic activity and permeability barrier of the encapsulated probiotics. The final pH of the fermented skim milk containing free cells was a 4.8 and with the encapsulated probiotics the pH was 5 after 15 h fermentation. When encapsulated *L. lactis* CM22 was used 129.29 ± 1.6 µg/L folate was produced after 10 h fermentation at 37 ºC. 144.54 ± 1.5 µg/L folate was obtained after 15 h fermentation and with free cells 162.23 ±1.8 µg/L total folate was present. The initial folate concentration in the skim milk was only 3.82 ± 1.3 µg/L. In the case of *L. lactis* CM28, with free cells the folate yield was 130.12 ± 2.1 µg/L and with encapsulated cells it was 96.59 ± 2.2 µg/L and 110.43 ± 2.4 µg/L respectively after 10 and 15 h fermentation.

Ice cream is also a good vehicle for probiotic delivery due to its composition and the pH of about 6 of ice cream provides an ideal condition for the probiotic survival. The pleasant flavour as well as the consumer acceptance gives an added advantage to ice cream to be developed as a functional food. Several studies have demonstrated the development of frozen yoghurt and ice cream using fermented mixes (Favaro-Trindade et al., 2006). The pH of the ice cream fermented by free cells for 8 h was 5. However, the time taken by the encapsulated cells to reach that pH was longer and the pH was 5.5 and 5 respectively after 10 h and 15 h fermentation. The slow uptake of nutrients and slow release of the metabolites across the encapsulation shell might have resulted in the increased fermentation time (Homayouni et al., 2007). Similar pattern in prolongation of fermentation by the encapsulated cells were observed by (Larisch et al., 1994) with alginate/poly-L-lysine beads containing *L. lactis* cells. Homayouni et al. (2008) also reported extended fermentation time with encapsulated cells of *Lb. casei* and *Bifidobacterium lactis.* Enumeration of bacteria was done post fermentation to ensure that the final product contains the MBV of 10^7 CFU/mL. The results indicated all the combinations had adequate number of viable cells (**Table 7.1**).

Table 7.1. Viable count of bacteria in skim milk and ice cream after fermentation by free and encapsulated bacteria

Sample	**Log CFU/mL in ice cream**	**Log CFU/mL in fermented skim milk**
CM22 10h	11.03 ± 0.08	11.5 ± 0.03
CM22 15h	11.04 ± 0.04	11.53 ± 0.02
CM22 FC	11.47 ± 0.03	10 ± 0.06
CM28 10h	11.28 ± 0.03	11.15 ± 0.04
CM28 15h	11.01 ± 0.04	11.04 ± 0.03
CM28 FC	10.98 ± 0.02	11.43 ± 0.05

22 10h – Encapsulated *L. lactis* CM22, 10 h fermentation; 22 15h – *Encapsulated L. lactis* CM22, 15 h fermentation; 22 FC - *L. lactis* CM22 (free cells), 8 h fermentation; 28 10h – Encapsulated *L. lactis* CM28, 10 h fermentation; 28 15h – *Encapsulated L. lactis* CM28, 15 h fermentation; 28 FC - *L. lactis* CM28 (free cells), 8 h fermentation; Initial inoculum (0 h) was 10^9 CFU/mL

Fig. 7.8 represents the folate production in ice cream by the free and encapsulated probiotics. The total folate produced by the encapsulated *L. lactis* CM22 was 173.80 ± 1.2

µg/L after 15 h fermentation and free cells produced 222.06 ± 2.1 µg/L after 8 h of fermentation at 37 ºC. The initial folate concentration in the ice cream mix was 18.32 ± 1.1 µg/L. In the case of *L. lactis* CM28, highest production of 172.35 ± 3.2 µg/L folate was obtained when encapsulated cells were allowed to ferment for 10 h whereas the free cells produced 150.6 ± 2.8 µg/L after 8 h fermentation.

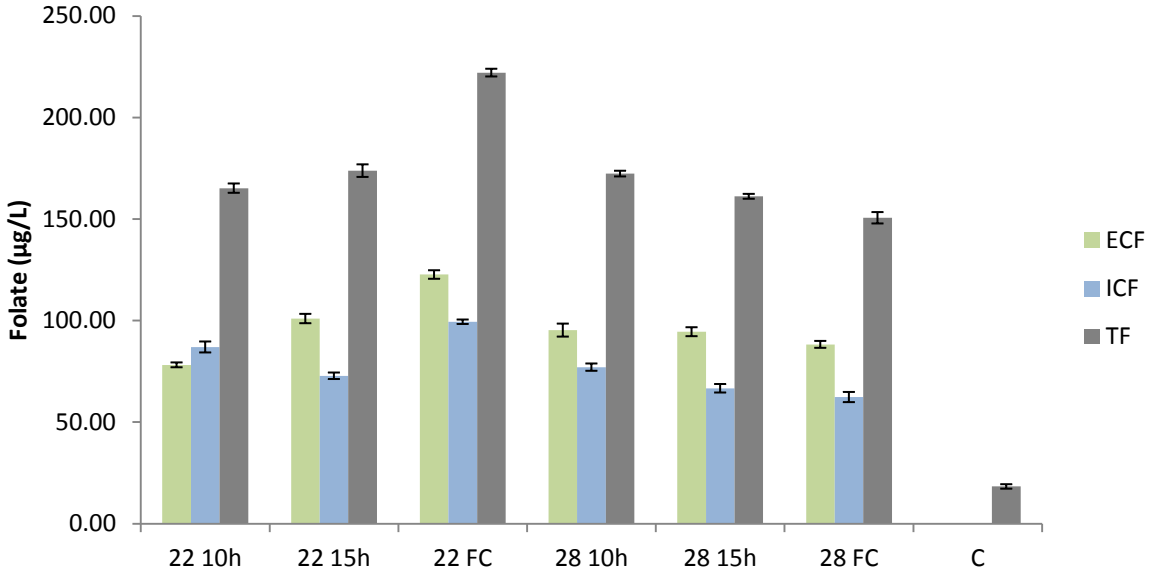

Fig. 7.8. Fermentative folate fortification of ice cream by free and encapsulated bacteria

22 10h – Encapsulated *L. lactis* CM22, 10h fermentation; 22 15h – *Encapsulated L. lactis* CM22, 15h fermentation; 22 FC - *L. lactis* CM22 (free cells), 8h fermentation; 28 10h – Encapsulated *L. lactis* CM28, 10h fermentation; 28 15h – *Encapsulated L. lactis* CM28, 15h fermentation; 28 FC - *L. lactis* CM28 (free cells), 8h fermentation; C – control (pasteurized ice cream)

ECF – Extracellular folate, ICF – Intracellular folate, TF – Total folate

Even though widely present in various foods the incidence of folate deficiency especially among women of child bearing age is a major concern. Rather than incorporating synthetic folate it is better to fortify the foods with natural folate. Encapsulation of probiotics using a suitable matrix, which increases its viability and stability and at the same time does not interfere with the production of the metabolite of interest, could open up new avenue in the development of functional foods. Enhanced production of exopolysaccahrides by encapsulated *Lactobacillus plantarum* was demonstrated by Ismail & Nampoothiri (2010). Shah & Ravula (2000) reported that microencapsulation increases the viability of *Lactobacillus acidophilus* MJLA1 and

Bifidobacterium spp. BDBB2 in frozen dairy products. The process of continuous production of pre-fermented milk with constant characteristics was described by Prevost & Divies (1987). Later Sodini et al. (1997) confirmed the feasibility of the process on an industrial scale.

Several studies on microbial folate production showed that the production and the extent of folate accumulation were not a function of species but were distinctive features of individual strains (Kariluoto et al., 2006; Sybesma et al., 2003b). The folate production using encapsulated probiotics can be further optimized considering the extended fermentation time for the encapsulated beads, the initial probiotic load of the beads, cell leakage etc. However, it was clear from the results that the encapsulated probiotics could thrive well in the simulated gastrointestinal conditions and produce folate almost comparable to that of free cells. Among the two *Lactococcus* strains, folate production by *L. lactis* CM22 was higher than *L. lactis* CM28 in skim milk and ice cream. A co-culture of the two strains can also be employed for encapsulation and subsequent folate fortification.

7.4. Conclusion

The study showed that both *L. lactis* strains, CM22 and CM28 are potential candidates for folate fortification of dairy and non dairy foods. The co-culture of the strains increased the folate content of apple juice which is otherwise a poor source of folate. The stability of the folate as well as the viability of the strains was satisfactory till the end of the refrigerated storage period. In the study it was also demonstrated that the encapsulated probiotics were capable of fermentative fortification of folate in skim milk and ice cream. The encapsulated beads could offer better results in terms of probiotic delivery due to enhanced stability in gastrointestinal conditions. Nevertheless, the conditions for folate production by the encapsulated probiotics can be further optimized to improve the folate yield. In addition, *in vivo* studies are necessary to confirm the efficacy of these formulations. The findings of the present study could lead to the development of novel probiotic functional foods with enhanced levels of folate and good shelf-life especially targeting the vitamin-deficient population.