CHAPTER 9

Summary and Conclusion

The significance of folate in maintaining human health and especially the protective effect towards various physiopathological conditions is recently being emphasized. Humans cannot synthesize folate *de novo* and hence rely exclusively on sufficient intake through diet by exploiting active transport systems. The requirement of folate is elevated during pregnancy and even a moderate deficiency might increase the risk of neural tube defects in the developing foetus. Suboptimal folate status is also linked with anaemia, cardio vascular diseases, osteoporosis, increased risk of breast and colorectal cancer, decreased cognitive performance, hearing loss etc. Regardless of its wide availability in various foods, the prevalence of folate deficiency is a growing concern owing to the vital roles the vitamin plays in metabolism. Folate producing probiotics has the potential for fortifying foods with the vitamin and can be a better substitute for fortification using synthetic folic acid.

With the perspective to develop a folate producing probiotic, the present study focused on isolating indigenous probiotic cultures. Four predominant folate producers were identified by 16S rRNA gene sequencing as *Lactococcus lactis* CM22, *L. lactis* CM28 (isolated from cow's milk), *Weissella cibaria* G4 (from chekkurnanis leaves) and an *Enterococcus* sp. P8 (from pickle). Salient probiotic features of these isolates including transit tolerance to simulated gastric juice, bile tolerance, surface binding properties (cell surface hydrophobicity, autoaggregation and coaggregation abilities, mucin binding and adhesion to intestinal epithelial cells), antibiotic sensitivity and antimicrobial activities against potential pathogens were evaluated by *in vitro* studies. The isolates displayed significant probiotic properties which varied among individual isolates unravelling the potential of these as probiotics. Based on the results summarized in chapter 2 on folate production and probiotic properties, *L. lactis* CM22 and *L. lactis* CM28 were selected for subsequent studies.

Fermented milk is reported to have several nutritional and health benefits including improvement of lactose intolerance, colonization of active culture in the digestive tract, control of gastrointestinal infections, stimulation of gastrointestinal innate and adaptive immune responses. Biofortification of milk with folate can be achieved by fermentatation

with folate producing LAB and is an effective rationale that could benefit people/population with the deficiency. With this initiative folate production studies were conducted in skim milk medium using *L. lactis* CM28. The strain was demonstrated to produce folate, accumulate the vitamin within the cells, and excrete it into the medium. Effective manipulation of medium additives such as folate precursors, prebiotics and reducing agents along with culture conditions enhanced folate levels in skim milk. The optimized medium constituted of skim milk (4% w/v) supplemented with glycine (6 µM/L), methionine (6 µM/L), mannitol (0.6% w/v), PABA (100 µM/L), glutamate (75 µM/L) and sodium ascorbate (0.2% w/v) with a fermentation time of 8 h at 37°C at static condition. Optimization resulted in more than fourfold increase in the extracellular folate (61.02 ± 1.3 µg/L) and total folate after deconjugation was 129.53 ± 1.2µg/L while the initial folate content in skim milk medium was only 3.82 ± 1.3 µg/L (control). The practicability of further scale up of the process was tested in a 5 L bioreactor where the total folate titre reached 141.9 ± 4 µg/L after 8 h fermentation at 37°C with an agitation of 50 rpm.

The effect of refrigerated storage on the viability of *L. lactis* CM28, pH, titratable acidity in terms of percentage lactic acid and the stability of folate was determined in order to gain knowledge before a decision of an eventual folate fortification. Fermented skim milk samples were evaluated for these parameters in 5 days interval for 15 days. The pH decreased from 4.74 to 4.42 and acidity increased from 0.28 to 0.48%. Less than a log unit reduction was observed in the viable count of the bacteria after 15 days and about 90% of the produced folate was retained in an active state during the storage period.

It was reported that instead of a monostrain probiotic a multistrain or multispecific probiotic could be more effectual possibly due to their synergestic action. Consequently we studied the effectiveness of a co-culture of *L. lactis* strains (CM22 and CM28) for folate production in skim milk. After fermentation by the co-culture a significant increase in folate level was obtained in skim milk (212.4 ± 3.8µg/L) compared to individual strains (folate production by *L. lactis* CM22 and CM28 was 163.9 ± 2.9 µg/L and 129.53 ± 1.2 µg/L respectively). The viability of cultures as well as folate stability was satisfactory during the storage period under refrigerated conditions.

In order to exert the desired beneficial properties, the probiotics should maintain high viability throughout the gastrointestinal tract. Hence, to increase their survival the *L.*

lactis strains were encapsulated by co-encapsulation using alginate and mannitol and by hybrid entrapment with skim milk, glycerol, $CaCO_3$ and alginate. The encapsulated cells showed improved survival in simulated gastrointestinal conditions compared to the free cells and hybrid entrapment provided better protection to the cells than co-encapsulation. After successive incubation in simulated gastric and intestinal juice the percentage survival of free cells of *L. lactis* CM22 were 51.1% while 56.34% and 62.74% cells remained viable in case of co-encapsulation and hybrid entrapment respectively. While 55.8% free *L. lactis* CM28 survived, hybrid entrapment significantly increased the viability to 68% and for co-encapsulated cells the survival was 61.1%.

The effectiveness of *L. lactis* strains (CM22 and CM28) in folate fortification of apple juice was evaluated and the increase in the intracellular, extracellular (easily bioavailable) and total folate was determined. Fermentation by the individual strains as well as the co-culture resulted in an increased folate titre in apple juice from 10.28 ± 0.7 µg/L to a highest of 114.15 ± 1.2 µg/L. Studies to check the efficacy of encapsulated *L. lactis* strains in fermentative fortification of skim milk and ice cream revealed an augmentation in folate level comparable to that of free cells. In skim milk fermentation, encapsulated *L. lactis* CM22 produced 144.54 ± 1.5 µg/L folate after 15 h fermentation and free cells produced 162.23 ± 1.8 µg/L after 8 h fermentation at 37 ºC.

Similarly the folate titre in skim milk fermented with free cells of *L. lactis* CM28 was 130.12 ± 2.1 µg/L and with encapsulated cells 110.43 ± 2.4 µg/L was obtained. In fermented ice cream the total folate produced by the encapsulated and free *L. lactis* CM22 was 173.80 ± 1.2 µg/L and 222.06 ± 2.1 µg/L respectively while the initial folate concentration in the ice cream mix was 18.32 ± 1.1 µg/L. A maximum of 172.35 ± 3.2 µg/L folate was produced with encapsulated *L. lactis* CM28 whereas the free cells produced 150.6 ± 2.8 µg/L. The improved gastrointestinal survival of the encapsulated bacteria offers an added advantage and could allow a wider application in the food market. The findings reported here may lead to the development of probiotic fermented functional foods enriched with folate that can boost the folate status of animals and humans.

L. lactis is one of the best studied LAB for which elegant and efficient genetic tools have been developed including dominant food grade selection markers. This can be utilized for metabolic engineering approaches for the controlled overexpression of selected genes involved in folate biosynthesis to increase the production. For that proper

understanding of the pathways that are being manipulated and the genes involved is obligatory. Three genes *folKE*, *folC* and *folA* were isolated from *L. lactis* CM28 and their identity was confirmed by sequencing and BLAST analysis. Subsequently *folKE* and *folC* were cloned into the expression vector pNZ8148 thereby placing them under the control of the inducible promoter *nisA*. The constructs were then transformed to the host strain *L. lactis* NIZO9000 containing *nisR/K* genes integrated into the chromosomal DNA for nisin induction. However, due to the time constraint the expression studies could not be completed but attempts are in progress. The results from those studies will endow with a basis for further improvement of functional foods which will have higher contribution to the daily recommended intake of folate.

Higher folate production might be achieved by controlled overexpression of the selected genes by the addition of sub inhibitory amounts of nisin (a food grade inducer) thus limiting the intake of the fermented product within a desirable limit (eg. 100 mL) to obtain the DRI of the vitamin. Besides this it is feasible to modulate the bioavailability of folate by controlling the degree of folate accumulation and release during fermentation. It is also possible to transfer the NICE system to the indigenous LAB isolates for overexpression of the genes by introducing a dual plasmid system employing the *nisA* promoter and *nisR* and *nisK* genes. However, the strains could be applied in food fermentations after substituting the antibiotic resistance marker with a food grade marker. And ultimately animal studies are to be conducted to prove the efficacy these recombinant strains.

Inadequate resources for producing and purchasing good quality foods are a barrier to achieve greater dietary diversity, especially among low income populations. Hence, several efforts are being made to make micronutrient rich foods available and affordable to a diverse group of population to combat deficiency. Emphasis is given on the use of fermented foods as a sustainable food for promoting the health and well being of people. Fermentation is generally an inexpensive process and requires a low-cost technology with the additional benefits of enhancing the nutritional and sensory profiles of food. Concurrently, fermentation fortification eliminates the need for downstream processing as the food can be consumed as such.