CHAPTER 5

Folate Production in Skim Milk by Co-culturing of *Lactococcus lactis* CM22 and CM28

5.1. Introduction

Most of the research with probiotics aims at unravelling the mechanism of action of one particular strain. Nevertheless, considering the fact that probiotic properties are highly strain specific, the functionality of multistrain or multispecies probiotic could be more effective than a monostrain probiotic in various aspects. Monostrain probiotics contain one strain of a particular species and multistrain probiotics contain more than one strain of the same species or, at least of the same genus whereas multispecies probiotics have strains belonging to one or preferably more genera (Timmerman et al., 2004). The important advantage is that the favourable characters of individual strains can be combined in one preparation and thereby enhance the benefit associated with it. Multistrain probiotics have increased chances of successful colonization in the gastrointestinal tract than monostrain cultures (Guo et al., 2010). The exchange of certain growth factors, such as amino acids, free peptides, formate and CO_2 results in positive interactions among the strains known as protocooperation (Driessen et al., 1982).

From several studies it was concluded that different strains of the genera *Lactobacillus, Lactococcus, Streptococcus, Bifidobacterium and Propionibacterium* exhibit symbiotic relationships towards each other which enhances their growth and metabolic activity (Timmerman et al., 2004). Lavasani et al. (2010) reported the synergistic immunosuppressive effect of different probiotic strains in an animal model. In their study, treatment with *Lactobacillus paracasei* DSM 13434, *Lb. plantarum* DSM 15312 or *Lb. plantarum* DSM 15313 prevented experimental autoimmune encephalomyelitis development in mice, but only a combination of all three lactobacilli resulted in an efficient therapeutic activity when given to mice with established disease. There are only a few reports regarding multistrain probiotic products composed of LAB strains with selected functions, and in one such study a combination of heat killed strains of *Lb. acidophilus* (LAP5, LAF1 and LAH7) showed an augmented antagonistic effect towards *Salmonella typhimurium* invasion in mice (Lin et al., 2007).

The aim of the present study was to evaluate and compare the efficacy of a probiotic co-culture comprising of *Lactococcus lactis* CM22 and *L. lactis* CM28 with that of individual strains in fermentative production of folate in skim milk medium.

5.2. Materials and Methods

5.2.1. Microorganisms and Culture Conditions

The two *L. lactis* strains (CM22 and CM8) were used for the folate production studies in skim milk medium. *Lb. casei* NCIM 2364 was used for folate detection by microbiological assay. The culture propagation and maintenance were mentioned in section 2.2.1.f.

5.2.2. Compatibility Test

The coexistence of the two *L. lactis* strains was checked by co-streaking and cross streaking (Daeschel, 1992; Guo et al., 2010). The two *L. lactis* strains were streaked perpendicularly across each other (cross streak) and parallel to each other (co- streak) in M17 agar supplemented with 0.5% (w/v) glucose and incubated at 37 ºC for 24 h. The growth pattern of the strains was observed and documented.

5.2.3. Folate Production Media

Modified skim milk medium optimized for each isolate were used for the study. The SM1 medium (optimized for *L. lactis* CM28) contained skim milk (4% (w/v)) supplemented with PABA (100 µM/L), glutamate (75 µM/L), glycine (6 µM/L), methionine (6 µM/L), mannitol (0.6% w/v), sodium ascorbate (0.2% w/v). In SM2 medium (optimized for *L. lactis* CM22) the skim milk (4% (w/v)) was supplemented with PABA (75 µM/L), glutamate (75 µM/L), glycine (6 µM/L), methionine (6 µM/L), mannitol (0.8% (w/v)) and sodium thioglycholate (0.2% (w/v)). The skim milk (4% w/v) was autoclaved at 121°C for 5 min and other supplements were filter sterilized and added into the skim milk after cooling.

5.2.4. Inoculum Preparation and Fermentation

The cultures were grown individually in FAA medium supplemented with a nutrient solution. The composition of the nutrient solution was given in section 3.2.3. The inoculums of both cultures (18 h old) were then mixed at various ratios and used for skim milk fermentation.

Folate fermentation was carried out in the two modified skim milk media (SM1 and SM2, 100 mL each) with different inoculum ratios (CM22: CM28 in 1:1, 1:2 and 2:1) to get a total inoculum size of 1% (v/v). The fermentation was performed at 37 °C for 8 h under static conditions without pH control. Folate analysis was done by microbiological assay as described in 2.2.2.a.

5.2.5. Storage Stability Studies of the Fermented Skim Milk

The fermented milk with highest folate content was stored under refrigerated conditions at 5°C for 15 days. The samples were collected at intervals and analysed for folate, pH, and lactic acid (%) in terms of titratable acidity (TA) and also determined the viable count of bacteria. TA is determined as per the method described in section 2.2.2.c. The viable count of bacteria was determined by serial dilutions in sterile saline and plating appropriate dilutions onto M17 agar plates supplemented with 0.5% (w/v) glucose. The plates were then incubated at 37 °C for 1- 2 days. CFU were enumerated and the viable count was expressed in log CFU/mL.

The data were calculated as mean ± SD determined from triplicate trials

5.3. Results and Discussion

5.3.1. Compatibility Test

The two *L. lactis* strains showed antagonistic activities against *Escherichia coli* and *Staphylococcus aureus* (section 3.3.4.3). Hence cross streaking and co streaking tests were conducted to check the ability of the two strains to coexist. It was observed that the strains did not show any antagonism against each other and hence were compatible (**Fig. 5.1**).

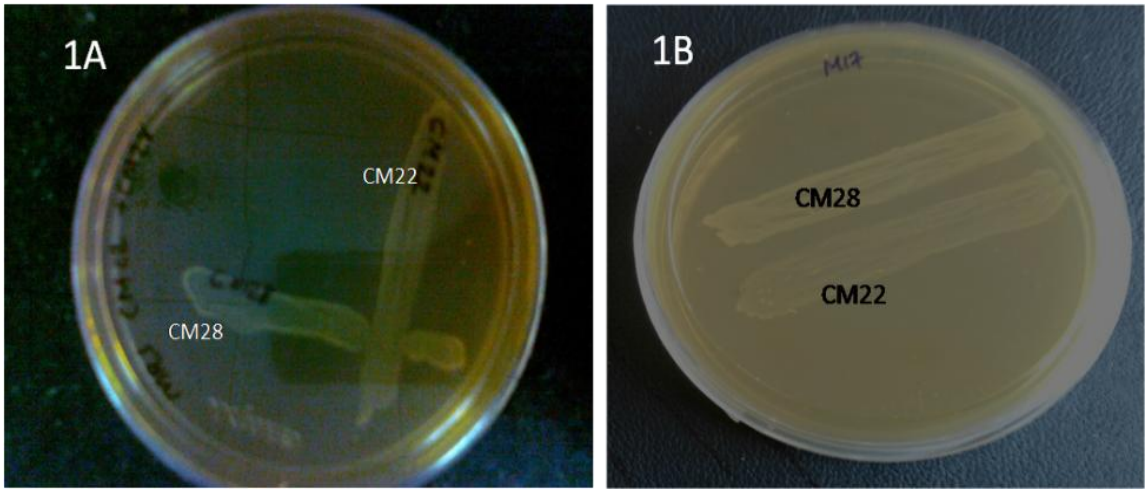

Fig. 5.1. Co-existence test (A) cross streaking (B) Co-streaking

In probiotic fermented products rather than a monostrain usually multistrain or multispecies culture are being used. When used in combinations the interactions among the different bacterial strains can result in inhibition or stimulation of microbial growth and metabolic activity (Vinderola et al., 2002). The isolates with antagonism might lead to loss of viability of other probiotic strains and thus could affect the functionality of the probiotic product and hence it is emphasized that strains used in multistrain and multispecies probiotics should be compatible or, preferably, synergistic (Timmerman et al., 2004).

5.3.2. Folate Production by the Co-culture in Fermented Skim Milk

Among the inoculum ratios, a highest production of 212.4 ± 3.8 µg/L folate (total) was obtained in SM1 medium with 1:1 inoculum ratio where the initial folate concentration in the skim milk was 3.82 ± 1.3 µg/L. The corresponding total folate production by the individual strains *L. lactis* CM22 and *L. lactis* CM28 was 163.9 ± 2.9 µg/L and 129.53 ± 1.2µg/L in 100 mL optimized skim milk media. The folate levels at various inoculum ratios are represented in **Fig. 5.2**.

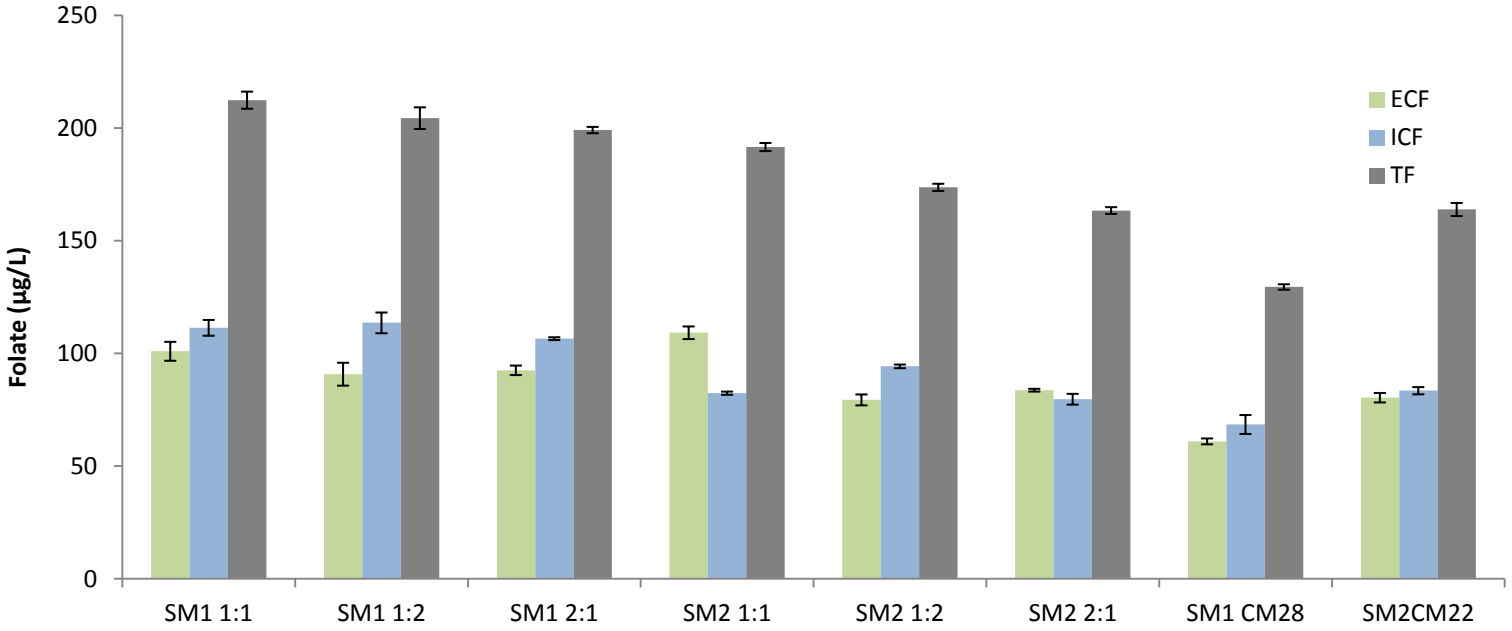

Fig. 5.2. Folate production in skim milk by the co-culture (CM22:CM28) at various inoculum ratios

SM1 1:1 – SM1 medium fermented by the co-culture at 1:1 ratio, SM1 1:2 – SM1 medium with 1:2 inoculum ratio, SM1 2:1- SM1 medium with 2:1 inoculum ratio, SM2 1:1 – SM2 medium with 1:1 inoculum ratio, SM2 1:2 – SM2 medium with 1:2 inoculum ratio, SM2 2:1- SM2 medium with 2:1 inoculum ratio, SM1 CM28 – SM1 medium fermented by CM28 (1% v/v), SM2 CM22 – SM2 medium fermented by CM22 (1% v/v)

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

In addition to elevated total folate levels, an increase in extracellular folate (101± 4.2 µg/L), was observed in fermented milk (SM1, 1:1 ratio) when compared to fermentation by individual strains. Extracellular folate of 109 ± 2.8 µg/L was obtained in SM2 medium with 1:1 inoculum ratio but the total folate was less (191.62 ± 1.8 µg/L) when compared to that in SM1 medium with 1:1 inoculum ratio (**Fig. 5.2**). It was earlier reported that when folate producing *Lb. plantarum* SM39 was co-cultured with a B12 producer, *Propionibacterium freudenreichii* DF13 an increase in the levels of both the vitamins was obtained in whey permeate medium (Hugenschmidt et al., 2011).

Crittenden et al. (2003) reported a six fold increase in folate concentration by fermentation using a combination of *Bifidobacterium animalis* CSCC 1941 and *Streptococcus thermophilus* CSCC 2000. But, fermentation of skim milk by a combination of folate producing strain (*B. animalis* CSCC 1941) and folate utilizing strain (*Lb. acidophilus* CSCC 2400) resulted in little net change in folate concentration of the skim milk. In another study, *S. thermophilus* and Lb. *bulgaricus* exhibited in the milk an interaction that is mutually favourable and when compared to mono-cultures a positive effect of the co-culture in terms of growth, acidification, production of flavours, exopolysaccharides, and of proteolysis was observed (Beal et al., 1994; de Souza Oliveira et al., 2012).

Milk is an ideal matrix for the survival of probiotics which further enhances the health benefits associated with them. Folate binding proteins (FBP) present in the milk could enhance the stability as well as the bioavailability of the produced folate (Verwei et al., 2003). It was suggested to combine FBP- rich foods with folate-rich foods to increase the bioavailability of natural folates in human diet. However, the effects of FBPs also depend on the interactions with other dietary components (Jones et al., 2003). Prebiotics are non digestible food ingredients that beneficially affect the host by selectively stimulating the growth of beneficial organisms (Manning & Gibson, 2004). In the present study, the presence of mannitol at optimized levels was beneficial for the growth as well as folate production by the co-culture. Mannitol is a sugar polyol with several beneficial properties like low calorie sweetener, antioxidant and is also included in the prebiotics group due to their indigestibility (Liong & Shah, 2006; Wisselink et al., 2002). It was reported that the supplementation of mannitol improved the growth of the probiotic cultures *Lactobacillus* sp. FTDC 2113 and *Lb. acidophilus* FTDC 8033 and also lactic acid

production in soymilk (Yeo & Liong, 2010). Mannitol could act as an osmoprotectant to the probiotics and thus increase their growth and survival (Jones & Versalovic, 2007). Sodium ascorbate was included in the medium as a reducing agent to prevent the oxidation of the highly labile folate. Sodium ascorbate stabilizes the produced folate during storage as well as extraction. It has been shown that the addition of ascorbic acid to ultra high temperature (UHT) pasteurized milk prolonged the storage stability of folates (Andersson & Öste, 1992).

The stability of folate during the storage period is an important parameter regarding the functionality of the product and it was observed that about 85% of the produced folate was stable after 15 days of refrigerated storage (**Fig. 5.3**). Lin & Young (2000) reported 2 -16% decrease in folate levels in fermented milk for the first week of refrigerated storage which continued to decrease gradually throughout the three week storage period.

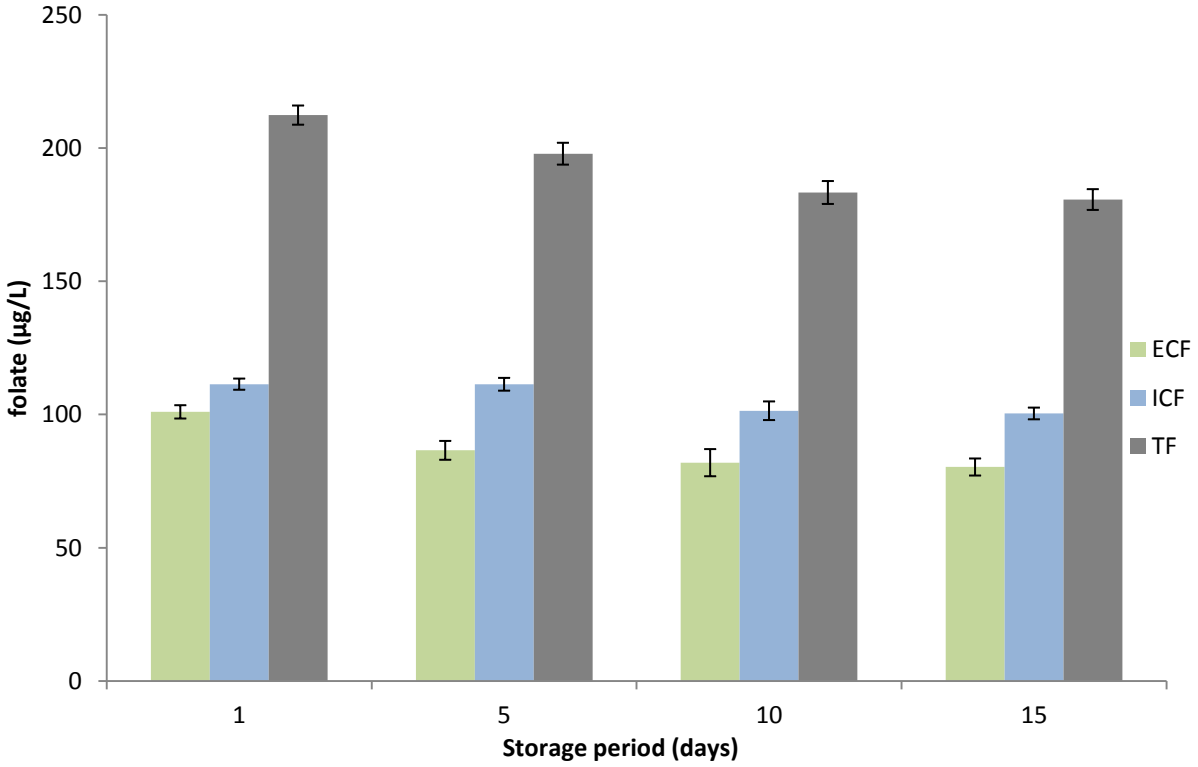

Fig. 5.3. Storage stability of folate in fermented skim milk medium

(SM1, 1:1 inoculum ratio)

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

It is recommended by the International Dairy Federation that the probiotic product should contain adequate amount of live bacteria (at least 10^7 CFU/g) at the time of consumption (Ouwehand & Salminen, 1998). Hence it is important to monitor and maintain the viability of the probiotics during storage period. Less than two log unit reduction was observed in the viability of the co-culture in fermented milk with a final value of 10.3 ± 0.11 log CFU/mL (**Fig. 5.4**). This decrease in viability could be due to post fermentation acidification (Damin et al., 2008).

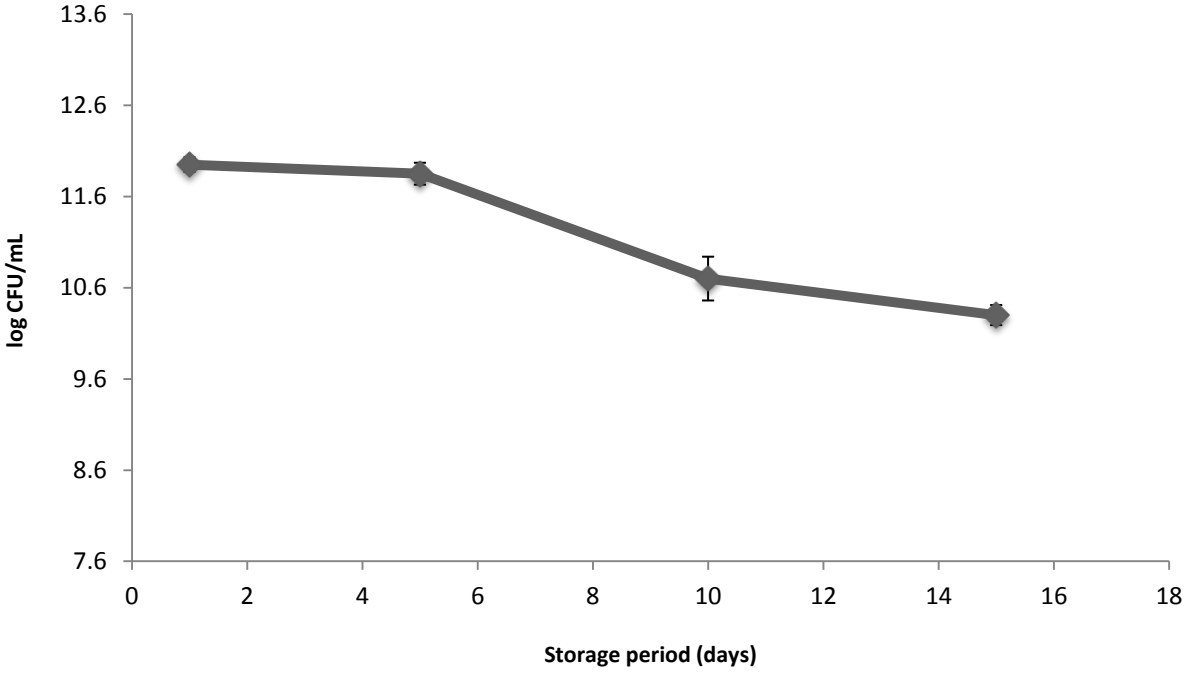

Fig 5.4 Changes in viable count of bacteria during refrigerated storage

In LAB fermented foods, lactic acid produced acts as the main preservative accompanied by a decrease in pH and increase in TA during fermentation (Gemechu, 2015). The pH of the fermented milk after 24 h of storage was 4.55 ± 0.01 which gradually decreased to 4.39 ± 0.01 after 15 days of refrigerated storage (**Fig. 5.5**). Gradual increase in TA was observed during storage indicating the post fermentation acidification (**Fig. 5.6**).

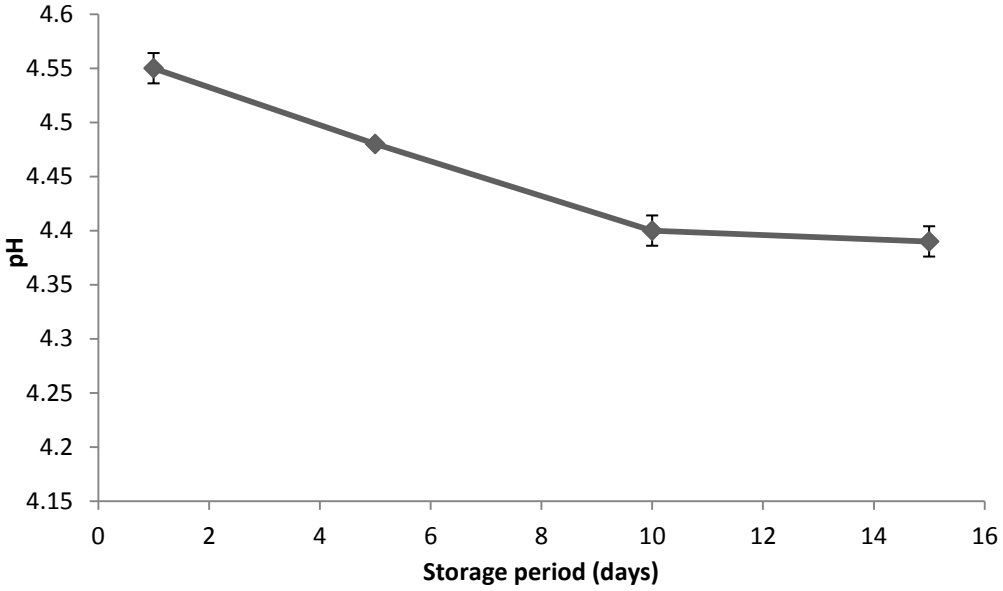

Fig 5.5 Changes in pH of fermented milk during refrigerated storage

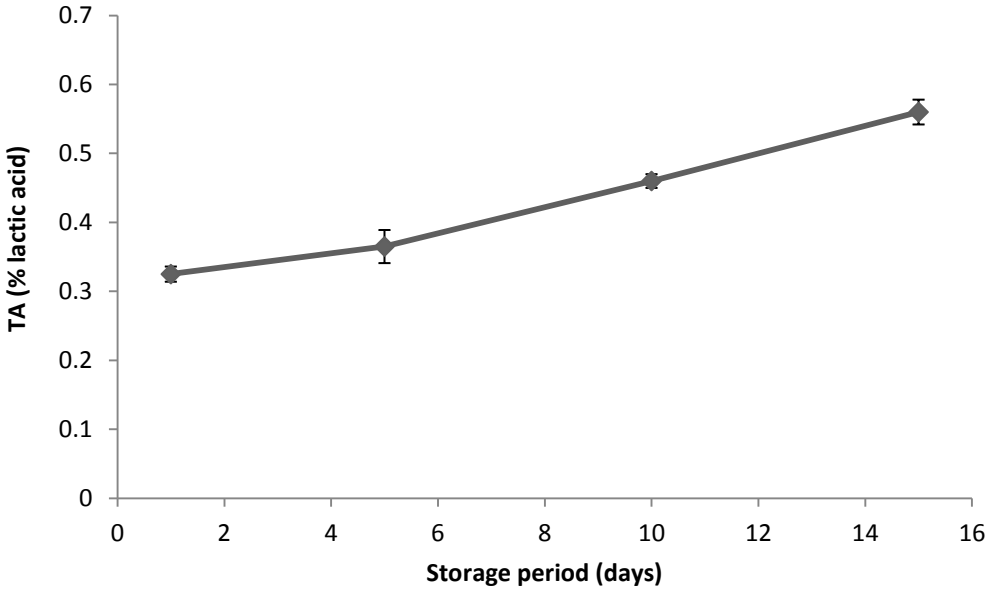

Fig 5.6 Changes in titratable acidity of fermented milk during refrigerated storage

These results are also in accordance with previous reports and the viability of probiotics, pH and acidity of the product were dependent on the species and strains of probiotics (Damin et al., 2008; Dave & Shah, 1997; Donkor et al., 2006). Guo et al. (2009) reported a continuous increase in TA and decrease in pH in milk fermented with *Lb. casei* Zhang on refrigerated storage up to 28 days while the fermentation in the samples with *Lb. acidophilus* NCFM, *Lb. casei* Shirota and *Bifidobacterium animalis* Bb12 slowed

down. The low pH and increased acidity could protect the milk against spoilage microorganisms and proliferation of pathogens thus increasing its shelf life (Widyastuti & Febrisiantosa, 2014).

5.4. Conclusion

Compared to single strain, multistrain probiotics may have superior functional characteristics possibly due to the synergistic effects between the strains. The study demonstrated that the co-culture of *L. lactis* strains improved the folate content of skim milk medium when compared to the individual strains. This could be further exploited to develop functional foods enriched with natural folate. Since the probiotic properties are strain specific and when different strains having different characteristics are combined they may be able to create a niche that might enhance colonization and adhesion of the surviving strains. Also, this approach may be extended to develop other mixed culture systems with improved probiotic properties.