Chapter 2 REVIEW OF LITERATURE

2.1 History of Folic acid and related work

Folate is an essential vitamin which is also water soluble in nature. Daily dose of this vitamin is essential due to the regular excretion of this vitamin from the body through urine or other body fluids. Keeping in view, the potential importance of this vitamin for the humans, these studies were focused with the objective of enhanced microbial production of folate by different microorganisms. For the better understanding of the studies related with this work, year wise systemic literature of this subject has been presented as follows:

1920-1929:

In early 1920s the scientist believed that folate deficiency and anemia were same. Later on, major findings about the existence of folic acid came in the lime light. First of all discovery by Minot and Murphy of a kind of nutritional anemia i.e. pernicious anemia came into the knowledge which can be corrected by feeding large amount of liver. Subsequently, the factor responsible for the correction was termed as "Intrinsic and extrinsic factor" by Castle (Vaughan, 1934).

1930-39:

Wills & Mehta (1930, 1931) observed that the Dietary Anemia in rats is prevented by yeast extract which lead to the further check the potential of compound present in yeast extract. Later on, Lucy Wills further observed that macrocytic anemia of pregnancy is corrected by a component of the yeast or Marmite, which later on known as folate (Wills, 1931). Vaughan also observed that anemia caused by the coeliac disease can also be corrected by the Marmite (Vaughan, 1932). After the continue research in this direction, Wills & Evans (1938) observed that purified liver extract did not have the potential to cure patients with nutritional, pregnancy and macrocytic anemia. However

injections of crude liver extract or autolysed liver extract can cure the tropical macrocytic anemia. It was discovered that Vitamin M corrects the nutritional anemia in monkeys (Day *et al*., 1938). Later on it was observed that vitamin M is another name of the folate. Stokstad and Manning (1938) mentioned that Factor S and Vitamin B_c was the factor present in the yeast which basically prevents the anemia.

1940-1949:

Hogan and Parrott (1940) observed the nutritional anemia in chickens. In the same year, Snell and Peterson named factor absorbed from yeast or liver as Norit eluate factor and it was also found to be growth factor for *Lactobacillus casei* (Snell & Peterson, 1940). After the further various kind of research on the folate, finally the name "Folic acid" is derived in 1941 as it was isolated from spinach. In Latin language term *"folium"* means leaf thus the term Folic acid is given to the substance which acts as growth factor for *Streptococcus lactis R* (*S. faecalis*) (Mitchell *et al.,* 1941). Fullerton (1943) and Watson & Castle (1943) observed that idiopathic steatorrhoea megaloblastic anemia can be corrected by the crude liver extract or yeast extract. Finally, pure crystalline structure of folic acid was synthesized by Bob Stokstad while working at Ledele laboratories (American Cyanid Company) situated in Pearl River, New York, USA (Hoffbrand & Weir, 2001). Later on, Wright & Welch discovered that folate conjugase is the enzyme which hydrolyses the folate polyglutamates into the microbiologically active monoglutamate form (Wright and Welch, 1943). Binkley *et al.* observed that potent source of vitamin B_c was yeast extract and concluded that only 2-5% form is active for the growth of *L. casei* so its enzymatic digestion is needed for the activity (Binkley *et al.,* 1944). First research project on obtaining pure crystalline form of folic acid was done by the team called as "Folic acid boys" under the supervision and guidance of Dr. Yellapragada Subbarow, Director of Research at the Lederle Lab, NY, USA. Further Angier *et al.* worked for the synthesis of folic acid and named it as pteroylglutamic acid (Angier *et al.,* 1945). Finally it was concluded that folic acid was made up of three subunits a pteridine ring, para amino benzoic acid and glutamic acid and was termed as (Pteroyl-glutamic acid or PGA).

After the synthesis of pure crystalline form of folic acid, it was clearly established that natural folates are usually different than the pteroylglutamic acid or folic acid in three respects i) additional glutamate chain in folates (poluglutamylation) ii) reduction to di or tetra hydrofolates and iii) addition of single carbon units or carbon substituted form for eg. Methyl ($-CH_3$), Formyl (-CHO), Methylene ($=CH_2$) and methenyl ($=CH_4$) attached at N_5 or N_{10} positions. Thus folic acid term is used to fully oxidized form chemical compound which is not usually present in the natural foods and it is generally chemically synthesized form. Whereas the term "Folate" denotes the large group of compounds both the natural folates (polyglutamylated and carbon substituted as well as folic acid) having the same basic vitamin activity as folic acid (Hoffbrand & Weir, 2001). In 1945 it was also observed by Day *et. al.,* (1945) that purified factor responsible for the growth of *Lactobacillus casei* is known as vitamin M. Subsequently it was observed that that Folate which is naturally present in liver is heptaglutamate (Pfiffner *et al.,* 1946).

1950-59:

Several attempts were made to discover the biochemical mechanisms of folate (Lanska, 2009).

1960-1969:

First report was published to establish the linkage between folate deficiency and neural tube defects (Lanska, 2009).

In 1968, a rapid disc assay method was developed for the determination of folic acid content in several dairy products, several fruits and vegetables (Vakil & Shahani, 1968). This method involved the measurement of zone of growth exhibition of *Streptococcus faecalis* around a disc containing folic acid solution or folic acid extract on a deficient agar medium. This disc assay method was a modification of the standard turbidimetric method. The disc assay method gave the results in 8-10 h as compared to 22-24 h in turbidimetric method. The diameter of growth zone had the logarithmic relationship with the concentration of folic acid. Folic acid content of Cottage, Cheddar and Swiss cheese were determined by this method and compared with the turbidimetric

method. This method was found to be fairly accurate and reliable but not widely accepted by the others.

1970-1979:

Later on the yeast concept for the folic acid given by Lucy Wills was further reviewed by the Daphne Roe in 1978 (Roe, 1978). After some time Bob Stokstad and his colleagues worked on the isolation, purification and characterization of many enzymes involved in the folate metabolism isolated from the mammalian cells (Stokstad, 1979).

1980-1989:

In Sweden, B vitamin content was analyzed in the samples of milk and fermented milk products by the standard chemical and microbiological procedures (Alm, 1982). This study was designed to check whether fermentation of milk enhances the nutritional content of the milk. Different kinds of milk samples were inoculated with the commercial mixture of *Streptococcus lactis, Streptococcus cremoris, Streptococcus diacetylactis* and *Leuconostoc cremoris* and incubated for 20-24 h at 20-21°C. Alm reported minor changes in B-vitamin content in milk after fermentation however the content of folic acid was significantly increased to approximately 120-140% except in the acidophilus milk.

Rao *et al.,* (1984) has been observed that increase of folate concentration was dependent on the incubation time ranging from the 8-24 h. In this study *S. thermophilus*, *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus bulgaricus* ATCC 11842 had been selected for the folate production ability in the skim milk. It has been observed that *S. thermophilus* and L*actobacillus acidophilus* enhanced the folate content of skim milk by more than 200% however *L. bulgaricus* reduced the folate content of skim milk that is already present in the milk within few hours. In most of the folate production studies, milk is chosen as the fermentation medium because milk is an excellent source of most of the B vitamins except folic acid and vitamin B_{12} (Gregory, 1975). Although *S. thermophilus* and *L. acidophilus* tends to enhance the folate content to 25 ng/mL and 22 ng/mL respectively but the folate started to decline after 36 h of incubation which may reflected due to the labile nature of folate in acidic environment. Further the precursors of

folate biosynthesis PABA and GTP were also added in the skim milk to evaluate the folate content however no effect on folate content has been observed on either PABA or GTP addition by any of the lactic cultures.

1990-1999:

Israel Chanarin made major contributions to the whole knowledge in the direction of folate metabolism and causes underlie the relation between folate deficiency and pregnancy and many bone marrow and other disorders. He and Irwin Rosenberg showed that dietary folates are reduced, methylated and deconjugated to 5-methyl tetrahydrofolate (THF) during absorption. He wrote three editions of the major monograph on the megaloblastic anaemias (Chanarin, 1990).

In the 1990s, US scientists worked on the daily recommended intake of folate and they realized that there was still a challenge for most of the population to meet their daily folate requirements, despite the availability of folate in foods and in supplements (Lanska, 2009). Centers for Disease Control and Prevention (1991) recommended that women who have the past history of NTD-affected pregnancy should consume daily 4000 μg of folic acid from starting at the day they begin planning a pregnancy. Subsequently, the U.S. Public Health Service (1992) recommended that all women of childbearing age consume 400 μg of folic acid to prevent NTDs daily through any ways fortification, supplementation, and diet. The Institute of Medicine (IOM) (1998) recommended that women planning of being pregnant should consume 400 μg of folic acid daily in addition from fortified foods or supplements besides through a normal diet. The U.S. Preventive Services Task Force (2009) published updated guidelines that reinforced these recommendations.

The Food and Drug Administration (FDA), USA published the regulation guidelines in which it was mentioned that addition of folic acid is highly required to breads, cereals, flours, corn meals, pastas, rice and other grain products is required specifically with the target to reduce the risk of neural tube defects in newborns in 1996 (Daly *et al.,* 1997).

The Food and Drug Administration mandated the fortification of enriched cereal grain flours with 140 g synthetic folic acid per 100 grams of grain which estimated to provide 100–200 µg of folic acid per day to women of childbearing age with the intension to reduce neural tube defects occurrence in newborns in 1998 (Crandall *et al.*, 1998).

Duthie, (1999) described the role of folic acid deficiency in humans in the development of cancer, especially colorectal cancer and breast cancer. He suggested the two principal mechanisms by which low folate concentration in humans may increase the risk of cancer. Folate deficiency reduced intracellular S-adenosylmethionine (SAM) which can ultimately alter the methylation of cytosine in DNA leading to improper activation of proto-oncogenes and introduction of malignant transformation which causes cancer. 5-methyltetrahydrofolate is essential for the conversion of methionine in the biologically active form S-adenosylmethionine which acts as the principal methyl donor in majority of biochemical reactions even also in methylation of cytosine in DNA. Thus, genes which are methylated at specific locations are neither transcribed nor translated in case of folic acid deficiency. This alteration or disruption in DNA methylation increases the malignant transformation. Subsequently, folic acid is also crucial for normal DNA synthesis and repair. Folate deficiency may also result in imbalance of DNA precursors, misincorporation of uracil into DNA and chromosome breakage. Folate is involved in the synthesis of purines and pyrimidine nucleoside thymidine. 5, 10-methylenetetrahydrofolate involved as methyl donor for thymidylate synthase enzyme which is responsible for the conversion of deoxyuridine monophosphate to thymidine monophosphate. Folate deficiency blocked the methylation of dUMP to TMP which causes the imbalance of DNA precursors i.e. excess dUMP in place of thymidine in the nucleotide pool. In this study, evidences of modulation of DNA by these mechanisms due to folate deficiency were given by cellular, animal and human studies.

2000-2005:

Lin and Young (2000a) in which folate synthesizing ability of yogurt bacteria *Streptococcus thermophilus* MC and ATCC 19258 and *Lactobacillus delbrueckii* ssp.

bulgaricus 448 and 449, has been evaluated in the fermented milk. Further the major effort of this study was to check the effect of addition of lactose or calcium chloride on the folate contents of fermented milk by these yogurt bacteria. Folate analysis was done by the HPLC method after the folate extraction from the sample using human plasma as source of folate conjugase. 2% lactose addition in reconstituted non-fat dry milk resulted in elevated folate level. Although folates increased by yogurt bacteria to only 3 to 7% when grown for 6 h and 12 to 198% 18 h. In case of extended incubation time folate synthesis declined for yogurt bacteria without the extra addition of lactose because of the folate utilizing behavior of the lactic acid bacteria along with the folate synthesizing capability especially when lactose as carbon source is exhausted (Rao *et al.,* 1984). The folate content was found to be decreased on 0.02% calcium chloride addition in reconstituted milk fermented with *S. thermophilus* MC or *L. bulgaricus* 448 or 449 except the *S. thermophilus* ATCC 19258 in which folate level was found to be elevated on calcium chloride addition. Thus calcium chloride was not recommended in general to enhance the folate content. The addition of 2% lactose also resulted in the increased the cell counts for yogurt bacteria grown for 6 and 18 h. However, addition of 0.02% calcium chloride did not give the significant results of the cell counts. However, the cell counts increased for both *L. bulgaricus* strains. The folate levels and cell counts were determined after refrigerated storage at 4°C for consecutive two weeks as shelf life of cultured yogurt was considered as two weeks. Folate content was found to decline by about 9 to 28% at the end of the second week. However, viable cell count of yogurt was found to remain quite stable for the 2-week for all the four studied strains. The inhibition of oxidant H_2O_2 cytotoxicity to Intestine 407 cells by folate was determined by the MTT colorimetric assay (Carmichael *et al.,* 1987; Crouch *et al.,* 1991; Mosmann, 1983; Twentyman & Luscombe 1987) which can cause oxidative damage of cells along with their nucleic acids. The cell viability of Intestine 407 cells was enhanced due to the inhibition of oxidant H_2O_2 cytotoxicity by folate and higher viability of Intestine 407 cells were observed when treated with higher concentration of folate.

Another study by Lin & Young, (2000b) reported a study in which folate levels and its stability during the storage was determined in fermented milk samples inoculated with the cultures of lactic acid bacteria. For this study *Bifidobacterium longum* B6 and

ATCC 15708, *Lactobacillus acidophilus* N1 and ATCC 4356, *Lactobacillus delbrueckii ssp. bulgaricus* 448 and 449 and *S. thermophilus* MC and 573 were selected. All the strains were grown in MRS medium except *S. thermophilus* which was grown in the M17 medium. Folate extraction from the samples was done using human plasma to deconjugate the polyglutamyl form into the monoglutamyl form. Finally folate was analysed quantitatively by the High performance liquid chromatography. Further the stability of folate was determined after one week of interval. For the HPLC, Hitachi L-6200 system was used and mobile phase used was HPLC grade methanol (15%) in 0.05 M KH_2PO_4. C_{18} hypersil column was used as analytical column and fluorescence detector was used. Reconstituted milk media was found to best among the selected production media. Results of this study demonstrated that lactic acid bacteria can be used widely to prepare the fermented dairy products with enhanced folate levels. Although strain selection along with the incubation time is very important criteria to consider for the dairy products as it has been observed that lactic acid bacteria not only synthesizes folic acid as well as utilize folic acid for their own growth and metabolism (Rao *et al.,* 1984). It was reported that folate level in fermented milk inoculated by *B. longum* was reduced by 18% in 2 weeks however only 9% decrease was observed in the fermented milk inoculated by *S. thermophilus*. By this it can be concluded that however rate of metabolism of lactic acid bacteria is very slow at 4°C however rate of folate utilization was higher than the synthesis at 4°C.

Shrestha *et al.,* (2000) observed the effect of different variables like autoclaving and centrifugation on the efficacy of the microbiological assay of folate. Spinach, fortified bread and two ready-to-eat breakfast cereals were examined for the folate estimation and extracted with or without autoclaving and centrifugation. Lower yield of total folate in all foods was observed after centrifugation and autoclaving. After digestion with protease and α-amylase, all food samples were deconjugated with either chicken pancreas or human plasma (trienzyme) or with simply conjugase alone (traditional single enzyme treatment). The tri-enzyme treatment was a significantly more effective over the single enzyme treatment only in fortified bread. Deconjugation with chicken pancreas showed the higher folate concentration than human plasma in all foods except spinach. In another study, comparison was made between the folate assay by cryoprotected frozen

Lactobacillus casei and serially subcultured inoculum of *Lactobacillus casei*. Cryoprotected cultures give faster results in shorter time and were less tedious. It showed better reproducibility and was more economical than the serial subcultured inocula. The effects of test tubes size and wavelength on the growth of culture were also investigated. *L. casei* grew faster in smaller tubes than the larger ones. The absorbance peak at 540 nm gave promising higher response than that at 620 nm.

Breithaupt, (2001) developed a sensitive and reliable method for the estimation of folic acid from the vitamin-fortified fruit juices and fruit drinks. Ion-pair reversed phase high-performance liquid chromatography (RP-HPLC) was performed for the detection after the solid-phase extraction using strong-anion-exchange material. Apple juice, cherry nectar, blackcurrent nectar and none vitamin fortified fruit drinks containing folic acid in the range of 0.30–1.40 mg/l were used for the folic acid estimation. Limits of detection and quantitation were found to be 0.04 and 0.06 mg/l, respectively. The method analyzed that the two samples contained significantly less folic acid than specified level.

Effect of cultivation conditions on folate production by lactic acid bacteria has been observed (Sybesma *et al.,* 2003). Several strains of lactic acid bacteria were screened for the ability to produce intracellular and extracellular folate. In this study, all the used strains of *Lactococcus lactis*, *S. thermophilus* and *Leuconostoc* sp. were found to produce folate however among the selected *Lactobacillus* sp. only the *Lactobacillus plantarum* was able to produce folate. *Lactococcus lactis* MG1363 was studied as model organism for metabolic engineering in this study however *S. thermophilus* B119 was used for the direct dairy applications. Folate production by both of these microorganisms was analyzed under different growth conditions. Excretion of folate in the medium was also studied which may be dependent on the length of the polyglutamate chains. Lengthier the polyglutamate chain of folate, higher is the retention of folate within the cell. In *L. lactis* no influence of pH has been observed on the intracellular and extracellular folate distribution however in *S. thermophilus*, pH influenced the intracellular and extracellular folate distribution. Folate production was increased with increasing the pH in continuous culture. Hemin addition in the growth medium was also found to enhance the folate production may be due to the stimulation of direct oxidation of NADH by oxygen. Folate

production was also stimulated in presence of growth inhibiting substances such as antibiotics and high salt concentrations. In *L. lactis* it was found that folate can be synthesized even in absence of PABA however folate production was enhanced by the PABA addition which indicated that the *L. lactis* had the ability to synthesize the folic acid itself. This study may lead to the conclusion that dairy products manufacturers can implement the significant folate production by simply selection of high folate producer strains and by identification of best possible cultivation conditions of those particular strains.

Crittenden *et al.,* (2003) reported a study in which thirty-two bacterial strains were isolated from yoghurts and fermented milks and were checked for their ability to either synthesize or utilize folate during skim milk fermentation. The aim of this study was to check how the bacterial strain affected the folate level during fermentation and combination of which kind of microorganism can enhance the folate level of fermented dairy products to the maximum level of folate. The organisms isolated were the traditional yoghurt starter cultures like *Lactobacillus delbrueckii subsp. Bulgaricus, Streptococcus thermophilus,* probiotic *lactobacilli, bifidobacteria*, and *Enterococcus faecium*. Microbiological bioassay according to method stated earlier (Keagy *et al.,* 1985) was used to analyse the folate quantity. Among them *S. thermophilus, bifidobacteria,* and *E. faecium* were found to be folate producers. *S. thermophilus* was found to be the best folate producers among them which elevated the folate level fermented skim milk from 11.5 ng/L to between 40-50 ng/L. *Lactobacilli* were found to be folate consumer rather than folate producers thus depleted the folate already existed in the skim milk. Fermentation time for both *Lactobacilli* and *Streptococci* was varied in the range of only 14 h however for *Bifidobacteria* it was more than 40 h. *Bifidobacterium* sp. and *E. faecium* almost enhanced the folate level roughly double fold however *B. breve* 5181 enhanced the folate level to maximum extent. Co-cultivation or mixed culture fermentations for folate production was reported as additive in nature. Mixed culture fermentations using a combination of *Bifidobacterium animalis* and *S. thermophilus* enhanced the folate level by six-fold than the original level. Mixed culture of *Bifidobacterium* and *Lactobacillus* resulted only in little increase however a combination of *B. lactis, L. acidophilus* and *S. thermophilus* which is used as traditional yogurt starter

culture enhanced the folate level greater than the potential of the individual strain as starter culture. This kind of study resulted in the conclusion that enhanced folate content in yoghurts and fermented milk might be possible by the careful selection of inoculum of individual culture or mixed cultures. So that folate levels can reach upto a certain level in terms of recommended daily intake.

Holasova *et al.*, (2004) demonstrated the evaluation of folate production ability of several procured strains of *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Propionibacterium freudenreichii* subsp. *shermanii*. Ultra Heat Treated (UHT) milk containing 1.5% fat treated with additional laboratory sterilization was used as a substrate for the production studies. Fermentation temperatures considered were 37°C and 30°C in case of *Propionibacterium*. Samples were incubated at static condition and were taken at various time intervals. In this study, mainly the 5-Methyltetrahydrofolate (5-MTHF) concentrations of the samples were determined using HPLC method described previous (Vahteristo *et al.*, 1996; Finglas *et al.*, 1999). All strains of *Streptococcus thermophilus* showed increased 5-MTHF production of around more than six-fold increase in comparison with control (increase =3.69 μg 5-MTHF/100 g) after 12 h fermentation. However, *Bifidobacterium longum* strains were enhanced the folate content upto maximum 73% in terms of 5-MTHF content (increase = 0.48 μg 5-MTHF/100 g) after 12 h fermentation thus they were considered as mild folate producers. *Propionibacterium freudenreichii* subsp. *shermanii* strains did not significantly influence the 5-MTHF content during the milk fermentation. Maximum 5-MTHF concentration was found between 6-12 hours of fermentation time in all the tested strains. Individual strains even within a species produced large and significant difference in the 5-MTHF production. Thus it can be concluded that by a careful selection of the folate producing strains, an elevation of the natural folate content can be achieved in the fermented milk.

From the above studies it has been observed that most of the folate production studies were reported mainly in the MRS, M17 and reconstituted skim milk media. Later on, Kariluoto *et al.*, (2004) reported a study in which folate production was determined in rye sourdoughs by the yeast and some strains of bacteria. Fermentation of rye dough was done by the three sourdough yeasts, *Candida milleri* CBS 8195, *Saccharomyces*

cerevisiae TS 146, and *Torulaspora delbrueckii* TS 207; a control, baker's yeast *S. cerevisiae* ALKO 743; and four *Lactobacillus* spp., *L. acidophilus* TSB 262, *L. brevis* TSB 307, *L. plantarum* TSB 304, and *L. sanfranciscensis* TSB 299. Growth medium used for all the cultures was the yeast extract–peptone–glucose medium. Small-scale fermentation method using rye flour was also used that resembled the sourdough fermentation step used in rye baking. Total folate contents were determined by the microbiological assay using *Lactobacillus rhamnosus* (ATCC 7469) as the growth indicator microorganism. It has been observed that all the studied microorganisms did not excrete significant amount of folates into the YPG medium as cell fractions of the cultures had greater amount of folate. Intracellular folate content was found to be higher in *S. cerevisiae* ALKO 743 and *C. milleri* CBS 8195 than the two other yeasts. Folate content of sterilized rye flour–water mixtures was increased from 6.5 µg/100 g to 23 µg/100 g by the yeast cells at 19-h, however lactic acid bacteria reduced the folate content to 2.9- 4.2 µg/100 g. All the tested four strains of lactic acid bacteria *Lactobacillus bulgaricus, L. casei, L. curvatus, L. fermentum, L. helveticus, Pediococcus spp*. and *Streptococcus thermophilus* depleted the folate contents after fermentation that varied between 2-10.4 µg/100 g. Fermentation of sterile rye flour with the combination of yeast and lactic acid bacteria i.e. co-cultivation showed minimal effect on folate content production with the yeast alone. Thus it was concluded that the folate content after co-cultivation was mainly due to folate synthesis by yeasts. Although the Fermentation of non-sterilized flour–water mixtures (containing naturally occurring bacteria) as such resulted in three-fold increases in the folate contents in comparison to additionally added lactic acid bacteria which turned into the folate consuming nature. Two folate producing bacteria among the endogenous bacteria which favored the increased production on isolation from non-sterilized flour and on characterization, identified as *Enterobacter cowanii* and *Pantoea agglomerans*.

Another study by Holasova *et al.,* (2005) in which enhancement in natural folate content of fermented milk products by the fermentation and effect of addition of fruit component were evaluated. Mainly the 5-methyltetrahydrofolate (5-MTHF) content of the fermented milk was calculated. Pasteurized milk was inoculated with the butter starter and the selected strains of *Streptococcus thermophilus* in combination with

Bifidobacterium longum or *Propionibacterium freudenreichii* subsp. s*hermanii* and was incubated at 30°C and 37°C for 12 and 18 h at static condition. 5-MTHF content was evaluated by the HPLC method in reversed phase with fluorimetric detection described as in their previous study after deconjugation with hog kidney conjugase for folate extraction and SPE SAX purification. Co-fermentation with *Streptococcus thermophilus* No. 144 and *Propionibacterium freudenreichii* subsp. S*hermanii* No. 160 resulted in the highest increase of 5-MTHF content 4.03 ± 0.44 μg/100 g at 37°C after 12 h of fermentation. Nine commercial samples of fruit components used in dairy industry which contained between 0.17–9.11 μg 5-MTHF/100 g were taken. Pineapple, sour cherry, apricot, and apple components contained very low amounts of 5-MTHF i.e. less than 1 μg/100 g. Strawberry component among all the tested component was proved to be the best source of folate content of 9.11 μg 5-MTHF/100 g. The values of folate dependent on the ripening state of strawberry as folate content of fresh fully ripe strawberries was found in the range of 25.5-54.0 μg/100 g fresh sample, i.e. 272 and 554 μg/100 g of dry matter. The highest 5-MTHF content was found in the varieties *El-santa* and *Honeyoe* and lowest content in *Senga Sengana*. Fully ripe strawberries of the *Senga Sengana* variety contained 63% more 5-MTHF than the unripe berries. Thus fully ripe strawberries of the variety *Elsanta* and *Honeyoe* are recommended in this study for getting the maximum folate content. In short the folate content of fermented milk product may be enhanced by 4.8 μg/100 g i.e. 69% of the original value and addition of fruit component may resulted in 31% enhancement of folate content.

Green *et al.,* (2005) demonstrated a study in which relation between blood folate and reduced homocysteine concentration was established. It has been suggested that daily consumption of 400 μg folic acid at the child bearing age is recommended for the prevention of neural tube defects in newborns either through the consumption of supplements and fortified foods. In this study effect of daily consumption of milk fortified with 375μg folic acid on blood folate level was checked. It was found that fortified milk increased the blood folate and simultaneously reduced the homocysteine level in women of childbearing age. Seventy-three non-pregnant women of 18-47 years were randomized to consume either 75 g/day of a fortified or unfortified (control) milk powder for 12 weeks. Women who consumed the folic acid fortified milk had higher red

blood cell and plasma folate concentrations than in the control group. Women consuming fortified milk had lower plasma homocysteine concentration (14%) at week 12 than the women consuming control milk. Daily consumption of fortified milk would be essential to reduce the risk of neural tube defects.

2006-2010:

The World Health Organization and the Food and Agricultural Organization of the United Nations published guidelines to help countries to set the Target Fortification Level, the Minimum Fortification Level, the Maximum Fortification Level and the Legal Minimum Level of folic acid to be used to fortify flour with folic acid (Annex D, Guidelines on food fortification, 2006)

Folate production ability of 76 *Bifidobacterium* strains was investigated by the Pompei *et al.,* (2007). As most of used microbial strains were folate auxotroph hence, folate free semisynthetic medium SM7 was used for the cultivation and evaluation of the folate production studies. Only the 17 strains of human origin were able to grow well in the folate free medium and produced significant folic acid concentration in the range of 0.6-82 ng/mL. Effect of addition of exogenous folate varying from 10-100 µg/L and PABA 0-100µM were also evaluated. Folate concentration was analysed by the microbiological assay using *Enterococcus hirae* ATCC 8043 in the Bacto folic acid assay medium. Among these, highest folate production was reported with the *B. adolescentis* MB11, MB 115, MB227 and MB239. Addition of exogenous folate had negative effect on the folate production studies using all the strains except in *B. adolescentis* MB 239. Again addition of PABA (0.3 mM) increased the maximum folate production. Further, folate production was decreased to a certain level when the PABA concentration was enhanced to greater than 0.3 mM. Finally, folate production by *B. adolescentis* MB 239 was studied in depth with batch and chemostat experiments. Folate production was found to be growth associated from this study. In the batch study, pH did not significantly influence the folate production. This study suggested the folate production by the *B. adolescentis* did not influence by the external folate addition, PABA concentration, pH

and carbon sources. After the extensive work, this study provided the new prospective about the folate production by the probiotic strain in the intestinal range.

Hjortmo *et al.,* (2008a) in which they investigated the effect of growth medium and cultivation conditions on folate content in a yeast strain of *S. cerevisiae*. It has been known since long time that yeast has high folate content per unit biomass as compared to other folate sources (Witthoft *et al.,* 1999). However this is the first kind of study in which effect of growth conditions on folate content of yeast has been studied as no studies till date raised the questions regarding the folate content in yeast on changing the growth conditions. Thus this study has the importance itself to develop the production process for yeast folates and which lead to increasing folate content foods fermented by the yeast. In this study, Folate content of a *Saccharomyces cerevisiae* strain was monitored in synthetic growth medium (YPG) and a molasses based medium during aerobic batch fermentation. In synthetic growth medium, large differences has been observed in intracellular folate content at different growth phases. It has been suggested that folate content was largely affected by the physiological state of the cells. It was confirmed in chemostat cultures where increase in growth rate (r^2=0.998) would affect the total intracellular folate content, indicating high growth rate i.e. respiro-fermentative growth is the most favorable phase to obtain high folate content. However in complex media, folate content (15–40 μg/g) was found very less throughout the batch growth. The results showed the influence of cultivation medium on folate content in yeast. Further the specific components were added in synthetic medium during batch experiments like a raw mixture of peptides and amino acids (peptone) which depleted the folate levels extensively by (90%) whereas amino acids addition one-by-one only had little effects on the intracellular folate content. Furthermore, PABA, folate or nucleotides supplementation in synthetic medium did not change the intracellular folate content. This work demonstrated the first step for the optimization process for production of natural folates used for fortification purposes, as well as to establish a fundamental understanding of yeast folate requirements with respect to the environmental conditions.

Another study by Hjortmo *et al.,* (2008b) reported a study in which they stated that folate content of fermented food which was inoculated with yeast can be increased

by using a proper yeast strain and its appropriate cultivation conditions during food fermentation. *Saccharomyces cerevisiae* strain CBS7764 enhanced the folate levels to 3 to 5-fold of white wheat bread when cultured in defined medium and harvested in the respiro-fermentative phase of growth prior to dough preparation (135–139 µg/100 dry matter). However white wheat bread prepared with commercial Baker's yeast can only enhanced upto 27–43 µg/100 g. Thus an alternative strategy is to produce bread containing high level of natural folate by choosing a proper yeast strain and suitable cultivation conditions rather than synthetically produced folic acid.

Iyer *et al.,* (2009) described a method of microbiological assay using *Lactobacillus rhamnosus* with an additional tri-enzyme extraction using protease, α-amylase and conjugase for the detection of folate in casein based media for its applicability in milk from different Indian milk species. By this method average value of folate found as 10 µg/L, 44 µg/L, 56 µg/L, 60 µg/L respectively in goat, cow, sheep and buffalo milk. Thus buffalo milk was found as richest source of folate by this method. Later on efficiency of this method was validated with the other methods available in the literature. Finally it has been observed that although this is a labor intensive method but it was highly selective, sensitive and reproducible method and gave the total folate content of the sample.

Further a study by Tomar *et al.,* (2009) based on the modified microbiological assay using *Lactobacillus rhamnosus* with additional trienzyme treatment using protease, α-amylase and human plasma conjugase and folic acid *casei* as the basal medium. Among the twelve strains of *S. thermophilus* were screened for the highest folate production in which NCDC 177 was found to produce highest folate production (34.28 µg/L) followed by the NCDC 199 (27.34 µg/L) and NCDC 303 (19.36 µg/L). However, NCDC 80 and NCDC 312 were found to deplete the folate level of the skim milk medium which indicated the consuming nature of these strains. Further the conventional optimization of culture conditions and medium additives were done to enhance the folate level. Optimum conditions for the maximum folate production were as follows: incubation temperature 42°C, incubation time 48 h, lactose 1%, PABA 100µM and NaCl

0.8% in skim milk medium. On implementing these culture conditions, folate concentration was found to increase by 62%.

Another study by Kariluoto *et al.,* (2010) showed folate production studies by the bacteria isolated from the oat bran. Three commercial oat bran products were chosen for the isolation of bacteria and finally twenty bacteria were isolated and tested for their folate production ability. The isolated bacteria and some reference microorganisms were grown in YPG medium and the amount of total folate i.e. intracellular (separated cell mass) and extracellular (supernatant) was determined by microbiological assay. Some strains showed the large amount of intracellular (up to 20.8 μg/g) and extracellular (up to 0.38 μg/g) folate production. Effect of temperature and pH was also studied and it has been observed that intracellular folate content was higher generally at the 28°C incubation temperature than at 18 °C or 37 °C and at pH 7 than at pH 5.5. Folate vitamer distribution was determined by HPLC for eight bacteria including one isolated from rye flakes. It has been reported that vitamer distribution was remarkably different with the individual microorganisms and changes of growth phase from exponential to stationary phase thus it can be concluded as vitamer distribution is dependent on the strain and the growth phase. The main vitamers identified from the eight studied strains were tetrahydrofolate, 5, 10-methenyltetrahydrofolate, 5-methyltetrahydrofolate and 5-formyltetrahydrofolate. The best folate producers were identified as *Bacillus subtilis* ON4, *Chryseobacterium* sp. NR7, *Janthinobacterium* sp. RB4, *Pantoea agglomerans* ON2, and *Pseudomonas* sp ON8. However, the folate excreted into the medium was found to be highest for *B. subtilis* ON5, *Chryseobacterium* sp. NR7, *Curtobacterium* sp. ON7, *Enterococcus durans* ON9, *Janthinobacterium* sp. RB4, *Paenibacillus* sp. ON10, *Propionibacterium* sp. RB9, and *Staphylococcus kloosii* RB7. Overall elevation in the folate amount during bacterial growth was found to be mainly associated with the proportional decrease of 5-formyltetrahydrofolate and proportional increase in the 5-methyltetrahydrofolate content. Folate vitamer distribution in the folate produced by the cereal isolated bacteria was first of all reported in this study which was found to be in agreement with the study of Lin & Young (2000b) in which the main folate vitamers produced from the *Bifidobacterium*, *Lactobacillus* and *Streptococcus* was also similar.

Hugenschimdt *et al.,* (2010) reported a study on the lactic acid bacteria (LAB) and propionic acid bacteria (PAB), which are known for their production ability of several important nutraceuticals. In this, 151 LAB and 100 PAB isolates of different origins (fermented foods and feeds) were screened for extracellular folate production and intracellular vitamin B_{12} production. For both the studies, supplemented whey permeate was chosen as the medium and analysis of folate was done by standardized microbiological assay using *Lactobacillus casei* ATCC 7469 as indicator organism and vitamin B_{12} by the HPLC method. In this study organic acid quantification and sugar analysis was also done. Extracellular folate production in SWP medium by the all selected LAB and PAB strains was analyzed after 24 h and 72 h of incubation. Most of the LAB and PAB showed the folate production upto 27ng/mL in SWP medium. However, among all the selected strains best producers were *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus brevis* and *Lactobacillus fermentum* which exhibited the higher extracellular folate productions. Maximum yield of folate was found to be 397±60 ng/mL by *L. plantarum* SM39. The folate yield reported in this study was eight times higher in comparison to the other studies reported by the others as 48 ng/mL and 41 ng/mL respectively by the LAB and PAB (Hugenholtz *et al.,* 2002; Sybesma *et al.,* 2003). PAB strains showed the extracellular folate production (<14 ng/mL) which was much lower than by LAB strains (<397 ng/mL). *Propionibacterium freudenreichii* DF15 produced the highest vitamin B12 production i.e. 2.5 mg/mL. Extracellular folate production by this study was found to be strain as well as species specific thus many strains of different origin might be tested for the folate production efficiency. Thus screening of large biodiversity of LAB and PAB can led to the identification of high natural folate producers and vitamin B12 producer strains for applications in fermented foods.

Dana *et al.,* (2010) demonstrated a study in which *Lactobacilli* of traditional fermented milk origin were evaluated for folate production efficiency. It is well known fact that milk or dairy products are good sources of B vitamins which are generally produced by probiotics. The aim of this study was to find suitable strains having capability of high folate production, their isolation and identification from the traditional fermented milk which was procured from two different provinces located in the west of

Iran i.e. Ilam and Lorestan. *Lactobacilli* were isolated according to the method described in ISO 7889 standard procedure. The isolated bacteria were primarily identified on the basis of catalase reaction and gelation liquefaction and nitrate reduction etc. tests and characterized phenotypically. Folate production ability of the isolated strains was checked by the fermentation of skim milk. In this study they had used a kit for the total folate assay and used the *Lactobacillus rhamnosus* strain as control because growth of *L. rhamnosus* is dependent on the content of folate in the growth medium. Folate production by the studied strains was found in between the 2.8 to 66.6 µg/L. Then the two highest folate producing strains were selected and identified by the 16S rRNA gene sequencing and finally by constructing a phylogenetic tree. Finally the maximum folate producer strains were identified to be the similar to *Lactobacillus crustorum* by using both the biochemical and preliminary molecular analysis. Therefore, these two strains could be used as high folate producing probiotics in the dairy industry.

Nor *et al.*, (2010) demonstrated the screening of some lactic acid bacteria (*Lactococcus lactis* NZ9000, *Lactococcus lactis* MG1363, *Lactobacillus plantarum* I-UL4 and *Lactobacillus johnsonii* DSM 20553) for their ability to produce intracellular and extracellular folate. All the strains were cultivated in modified MRS medium that contains addition of PABA in the 0.01 µM concentration. Cells and supernatant recovered from the fermented broth were used to measure respectively the intracellular and extracellular folate content. Folate analysis was performed by the microbial assay using *L. casei* ATCC 7469 in folic acid *casei* medium. *L. plantarum* I-UL4 was found to be the highest folate producer (36.36 mg/L) as compared to other tested strains. Cell and substrate concentrations were also analysed in this study and after evaluation it was suggested that substrate was mainly consumed for the cell growth rather than the folate synthesis. It has also been stated that higher cell growth tends to increase the viscosity of the medium thus reduce the availability of substrate for consumption and there was reduction in folate production efficiency. In the next phase of this study, conventional and statistical experimental designs (Central Composite Design) were used to optimize the medium formulation for the better growth and folate biosynthesis of *L. plantarum* I-UL4. Lactose, maltose and glucose were selected as carbon source optimization in which lactose showed the highest folate production (36.19 µg/L), although it grew well on all

the carbon sources. For the nitrogen source optimization, yeast extract, meat extract and peptone were used. Among these, meat extract was found to be best nitrogen source for folate production (47.01µg/L) however yeast extract was found to be best for the maximum cell concentration. Further the manual optimization of lactose, meat extract and PABA concentration was done to get the maximum folate concentration. Later on, the optimal values of these three important factors i.e. lactose, meat extract and PABA were determined by response surface methodology (RSM). Best conditions suggested in this study for the optimal growth of *L. plantarum* I-UL4 and folate production were the lactose 20 g/L, meat extract 16.57 g/L and PABA 10 µM. After implementing these optimized medium conditions compared to standard MRS medium, folate production by *L. plantarum* I-UL4 was found to be enhanced from 36.36 to 60.39 mg/L.

Herranen *et al.*, (2010) reported a study with the aim of identification of endogenous bacteria present in commercial oat bran and rye flake products in order to check their folate production ability while maintaining the soluble dietary fibers in physiologically active and unhydrolyzed form. In this study, 42 and 26 bacteria were isolated respectively from three different oat bran products and from one rye flake product. The bacteria were identified by 16S rRNA gene sequencing analysis. The most common bacterial genus found in oat bran was *Pantoea, Acinetobacter, Bacillus*, and *Staphylococcus*. *Pantoea* species was also the dominant genus in rye flakes. Folate production ability was analysed at the stationary growth phase from aerobic cell cultures. The amount of intracellular and extracellular folates was determined separately from the cell mass and the supernatant by microbiological assay. The best folate producers isolated from oat bran belonged to the genera *Bacillus, Janthinobacterium, Pantoea*, and *Pseudomonas*, and those isolated from rye flakes are *Chryseobacterium, Erwinia, Plantibacter*, and *Pseudomonas*. Extracellular folate contents were found to be higher for *Bacillus, Erwinia, Janthinobacterium, Pseudomonas,* and *Sanguibacter*. This work concluded that endogenous bacteria were better folate producers in comparison to lactic acid bacteria. This work gave the first insight into the potential ability of folate production by endogenous microflora thus modulation of the nutrient levels of oat and rye based cereal products.

Gangadharan *et al.,* (2010) reported a study about the folate producing ability of the lactic acid bacteria isolated from cow's milk and evaluation of probiotic characteristics. The probiotic potential of newly isolated lactic acid bacteria from cow's milk was checked in terms of tolerance to low pH, phenol, bile and NaCl and antimicrobial activity against potential pathogens and by antibiotic resistance study and cell surface hydrophobicity. Further efficacy of the selected isolates for folic acid production was evaluated by microbiological assay. Later on, quantitative determination of various forms of folate was also done by the high performance liquid chromatography on C18 Nova-Pak analytical column. Two best folate producing isolates, CM 22 and CM 28 were identified as probiotic strains and selected for folate production in skim milk medium and the net folate yield was found to be 12.5 ng/mL and 14.2 ng⁄mL respectively. HPLC chromatogram revealed that THF and MTHF were the major form synthesized by CM 28 and THF was the major form synthesized by the CM 22. These two isolates (CM 22 and CM 28) resembled more than 98% to *Lactococcus subsp. cremoris* and *Lactococcus lactis* subsp. *lactis* respectively by 16S rRNA sequencing.

Iyer *et al.,* (2010a) reported a study in which optimization of cultivation conditions and medium components were done by response surface methodology to enhance the folate production by *S. thermophilus* strain RD102. RSM was carried out using a 2^3 central composite design and surface modelling method. As in this study folate production was found to be growth-associated which was found in agreement with the reported study (Sybesma *et. al.,* 2003) so both folate production and growth were considered as the desired responses for the optimization. Incubation period, p-amino benzoic acid (PABA) and lactose were selected as factors based on preliminary investigations (Tomar *et. al.,* 2009). The optimum time of incubation was found to be 72 h and concentrations of PABA and lactose were found to be 300 µM and 3% respectively. The optimized conditions obtained from RSM resulted in elevation of folate production by 26% as compared to the control. Increase in folate production was also observed in this study along with the increase in cell growth. Thus experimental factorial design and response surface analysis can be applied for the determination of optimal operating conditions to obtain a higher folate production by *Streptococcus thermophilus*.

Therefore, this study leads to the direction for developing strategies to enhance the folate level upto a maximum extent.

The probiotic potential of two high folate producing strains, *Streptococcus thermophilus* strains (RD102 and RD104) was done by *in vitro* and *in vivo* test (Iyer *et. al.,* 2010b). Acid and bile tolerance study (Clark *et al.,* 1997; Gilliland *et al.,* 1984), cell surface hydrophobicity (Rosenberg *et al.,* 1980), bile salt deconjugation (Taranto *et al.,* 1995), β-galactosidase activity using ONPG disc assay, antibiotic sensitivity (Performance standards for antimicrobial disk susceptibility tests, 2007) and gastrointestinal stress tolerance test (Charteris *et al.,* 1998b) are the *in vitro* methods to analyze the probiotic efficiency however *in vivo* feeding test were also performed with the mice to check the characteristics. These strains were able to survive even at pH 2.5 and with 2% bile salt with a significant bile salt hydrolase activity, positive cell surface hydrophobicity and showed sensitivity to most of the clinically important antibiotics. These strains showed a viable count of 5 log cfu/mL and 7 log cfu/mL, respectively in simulated gastrointestinal stress tolerance test at pH 2.0 and 2% bile salt. Strains showed a viable count of about 7 log cfu/g in faeces and 6 log cfu/g in large intestine, respectively during the *in vivo* feeding trial in mice. Thus, these strains were found to possess probiotic properties and have the potential to develop the functional foods as source of novel probiotics.

2011-2015:

A review report on folic acid fortification, its history and its effects was published (Crider *et al.,* 2011). Implementation of mandatory fortification in different countries has been given in this article. In United States, fortification of folic acid at the level of 400 µg/100g in breakfast cereals or other food items is mandatory since 1998. Canada and Costa Rica has also mandated the fortification in the same year but the amount varies as 150 µg/100g and 180 µg/100g respectively. In Chile, folic acid fortification was mandated in 2000 at the level of 220 µg folic acid per 100g of food. Later on in 2003, South Africa also implemented the mandatory fortification of folic acid at the level of 150 µg/100g.

Another study by Gangadharan and Nampoothiri, (2011) in which *Lactococcus lactis ssp cremoris*, an isolate from raw cow's milk resulted in the elevated folate level (164 ng/ml, deconjugated folate) in skim milk by effective manipulation of medium additives and cultivation conditions. Fermented milk was considered as potential matrix for folate production because of the presence folate binding proteins in milk which improves the folate stability as well as folate bioavailability (Verwei *et al.*, 2003). PABA and glutamate addition individually from 25-75 µmol/L resulted in elevated levels of extracellular folate however further increase lowered the folate production. Highest folate production (17.2±0.7 ng/mL) was found at 8 h of fermentation by *L. lactis* after that production gradually decreased. Optimum temperature for maximum production was found at 37°C rather than 30°C. Sorbitol and mannitol addition in fermented milk showed that mannitol addition had superior effect on folate production (37.2±1.3 ng/mL) as compared to sorbitol due to the nature as efficient osmolyte thus acts as protector of *L. lactis* cells (Efiuvwevwere *et. al.,* 1999). Sodium thioglycolate and sodium ascorbate addition also resulted in the enhanced folate production due to the antioxidant nature of ascorbates and thiols (Gregory, 1996). Further scale up of the folate production in 5 L bioreactors resulted in the maximum titer of 187 ng/ml deconjugated folate. *L. lactis* was also used for the fermentative fortification of juices and proved to be an excellent source for the folate enrichment in cucumber and water melon juice. Initially the folate level of cucumber and melon juice was 10±0.2 and 18±0.9 ng/mL respectively which elevated to 60±1.9 and 26±1.6 ng/mL respectively after fermentation. PABA and glutamate addition (25µM) did not affect the folate level of cucumber juice but increased the folate level of watermelon juice (36±2.3 ng/mL).

Hugenschmidt *et al*., (2011) reported a study in which a high folate producer *Lactobacillus plantarum* SM39 was cultured in combination with a vitamin B_{12} producer, *Propionibacterium freudenreichii* DF13 in order to get both the folate and vitamin B_{12} as both the vitamins have independent metabolisms. Both the strains were co-cultured in whey permeate medium supplemented with yeast extract (SWP) in order to produce highest levels of both vitamins. Addition of 5 mg/L cobalt chloride, 15 mg/L DMBI, and 10 mg/L PABA in SWP medium followed by a two-step fermentation under optimal conditions, i.e. three days anaerobic/four days aerobic, led to highest vitamin B_{12} and

folate yields i.e. 751±353 ng/mL and 8399±784 ng/mL respectively. In this study, Folate yields were observed to be more than 10-fold higher than maximum values reported for natural fermentations using a genetically optimized strain. Supplementation of whey permeate with 5 mg/L cobalt(II) chloride, 15 mg/L DMBI, and 10 mg/L PABA resulted in the ratio of folate and vitamin B_{12} in 11:1 which can be possibly enhanced by further optimization to produce a vitamin supplement with a suitable balance of folate and vitamin B_{12} of 170:1 suggested for human consumption. The fermented medium containing high levels of natural folate and vitamin B_{12} content could be used to prepare the functional foods by fortification with either natural vitamins or the nutritional supplement.

Iyer & Tomar (2011) demonstrated a study in which dietary effect of folate rich fermented milk produced by the *S. thermophilus* RD 102 and RD 104 had been observed on the hemoglobin level using a murine model. Folate functions as a carbon carrier in the formation of heme i.e. iron-containing non-protein portion of hemoglobin. Thus its deficiency in humans leads to anemia. Thirty-two albino mice 30±10 day old were provided a basal diet for consumption (i.e., a synthetic anemic diet, n= 8, group I, control 1), a basal diet with skim milk (n= 8, group II, control 2), a basal diet with fermented skim milk produced by folate plus RD 102 (n= 8, group III, test 1), and a basal diet with fermented skim milk produced by folate plus RD 104 (n= 8, group IV, test 2) in a 6-week, double-blind, placebo-controlled study. Hemoglobin concentrations were estimated by the Drabkin–Austin cyanmethemoglobin method (Drabkin & Austin, 1932) during the prefeeding (10 d), feeding (20 d), and postfeeding (10 d) trials, respectively. The results indicated that the test groups (III and IV) which consuming folate-rich fermented milks prepared by high-folate producing *S. thermophilus* RD 102 and RD 104 strains showed a significant elevation in hemoglobin level compared to the control groups (I and II) consuming anemic diet and skim milk respectively. Thus, folate-rich fermented milks produced by the *S. thermophilus* strains had positive potential to significantly increase the hemoglobin level of blood and hence offers a novel approach to fortify dairy products with natural folate.

Another study by Iyer *et al.,* (2011) in which bio-prospecting study of folate producing strains of *Streptococcus thermophilus* was done which were isolated from milk and different fermented milk products of Indian origin. 209 different samples were taken from which around 500 randomly colonies were obtained. Among these, 117 isolates were characterized and identified as *S. thermophilus* by classical biochemical and molecular characterization. Occurrence frequency of *S. thermophilus* was found to be highest in the dahi followed by yogurt and lassi and a very low frequency was observed in milk and cheese. About 15% of studied strains were found to produce folate in the range of 40–50 μg/L, 35% strains in the range of 20–30 μg/L, and the remaining strains produced in the range of 4–16 μg/L on quantitative screening of folate using a microbiological assay along with the trienzyme extraction of the samples. Phenotypic characterization of the of the ten highest folate producers was done by the species specific polymerase chain reaction (PCR) based on the Lac Z gene (Lick *et al.,* 1996). Comparative analysis of the random amplification of polymorphic DNA PCR fingerprint profiles was used to characterize interspecific diversity. The Lac Z gene sequence of two of the highest folate producing isolates RD 102 and RD 104 were PCR amplified and sequenced and submitted to NCBI GenBank database under following accession numbers of FJ161697 and FJ161698.

Divya *et al.,* (2012) demonstrated a study in which five new lactic acid bacteria were isolated from milk products and vegetables on MRS agar supplemented with 1% $CaCO_3$ at 37°C for 48 h and later on identified by 16S rRNA sequencing as *Weissella cibaria, Enterococcus faecium,* and three different strains of *Lactobacillus plantarum*. Essential probiotic properties of these isolates such as tolerance to phenol, low pH, high sodium chloride, and bile salt concentration were checked *in vitro*. Efficiency of adherence to mucin and cell surface hydrophobicity was also evaluated by *in vitro* studies. Antimicrobial activities against some potential pathogens were checked antibiotic sensitivity of these strains against 25 different antibiotics was also tested to confirm the probiotics efficiency. Further the folate production ability was also checked in which *Weissella* and *Enterococcus* were revealed as substantial producers of folic acid. The folate level in the fermented samples was determined by microbiological assay using *Lactobacillus casei* NCIM 2364 as indicator strain. Antifungal activity was also checked

and three strains of *L. plantarum* showed positive significant inhibitory activity against some common fungi that is responsible for food stuffs contamination thus indicated the potential use of strains as a bio preservative of food material. Antifungal activity of these strains may be due to the production of organic acids, H_2O_2 and cyclic peptides etc. Thus these two strains *W. cibaria* and *E. faecium* can be attributed for the functional food production. Similarly three strains of *L. plantarum* can be used to formulate the potent bio preservatives in food.

Padalino *et al.,* (2012) demonstrated folate production ability of *Bifidobacterium catenulatum*, *Bifidobacterium adolescentis*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* were investigated in milk and complex media. Moreover, the main focus of the study was to check the effect of two prebiotics addition, fructooligosaccharides and galactooligosaccharides, on folate biosynthesis. Bacterial growth kinetics of all studied strains was also observed in both complex medium and in non-fat milk with and without FOS (1.0 g/100 mL) and GOS (1.0 g/100 mL) addition. The growth curves showed almost a similar pattern in all the studied strain with stationary phase after approximately 10 h of incubation time and higher number of colony forming units was observed in complex medium than in milk except in *S. thermophilus*. Prebiotic addition resulted in higher number of bacterial counts than in medium without prebiotic additive. Folate levels were tested in both nonfat milk and complex media generally highest production was found within the 6-10 h of incubation time in a prebiotic free medium. Levels of the dominant folate forms produced i.e., tetrahydrofolate and 5-methyltetrahydrofolate, were also determined using high performance liquid chromatography after 0, 6, 10 and 24 h incubation. *B. catenulatum* (28.82 ±2.02 µg/ 100mL) and *S. thermophilus* (19.03 ±1.95 µg/100 mL) produced the highest level of folate respectively in complex media and milk. Lowest level of folate was produced by the *L. plantarum* in 10 h. The inclusion of any of the prebiotics FOS and GOS in the culture medium did not significantly stimulate the synthesis of folate by any of the five strains studied, although it increased only the rate of bacterial growth. The reason of the lower folate production on FOS and GOS addition may be due to the greater amount of the acetic acid and lactic acid production in presence of oligosaccharides (Chick *et al.,* 2001). Thus it may be established that there might be negative relationship

between the folate production and acidic pH of the medium and destruction of labile folate forms in the acidic environment.

Mousavi *et al.,* (2013) reported a study in which effects of medium and culture conditions on folate production by *Streptococcus thermophilus* BAA 250 was studied. The bacterial strain was cultivated in modified M17 medium containing yeast extract (2.5 g/L), peptone from meat (5 g/l), peptone from casein (5 g/l), $MgSO_4$ (0.25 g/l), K_2HPO_4 (0.6 g/l), PABA (0.1 mg/l), lactose (5 g/l) and ascorbic acid (0.5 g/L) (Galia *et al.*, 2009; Sybesma *et al.*, 2003; Zisu & Shah, 2003). Effect of carbon sources such as glucose, maltose and sucrose and organic nitrogen sources as meat extract, yeast extract, peptone/casein or peptone/meat extract and inorganic nitrogen sources as ammonium nitrate and ammonium sulphate were studied in the shake flask fermentation. Lactose (3g/L) and yeast extract (20g/L) respectively were found to be the best suitable carbon and nitrogen sources for maximum folate production by *S. thermophilus* BAA-250. PABA had no significant effect on folate production in the concentration greater than the 1 μM by *S. thermophilus*. The optimum pH was found to be 7.0 for maximum folate yield and productivity of 54.53μg/L and 2.27μg/L.h, respectively. Optimum folate concentration of about two fold increase in folate yield obtained in the presence of lactose and yeast extract in a controlled pH of 7 during batch fermentation in bioreactor. However it has been suggested that pH has no significant effect on the intracellular and extracellular folate distribution and excretion into the medium. Further the kinetic studies of folate production indicated that it is growth-associated process in case of *S. thermophilus*.

Laino *et al*., (2013) reported a study for the production of folate bio-enriched yogurt by some selected lactic acid bacteria. Folate producing *Lactobacillus delbrueckii subsp. bulgaricus* (3 strains) and *Streptococcus thermophilus* (2 strains) were taken for this study and used in different combinations to prepare 15 different yogurts. Samples were taken at different time intervals during the fermentation process and analysed for folate concentrations using a modified microbiological assay (Horne & Patterson, 1988). The yogurt elaborated with the combination of strains CRL871+CRL803+CRL415 resulted in significantly higher folate concentrations (180±10 mg/L) when incubated at

42°C which implies that almost a 250% increment was observed with respect to non-fermented milk and about 125% increment as compared to commercial yogurts. No significant reduction in folate concentration was observed during 28 days of storage at 4°C. Thus this kind of novel folate bio-enriched yogurts can be prepared by the proper combination of the native folate producing starter cultures which can be able to satisfy the demands of consumers.

Iyer & Tomar, (2013) had given a research report in which concurrent determination of folate versus folic acid in milk was done by several different analytical methods such as microbiological assay with *Lactobacillus rhamnosus* as the indicator assay organism, Enzyme Linked Immuno Sorbent Assay (ELISA) by competitive binding rapid ELISA kit (RIDASCREEN®) and high-pressure-liquid chromatography (HPLC) for detection of the different folate forms and its level present in the sample. Different samples of fresh raw pooled cow, buffalo, sheep and goat milk were taken for the study. Sample preparation was done by the method described by the Keagy, (1985) with additional tri-enzyme treatment (Iyer *et al.,* 2009; Tomar *et al.* 2009). It has been noted that microbiological assay with *L. rhamnosus* gave almost similar response to most folate isomers extracted by the tri-enzyme treatment thus estimated the total folate content compared to the other two methods which specifically estimated the folic acid content of the sample. In case of ELISA, major specificity was limited to mainly folic acid and dihydro folic acid thus it showed a lower response in case of other folate derivatives. The results of the ELISA methods were found to be in agreement with other assay techniques thus validating the reliability of the method. HPLC chromatogram of milk samples resulted in several peaks which indicated the different forms of compounds present in the sample. HPLC is highly specific, and so any peak emerging in chromatogram from the analytical column represented a specific folate derivative (Pffeiffer *et al.*, 1997). In this study, it has been demonstrated that estimation by HPLC with UV detector was highly specific and hence only folic acid could be detected without any cross reactivity. Thus, HPLC was observed to be the most sensitive method among the different methods for determination of folic acid and its different derivatives and can efficiently determine the folic acid fortification level. However, microbiological assay remained highly sensitive,

efficient and reproducible method for estimation of total folate so can be potentially applied use for dietary folate estimation.

Ahire *et al.,* (2013) reported a study in which isolation of potential folate producing strains were done from the conventional household fermented milk using microbiological techniques. *Lactobacillus helveticus* CD6 was identified as potential folate producer. Screening for folate production was performed by the disc assay diffusion method given by the Vakil and Shahani, (1968) with some modifications. Folate analysis was done by the HPLC methods using C18 reverse phase column. It has been observed that isolate CD6 mainly showed the production of 5-methyl tetrahydrofolate that is biologically active form. Further the antioxidative potential and probiotic potential of the folate producer strain has been checked. It was concluded in this study that folate producer *Lactobacillus helveticus* CD6 can serve the purpose of antioxidative potential along with the probiotic effect.

Laino *et al.,* (2014) investigated the folate production ability of 55 strains from different *Lactobacillus* species. In order to evaluate folic acid production efficiency, lactobacilli were cultivated and propagated in the folate-free culture medium (FACM) seven times. Most of the tested strains needed folate for growth thus the *Lactobacilli* showing good growth were considered for the folate production studies. The folate production and the extent of extracellular and intracellular vitamin accumulation were particular features of individual strains. For the identification and physiological characterization of the best producer strain API 50 CH system was used. Finally strain was identified as *Lactobacillus amylovorus* CRL887 and it was selected for further studies due to its ability to produce significantly higher concentrations of folate (81.2 ±5.4 μg/L). The growth kinetics study and vitamin production in folate free complex medium showed the ability of strain to grow even in absence of folic acid and growth associated folate production as mostly the production occurred in the exponential growth phase of the strain. However it has been observed that this strain did not have the capability to enhance the folate content of the milk even after 24 h of incubation. The safety of this newly identified folate producing strain was evaluated on healthy adult BALB/c experimental mice. No bacterial translocation and no undesirable side effects

had been detected in liver and spleen even after 7 days of CRL887 consumption. This strain was then co-cultured with previously selected folate producing starter cultures (*Lactobacillus bulgaricus* CRL871, and *Streptococcus thermophilus* CRL803 and CRL415) (Laino *et al.*, 2013; Holasova *et al.*, 2004; Holasova *et al.*, 2005, Wouters *et al.*, 2002) which resulted the high folate concentrations (263.1 ± 2.4 µg/L) in yogurt in 4 h of incubation which can provide almost 15% of the recommended dietary allowance in single serving. These are the first results reported in which *Lactobacillus amylovorus* strain can be successfully used as co-culture for development of natural folate enriched fermented milk.

Another study by Divya and Nampoothiri, (2014) in which a folate producing probiotic lactic acid bacterium isolated from cow's milk and identified as *Lactococcus lactis* CM28 by 16S rRNA sequencing was used to fortify skim milk in order to enhance its folate level. Optimization of culture condition like incubation time and temperature along with some medium additives such as folate precursors (PABA and glutamate), prebiotics (sorbitol, mannitol and FOS) and reducing agents (sodium ascorbate, sodium thioglycholate and cysteine hydrochloride) was done to find out suitable factors for enhanced folate levels in skim milk at both stationary and shaking (100 rpm) condition. Optimization results are incubation time of 8 h, incubation temperature 37°C, 0.6% mannitol addition, 100 µmol/L PABA and 75 µmol/L glutamate, 0.2% sodium ascorbate, glycine (6 µmol/L), methionine (6 µmol/L), mannitol (0.6 %). A four fold increase in the extracellular folate (61.02±1.3 µg/L) was detected with the optimized conditions of skim milk medium and after deconjugation, the total folate concentration was 129.53±1.2 µg/L. The effect of refrigerated storage on the folate stability, viability of *L. lactis*, pH, and finally titratable acidity (TA) in terms of percentage lactic acid was determined. Only a slight variation in pH (4.74±0.02 to 4.415±0.007) and acidity (0.28±0.028 to 0.48±0.014 %) were noted during folate fermentation. Probiotics should survive the acidic environment of the stomach and reach the intestine in high numbers upon ingestion and adhere to the intestinal walls and multiply to exert probiotics health benefits on the host. According to the recommendation of the International Dairy Federation the minimum number of probiotic cells at the time of consumption should be $\geq 10^7$ CFU/g (Ouwehand & Salminen, 1998). It is also important that the probiotic product retains its

functionality throughout the storage period (Daneshi *et al.,* 2013). In this study, folate levels, pH, TA and viable counts of the fermented milk were measured initially and then at every 5 days till 15 days. Approximately 90 % of the folate was found to be stable even after 15 days. The viable count of bacteria decreased to 6×10^{10} CFU/ml (10.78 log CFU/ml) after 15 days which were sufficient enough to exert health benefits on the host. It was observed that the TA slightly increased and pH decreased over the storage period. The decrease in pH or post acidification of fermented milk on cold storage was due to β-galactosidase activity (Kailasapathy, 2006). This in turn affects the viability of the probiotic bacteria. Only less than a log unit reduction was noted in the viable count of the probiotic even after 15 days of storage.

Divya & Nampoothiri, (2015) reported a folate production study by the immobilized cells of the two isolated bacteria from cow's milk. Two lactic acid bacteria (LAB) were identified as *Lactococcus lactis* strains and designated as *L. lactis* CM22 and *L. lactis* CM28. They were immobilised in co-encapsulation matrix. Further the survival study of free cells and encapsulated cells was done in simulated gastric juice and simulated intestinal juice. Results indicated that encapsulated cells showed better survival ability in simulated gastrointestinal conditions as compared to the free cells. The percentage survival of probiotics encapsulated by hybrid entrapment method was 62.74% for *L. lactis* CM22 and 68% for *L. lactis* CM28. Studies to check their efficacy in fermentative fortification of skim milk and ice cream revealed an enhancement in folate level.

Previous studies suggested that Bifidobacteria have the ability to produce folate, a vital vitamin for humans. In another study, a total of 44 strains, including 12 species and 7 subspecies, of bifidobacteria were checked for the folate production in a medium which containing only the required levels of folate for growth of the tested strains (Sugahara *et al.,* 2015). All the human-residential bifidobacteria (HRB) showed the capability to produce folate *in vitro.* Whereas most strains of non-HRB did not had the potential for folate production except the *B. thermophilum* and *B. longum ssp. suis* strains. *In vivo* folate production by both HRB and non-HRB was further evaluated and confirmed using mono-associated mice. Folate concentrations in fecal samples, hemoglobin levels of

blood and mean corpuscular volumes were found to be significantly higher in the mice colonized with a folate producer HRB strain, *B. longum subsp. longum*, than the mice colonized with a nonproducer, *B. animalis subsp. lactis*. These results confirmed that *in vitro* folate production ability was higher in HRB strains in comparison to the non HRB strains in a medium containing essential levels of folate for growth and suggested the utilization of HRB strains for the potential folate delivery in hosts.

Kodi *et al.,* (2015) demonstrated the ability of folate production by the *Lactobacillus* species Lcf10 which was isolated from fermented milk products. Probiotic properties of the strain were also evaluated. Probiotics evaluation studies showed the positive results which confirmed the possibility of administration of these strains in the form of probiotic beverages. It showed positive antimicrobial activity against enteric pathogens like *E. coli, Staphylococcus aureus*, *Proteus mirabilis*, *Bacillus cereus*. Hence probiotic curd can be prepared by using Lcf10 during curd fermentation that will produce the cheaper and healthier functional food for the consumer benefits.

2.2 OBJECTIVES

The research work was undertaken with the aim to produce Folate (folic acid), a water soluble vitamin, which has significant role in the prevention of various disease like neural tube defects, cardiovascular disease and cancer. The work has been initiated to explore the possibility of selecting suitable microorganisms having higher production efficiency and their exploitation for the maximum production of natural folate and its fermentative fortification in food items.

The first phase of the work involved the selection of suitable microorganisms for the folate production on the basis of literature survey as well as increased production of folic acid.

The second phase of work concerned mainly with the evaluation of probiotic potential of folate producing strains by *in vitro* methods. This evaluation was essential because the natural fermentation media was used throughout the studies.

The third phase of work dealt with the optimization of several parameters, *viz*. inoculum development, medium composition, growth parameters, different physical parameters like pH, temperature, incubation time and various carbon sources, etc. and validation of optimized parameters. Both conventional and response surface methodology was used. Response surface methodology was done in two stages; Plackett-Burman design, to identify the significant factors for folate production and later on, the significant parameters were optimized by using a central composite design.

The fourth phase of work dealt with the folate production studies using immobilized cells.

The fifth phase of work mainly concerned with fermentative fortification of food products which was done by probiotic microbial strains and to check the stability and shelf life of fortified food products after storage.