CHAPTER 1
Introduction and Review of Literature

1.1. Introduction

Folate (vitamin B9) is involved in several vital metabolic reactions including DNA replication, repair and methylation; nucleotide synthesis and amino acid inter conversions. Mammalian cells cannot synthesize this vitamin and hence it is an essential component in human diet. The daily recommended intake (DRI) is 200 - 400 µg for adults and for pregnant women and women of child bearing age, a higher dose (600 µg) is recommended. The protective role of folate against neural tube defects in newborns is well established (Lucock, 2000; Pitkin, 2007). Low folate status has been linked to a wide variety of disorders including cardiovascular diseases, anaemia, Alzheimer's disease, poor cognitive performance, osteoporosis and certain forms of cancer (Boushey et al., 1995; Brattstrom, 1996; Ames, 1999).

Despite its wide availability, folate deficiency still exists even in developed countries. This resulted in the implementation of mandatory folate fortification programmes by many countries. Some studies have shown that high intake of folic acid, the chemically synthesized form, but not natural folates may cause certain adverse effects including masking the symptoms of vitamin B12 deficiency, bowel cancer, and arthritis (Iyer & Tomar, 2009). Natural inhibitors present in the food along with the physiological conditions in the gut could contribute to the decreased bioavailability of folate. The various methods of cooking also cause thermal degradation as well as leaching of folate into the cooking water resulting in significant loss of the vitamin (McKillop et al., 2002). Fermented foods have higher concentration of naturally occurring folate produced primarily by lactic acid bacteria (LAB). Since LAB are food grade bacteria with generally recognized as safe (GRAS) status, focus has been shifted to folate production by LAB and foods fermented by LAB with enhanced folate levels. The proper selection of folate producers is necessary to increase the levels of natural folate in fermented food.

1.2. Objectives of the Study

The main focus of this study is to explore the potential of newly isolated lactic acid bacteria (LAB) to produce folate. It comprises the following objectives,

➢ Isolation and identification of folate producing LAB from various sources

➢ Characterization of probiotic properties of significant folate producers

➢ Fermentative production of folate by the selected isolates – optimization of the production conditions and standardization of folate detection from the fermented samples

➢ Improvement of gastrointestinal survival of the isolates by encapsulation

➢ Folate fortification studies using free and encapsulated LAB

➢ Identification and isolation of key genes involved in folate biosynthesis for molecular studies

The thesis is framed into nine chapters. First chapter is introduction and detailed review of literature covering the importance of folate, its structure, role in metabolism and an overview regarding the use of probiotics to combat folate deficiency. In the second chapter the general methodologies employed throughout the study were described. Third chapter deals with the isolation, identification and probiotic characterization of folate producing LAB. Chapter four discusses the fermentative production of folate in skim milk by the isolate *Lactococcus lactis* CM28. In chapter five, a co-culture of *L. lactis* CM22 and *L. lactis* CM28 was employed for folate production using skim milk based medium. Chapter six demonstrates encapsulation of probiotic LAB for improving the gastrointestinal survival and chapter seven deals with the use of free and encapsulated *L. lactis* cultures for folate fortification. Isolation, identification and cloning of selected folate biosynthetic genes from the indigenous isolate are described in chapter eight. Conclusions drawn from the present study and the scope for further studies are integrated in chapter nine. This will be followed by a bibliography section and annexure on major instruments used, culture media composition etc.

1.3. Review of Literature

1.3.1. Folate Discovery

The history of folic acid dates back to the work of Lucy Wills during the late 1920s and early 1930s in India. In 1931, Lucy Wills made a key observation and found out that macrocytic anaemia in pregnant women was prevented by yeast or yeast extract (marmite) added to the diet (Wills, 1931). Later in 1938 Wills and Evans reported that patients with tropical macrocytic anaemia were cured by injections of crude liver extract or by feeding autolysed yeast extract. Folic acid received its name from the Latin word folium (folium = leaf) when it was isolated from spinach leaf by Mitchell and others (Mitchell et al., 1941). Furthermore, in 1943 Bob Stokstad isolated the pure crystalline form and was able to determine its chemical structure while working at the Lederle Laboratories of the American Cyanamid Company (Shane & Carpenter, 1997). Folic acid was subsequently synthesized in 1945 as a yellow crystalline powder of molecular weight 441 with the chemical name pteroyl glutamic acid (PGA) by Dr Yellapragada Subbarao and others. It was found that the PGA molecule consisted of a pteridine ring, para aminobenzoic acid (PABA) and glutamic acid. Natural folates are polyglutamates with additional single carbon units (methyl, formyl, methylene and others) attached to the N_5 or N_{10} nitrogen atoms and will be reduced to di- or tetra- hydro forms (Hoffbrand & Weir, 2001).

Further research on its metabolic functions revealed the efficacy of folic acid in curing megaloblastic anaemia of sprue, celiac disease, pregnancy and malnutrition. Subsequent studies in this area revealed the biochemical mechanism of action folate and the association of folate deficiency with various diseases. Though effective against megaloblastic anaemia, folic acid could only temporarily relieve the symptoms of Addisonian perinicious anaemia and the neurological symptoms worsened as in the case of untreated patients (Amill & Wright, 1946; Moore & Bierbaum, 1945; Vilter et al., 1946). When vitamin B12 injections were given to the patients the problems were corrected. Later it was discovered that in the case of Addisonian perinicious anaemia deficiency of vitamin B12 induced a secondary folate deficiency. Though the terms 'folate' and folic acid are often used interchangeably 'folic acid' refers to the fully oxidised chemical compound PGA and 'folate' denotes the various derivatives naturally found in food.

1.3.2. Folate Structure

Folic acid or PGA, the synthetic analogue used for food fortification and nutritional supplementation is comprised of PABA linked to a pteridine ring at one end and L-glutamic acid at the other end. Naturally occurring folates exist in various chemical forms which differ in the extent of reduction state of pteroyl group, number of glutamate residues and the nature of substituents on the pteridine ring. They include 5-methyltetrahydrofolate (5-MTHF), 5-formyltetrahydrofolate (5-formyl-THF), 10-formyltetrahydrofolate (10-formyl-THF), 5,10-methylenetetrahydrofolate (5,10-methylene-THF), 5,10-methenyltetrahydrofolate (5,10-methenyl-THF), 5-formiminotetrahydrofolate (5-formimino-THF), 5,6,7,8-tetrahydrofolate (THF) and dihydrofolate (DHF) (LeBlanc et al., 2007) (**Fig.1.1**).
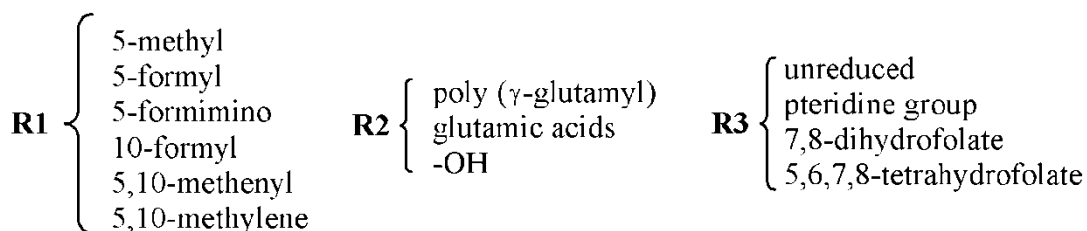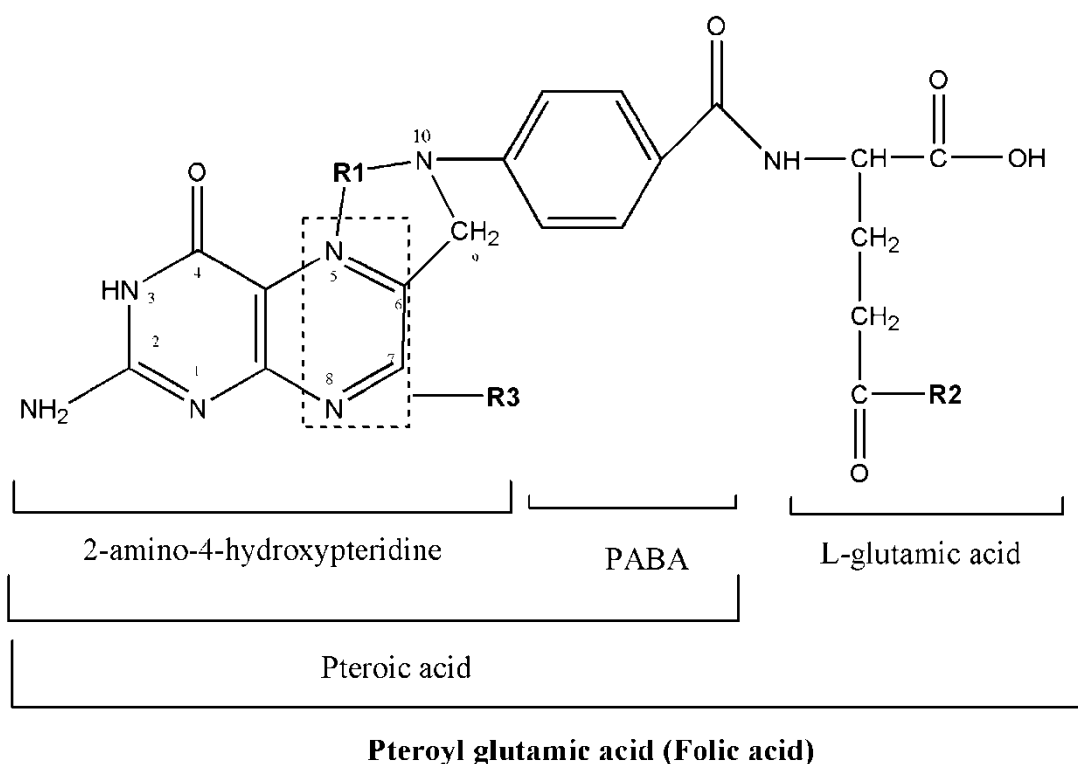

Pteroyl glutamic acid (Folic acid)

R1	R2	R3
5-methyl	poly (γ-glutamyl)	unreduced
5-formyl	glutamic acids	pteridine group
5-formimino	-OH	7,8-dihydrofolate
10-formyl		5,6,7,8-tetrahydrofolate
5,10-methenyl		
5,10-methylene		

Fig.1.1. Structure of folic acid and folate derivatives

1.3.3. Folate Absorption

The polyglutamylated natural folates will be enzymatically converted to mono glutamyl folates by γ-glutamyl hydrolase or human conjugase that is present in the brush border of the small intestine prior to absorption. Monoglutamyl folates are actively transported across the proximal small intestine by a saturable pH dependant process. While higher doses of folic acid are absorbed by non saturable passive diffusion. Monoglutamatyl folates present in the portal circulation can be taken up by the liver where it is metabolized to polyglutamate forms and retained or will be released into the blood (Finglas, 2000; Milman, 2012). When released from the tissues into circulation the polyglutamayl folates will be reconverted to monoglutamyl folates. Cellular transport of folate is by membrane carriers or folate binding protein mediated systems (Finglas, 2000).

1.3.4. Bioavailability: Folate Vs Folic acid

Bioavailability of a nutrient is the amount that is readily available for metabolic processes or storage. The bioavailability of polyglutamyl folate is 60 – 80% compared with the monoglutamyl form. Synthetic folic acid is rapidly absorbed than the natural folates and the relative bioavailability of natural folates is only 50% compared to that of synthetic folic acid (LeBlanc et al., 2007). Folic acid competes with the natural folates for binding with enzymes, carrier and binding proteins. Folic acid receptors have high affinity towards folic acid than to 5-MTHF. So the presence of higher doses of folic acid in the blood may inhibit the transport of 5-MTHF (Smith et al., 2008). It was reported that the long term over supplementation of folic acid down regulates the intestinal renal uptake of folate (Ashokkumar et al., 2007). Folic acid is made biologically active by enzymatically converting it to 5-MTHF which is the predominant form of folate appearing in plasma. Folic acid is first reduced to DHF and then to THF by the enzyme dihydrofolate reductase. This process usually takes place in the gut mucosa where it will be further converted to 5-MTHF before entering the folate cycle (Scott et al., 2000). Consumption of folic acid above certain threshold doses results in the presence of unmetabolised or unaltered folic acid in plasma. The concentration of the unmetabolised folic acid varies among individuals depending on their dihydrofolate reductase activity (Smith et al., 2008). The bioavailability of dietary folates are dependent on various factors including food matrix, food processing, presence of folate antagonists, alterations in intestinal pH and intestinal deconjugation of polyglutamyl folates (Finglas, 2000; LeBlanc et al., 2007).

1.3.5. Folate Functionality

The main role of folate coenzymes in the body is to mediate the transfer of one carbon units in various metabolic pathways. These include amino acid metabolism, purine and pyrimidine synthesis and, the formation of S-adenosyl methionine (SAM). Polyglutamyl form of THF is the central folate acceptor molecule in one carbon metabolism (Bailey & Gregory, 1999). In methionine synthase reaction, methionine synthase utilizes 5-MTHF for the conversion of L-homocysteine to L-methionine. The reaction is catalysed by a B12 (cobalamin) containing methyl transferase. Most of the methionine synthesized will be converted to SAM, a universal donor of methyl group. The methionine synthase reaction also results in the regeneration of THF required for the formation of 5, 10-methylene-THF and 10-formyl-THF used directly in thymidylate and purine synthesis, respectively. An overview of one carbon metabolism is represented in **Fig.1.2**.
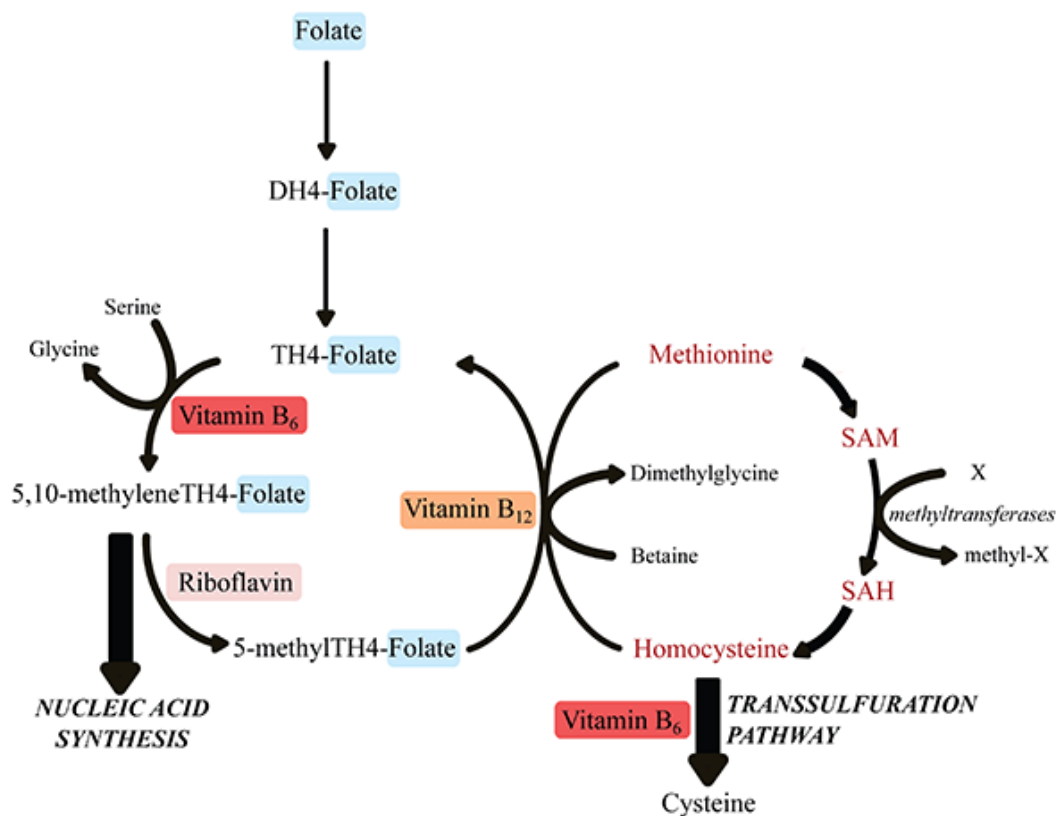

Fig.1.2. Folate functionality

Courtesy: http://flipper.diff.org/app/pathways

1.3.6. Health Implications of Folate Deficiency

a. Anaemia

One of the common and oldest disorders related to folate deficiency is megaloblastic anaemia due to ineffective erythropoiesis. Low levels of folate leads to prolongation of synthetic phase of cell division and retardation of germ cell maturation resulting in abnormal red cell precursors, megaloblasts. As a result the rate of delivery of new erythrocytes into circulation is reduced and anaemia gradually develops. Haematological manifestations may include an increase in mean corpuscular cell volume and a decrease in haemoglobin levels (Iyer & Tomar, 2009; Koury & Horne, 1994). The main risk factor is inadequate dietary intake, loss of folate during cooking, pregnancy and malabsorption. Oral or intravenous folate supplements for short term were found to be effective in correcting anaemia and in case of malabsorption long term treatment is needed (Aslinia et al., 2006; Iyer & Tomar, 2009).

b. Neural Tube Defects

Neural tube defects (NTDs) are one of the most common congenital anomalies in humans. Each year 3 - 4 lakh babies worldwide are born with NTDs (Gupta & Gupta, 2004). The prevalence of NTDs is high in India compared to other countries, occurring 4.5 per 1000 births (Allagh et al., 2015). The primordial nervous system begins to develop as a plate of cells during early embryonic stage and folds on itself to form a tube called neural tube. Closure of this neural tube occurs between 21-28 days post conception. Failure of neural tube closure results in NTDs. Anencephaly is the total or partial absence of brain tissue and calvarium, and is invariably lethal, with death either before or shortly after birth. Spina bifida causes paraplegia with paralysis of lower extremities and impaired bladder and bowel function (Pitkin, 2007). Several clinical trials established the role of maternal folate status in the development of NTDs. During pregnancy there is an increased requirement of folate as there is a marked increase in one carbon transfer reactions (Iyer & Tomar, 2009). The decreased maternal folate levels could be due to dietary deficiency or a genetic defect (e.g. methylene tetrahydrofolate reductase/MTHFR polymorphisms) in folate metabolism or both. Folate also prevents preterm delivery, placental abruptions, decreases growth retardation and increases birth weight (Gupta & Gupta, 2004). Folate supplementation prior to and during early pregnancy reduces the risk of NTDs in the developing foetus (Czeizel & Dudas, 1992; Dunlevy et al., 2007).

c. Cardiovascular Diseases

Elevated homocysteine levels were found to be an important risk factor in arteriosclerosis and ageing (Djuric et al., 2008). Several studies have established that folate deficiency is a major cause of hyperhomocysteinemia. Many clinical trials have showed that treatment with folates or folic acid significantly reduces plasma homocysteine levels. Folate acts as a substrate in the remethylation of homocysteine to methionine (Durand et al., 1998; Verhaar et al., 2002). Another possible reason for the beneficial role of folate against atherosclerosis is its antioxidant property. Direct interaction of 5-MTHF with the enzyme endothelial nitric oxide synthase (eNOS) may provide an explanation for the observed effects on oxidative stress and cardiovascular risk (Djuric et al., 2008; Verhaar et al., 2002).

d. Cancer

Folate deficiency has been implicated with the development of cancer especially colorectal cancer. Though the exact mechanism by which this modulation is mediated is not known, there appears to be several possible mechanisms through which a low folate status may increase the risk of malignancy. One of the cancer prevention properties of folate is attributed to its role in thymidylate and purine synthesis – nucleotides needed for DNA replication and repair. Folate deficiency may affect DNA stability due to uracil misincorporation, and chromosome breakage. Furthermore, optimum folate levels are necessary for the production of SAM, the universal methyl group donor. Suboptimal levels of folate can alter DNA methylation leading to inappropriate activation of proto-oncogenes and induction of malignant transformation (Duthie, 1999; Ulrich, 2007). MTHFR is a key enzyme that determines whether reduced folates are directed towards DNA methylation pathways or nucleotide synthesis. Polymorphisms in MTHFR gene in presence of low folate levels may result in an imbalance between DNA synthesis and methylation and may be responsible for folate related carcininogenesis (Stover, 2009).

e. Cognitive Diseases

Severe folate deficiency has also been linked to several neurological problems including depression, stroke and Alzheimer's disease (Iyer & Tomar, 2009; Luchsinger et al., 2007; Troen et al., 2008). SAM, the downstream metabolite of methionine is involved in various methylation reactions including the biosynthesis of monoamine

neurotransmitters, serotonin, epinephrine and dopamine. The deficiency of 5-MTHF, the active metabolite of folate, leads to low levels of SAM and neurotransmitters in the cerebrospinal fluid (CSF) and contributes to the development of depression (Miller, 2008). Several clinical trials demonstrated that administration of 5-MTHF to depressed patients with borderline or definite folate deficiency significantly improved the symptoms (Godfrey et al., 1990; Papakostas et al., 2014; Passen et al., 1993). Patients with severe hyper homocysteinemia exhibit a broad range of clinical manifestations, including neurological abnormalities such as mental retardation, cerebral atrophy, and seizures (Kruman et al., 2002; Van den Berg et al., 1995). Studies have shown that persons with low folate and increased homocysteine levels are at high risk of developing Alzheimer's disease. In a mouse model, increased DNA damage and hippocampal neurodegeneration was observed in amyloid precursor protein (APP) mutant transgenic mice, but not wild-type mice, with a folic acid deficient diet. The levels of amyloid β- peptide ($A\beta$) were unchanged in the brains of APP mutant transgenic mice. These results suggests that folate deficiency and elevated homocysteine levels impairs DNA repair and sensitizes neurons to $A\beta$ toxicity (Kruman et al., 2002). Further studies and clinical trials are necessary to better understand the preventive and therapeutic effect of folate in the development of neurodegenerative disorders.

1.3.7. Folate: Dietary Sources, Requirements and Fortification Programmes

Folate is found naturally in a wide variety of foods including legumes (beans, lentils, peas etc), green leafy vegetables (spinach), asparagus, broccoli, carrot, beets, citrus fruits (orange, grapefruit), tropical fruits (mango, pomegranate, guava, banana), avocado, seeds and nuts (sunflower seeds, almond, and peanuts), liver, and fermented dairy products (LeBlanc et al., 2007). Folate content of certain foods is given in **Table 1.1.**

Table 1.1. Folate content in selected foods

Food Item	Folate concentration (µg/100g)
Spinach (raw)	194.0
Broccoli	108.0
Avocado	81.0
Egg Yolk	146.0
Whole egg	47.0
Asparagus	191.0
Lentils (dried)	433.0
Banana	19.0
Mango	36.0
Milk (whole)	6.0
Almonds	56.0
Chick peas (dried)	557.0
Tomato	21.0
Carrot	14.0
Radish	27.0
Sunflower seeds	226.0
Beans, Mung (cooked)	159.0

Data compiled from different national food tables

High risk women (women with a previous pregnancy affected by NTD) are recommended to take 4000 µg/day when planning for a subsequent pregnancy (de Bree et al., 1997). The occurrence of folate deficiency across several populations and in countries at various stages of development regardless of its wide availability is a great concern. Reduced dietary intake is the main reason for low folate status. During pregnancy and lactation there is an increased need for folate. Certain medications and genetic factors also contribute towards folate deficiency. Folate deficiency is defined as a serum folate concentration <7 nM/L (~3 ng/mL) or a red blood cell folate concentration <315 nM/L (~140 ng/mL) (Crider et al., 2011). It was found that greatest reduction in NTDs was

observed at serum folate concentrations much higher than the defined value for folate deficiency (Daly et al., 1995). Following the findings that periconceptional intake of folate reduces the risks of NTDs; in 1992 the U. S. Public Health Service recommended that all women of childbearing age consume 400 µg of folic acid daily through fortification, supplementation, and diet. The recommended dietary allowance (RDA) of folate for various age groups is shown in **Table 1.2.**

Table 1.2. Daily folate requirements**;** Source: Eitenmiller et al., 2007

Age (years)	RDA (µg/day)	Tolerable upper intake level (µg/day)
Infants (0-1)	65 - 80	
Infants (1-3)	150	300
Children (4-8)	200	400
Children (9-13)	300	600
Teens (14-18)	400	800
Adults(19+)	400	1000
Pregnant women	600	1000
Breastfeeding women	500	1000

Considering various health issues associated with folate deficiency, several countries have implemented folic acid fortification programmes. In January 1998, the U.S. Food and Drug Administration (FDA) established mandatory fortification of folic acid to enriched breads, cereals, flours, corn meals, pastas, rice, and other grain products. Other countries, including Canada, Chile, Costa Rica, and South Africa have also implemented mandatory folic acid fortification programmes (Crider et al., 2011). A decrease in the prevalence of NTDs has been reported following the folic acid fortification programmes (Chen & Rivera, 2004; Ray et al., 2002; Sayed et al., 2008; Williams et al., 2002). Till now there is mandatory folic acid fortification in more than 50 countries. To date, all nationwide fortification programs have used the fully oxidized and chemical form, folic acid, rather than the chemically less stable reduced forms of folate that occur naturally in foods. Folic acid needs to be converted to THF following absorption by liver and if the

body's ability to reduce folic acid is exceeded unmetabolised folic acid will be found in circulation. This was confirmed by certain experimental studies where unmetabolised folic acid was found after consuming >200 μg of folic acid (Sweeney et al., 2007). Unmetabolised folic acid has been linked to cognitive impairment in older persons which in turn could be related to low vitamin B12 status. Recent research in human subjects suggests that folic acid supplements may increase the chance of multiple colorectal adenomas and breast cancer in postmenopausal women (Cole et al., 2007; Stolzenberg-Solomon et al., 2006). It has been reported that high folate status attributed by folic acid supplementation increases the chances of multiple births after *in vitro* fertilization, with its associated risks of infant and maternal mortality (Haggarty et al., 2006). Also, it was observed that increased concentrations of unmetabolised folic acid, not the natural folate, decrease the cytotoxicity of natural killer cells (Troen et al., 2006). Natural killer cells are considered as body's first line of defence against carcinogenesis by the destruction of arising clones of neoplastic cells (Wright et al., 2007). This suggests a possible route by which excess folic acid (but not circulating high concentrations of natural 5-MTHF) promotes existing premalignant and malignant lesions (Mason et al., 2007; Wright et al., 2007). Due to these potential risks associated with fortification with folic acid, fermented foods with elevated levels of natural folate could be a better alternative.

1.3.8. Folate Production by Food Grade Microorganisms

Milk is not a rich source of folate, however, many fermented dairy products contain significant amount of folate. The elevation of folate levels in fermented foods is credited to folate synthesis by the fermenting microorganisms (Lin & Young, 2000). Hence, the synthesis and utilization of folate by the microorganisms are the important factors determining the final folate concentration of various fermented foods. The United States Department of Agriculture (USDA) has recommended the consumption of at least 3 servings of milk products as part of a healthy diet. This could contribute up to 23% of the DRI of folate. Besides dairy products, microorganisms are capable of increasing the folate content in a wide variety of non dairy foods including wine, beer, bread, sour dough, fermented vegetables etc (Jägerstad et al., 2004; Leroy & De Vuyst, 2004). The increase in folate content during sour dough fermentation is mainly due to the growth of yeasts which synthesize the vitamin (Kariluoto et al., 2006; Kariluoto et al., 2004; Osseyi et al., 2001). This increase could compensate the folate loss during baking (Kariluoto et al.,

2004). Numerous studies have shown that certain LAB have the ability to synthesize folate where as some others may deplete them (Lin & Young, 2000; Rao et al., 1984). Depending on the starter culture present, the folate levels of the fermented product may vary considerably. For example, the amount of folate in cow's milk ranges from 20-60 µg/L whereas, its concentration in yoghurt could reach up to 200 µg/L (Wouters et al., 2002).

Several industrially important LAB including *Streptococcus thermophilus, Lactococcus lactis, Lactobacillus acidophilus, Lactobacillus plantarum, Leuconostoc lactis* are reported to be folate producers (LeBlanc et al., 2010). *S. thermophilus* strains are one of the dominant folate producers in milk, mainly producing 5-MTHF. Above six fold increase of 5-MTHF was obtained in yoghurts fermented with *S. thermophilus* for 12 h (Holasova et al., 2004). Significant increase in folate levels were reported in vegetables and corn flour when fermented with LAB (Jägerstad et al., 2004; Murdock & Fields, 1984). The ability of different LAB to produce folate varies remarkably. The synthesis and utilization of folate by these microorganisms are the important factors in determining the final folate levels (Iyer & Tomar, 2009). Most of the lactobacilli consume folate with a few exceptions like *Lb. plantarum.* It was reported that folate levels are influenced by the specific lactic acid bacterium, growth conditions and medium used. Thus, by the selection of suitable folate producing LAB as starter culture, yoghurt with elevated levels of folate could be produced (Sybesma et al., 2003b).

Bifidobacteria are one among the most important groups of gut microbiota and are widely used as probiotic. Several *Bifidobacterium* strains have been screened for their ability to produce folate in low folate or folate free medium. Significant differences in folate accumulation were observed among the tested species (Pompei et al., 2007). Also, some bifidobacteria did not produce folate when it was already present, while some others produced it despite of the vitamin concentration (Pompei et al., 2007). It was suggested that folate production was neither related to growth of the bacteria nor a trait of the species, rather it seemed to be highly strain dependent (Rossi et al., 2011). For most of the *Bifidobacterium* strains the accumulated folate was found to be extracellular (Deguchi et al., 1985; Pompei et al., 2007). In addition to LAB and bifidobacteria, different propionibacteria were also reported to be folate producers. The levels of folate produced by propionibacteria were comparable to that of *S. thermophilus*, the well known folate

producer. Similar to LAB and bifidobacteria, there were strain level variations in the amount of folate produced, secreted as well as retained. The retention and excretion of folate depends on the forms of folate produced (Hugenholtz et al., 2002a). One of the major limitations in the use of bifidobacteria and propionibacteria for folate fortification is their requirement of strict anaerobic conditions and possibilities of folate utilization by co-cultures when used as adjunct starter (Iyer & Tomar, 2009). Besides these bacteria certain strains of *Saccharomyces cerevisiae* were found to produce folate ranging from 1 to 4 mg/100 g yeast (Witthöft et al., 1999). Folate production by various microbial species in chemically defined folate free medium is represented in **Table 1.3.**

Table 1.3. Folate produced by microorganisms in chemically defined folate free medium

Microbial species	Extracellular folate (µg/L)	Intracellular folate (µg/L)	Total folate (µg/L)	Reference
LAB				(Sybesma et al., 2003b)
L. lactis subsp. *cremoris*	8 - 46	59 - 99	92 - 116	
L. lactis subsp. *lactis*	5 – 26	47 – 269	57 - 291	
L. lactis subsp. *lactis* biovar *diacetylactis*	14 - 21	65 - 84	79 - 100	
Lb. plantarum	27	18	45	
Lb. helveticus	-1 - 3	-1 – 90	2- 89	
Lb. acidophilus	0	1	1	
Lb. casei	-45	33	-13	
Lb. casei subsp. *rhamnosus*	-98	34	-63	
Lb. delbrueckii subsp. *bulgaricus*	12	41	54	
S. thermophilus	23 – 40	4 – 179	29 - 202	
Leuc. lactis	37	7	45	
Leuc. paramesenteroides	33	10	44	
Bifidobacteria				(Pompei et al., 2007)
B. adolescentis	1 - 65	10 - 40	70 - 110	

B. animalis	26	-	-	
B. dentium	29	-	-	
B. bifidum	1	-	-	
B. breve	1 - 3	-	-	
B. infantis	27	-	-	
B. catenulatum	3	-	-	
B. longum	2	-	-	
B. pseudocatenulatum	12 - 82	5 – 35	75 – 90	
Propionibacteria				(Hugenholtz et al., 2002a)
P. thoenii	28	8	36	
P. acidipropionici	58	-22	36	
P. jensenii	51	-11	40	
P. freudenreichii ssp. *shermanii*	0 – 93	-20 – 41	17 - 78	

L. = Lactococcus, Lb. = Lactobacillus, S. = Streptococcus, Leuc. = Leuconostoc, B. = Bifidobacterium, P. = Propionibacterium

Negative values indicate utilization from the medium

1.3.9. Folate Biosynthesis

Folate is synthesized by plants, fungi, certain protozoa, several archaea and bacteria likely through the same general biosynthetic pathway with some modifications (Bermingham & Derrick, 2002; Hanson & Gregory Iii, 2002; Levin et al., 2004; Rossi et al., 2011). Folate is a tripartite molecule composed of a pteridine ring, PABA and one or more L-glutamate residues. Folate biosynthesis is a complex pathway in which GTP is converted via seven consecutive steps into THF (Wegkamp et al., 2007). The pteridine part of folate is derived from GTP that is synthesized in the purine biosynthesis pathway. PABA is derived from chorismate via the biosynthetic pathway for aromatic amino acids, involving glycolysis, pentose phosphate pathway and shikimate pathway. The third component, glutamate is usually taken up from the medium.

The first enzyme in the *de novo* synthesis of folate is GTP cyclohydrolase I (*folE*, EC 3.5.4.16) which catalyses a complex reaction from GTP to 7,8-dihydroneopterin triphosphate through an intermediate with the release of formate. 7,8-dihydroneopterin triphosphate is then converted to the corresponding monophosphate by a specific pyrophosphatase. Dihydroneopterin aldolase (*folB*, EC 4.1.2.25) then acts on the product to give glycoaldehyde and 6-hydroxymethyl-7,8-dihydropterin, which is then phosphorylated by hydroxymethyldihydropterin pyrophosphokinase (*folK*, EC 2.5.1.15) to 6- hydroxymethyl-7,8-dihydropterin pyrophosphate. This is followed by the condensation of 6- hydroxymethyl-7,8-dihydropterin pyrophosphate and PABA catalysed by dihydropteroate synthase (*folP*, EC 2.7.6.3). The resulting 7,8-dihydropteroate is glutamylated by a bifunctional protein folate synthetase/polyglutamyl folate synthetase (*folC*, EC 6.3.2.12/17) producing DHF. DHF is then reduced to THF by the enzyme dihydrofolate reductase (*folA*, EC 1.5.1.3). Polyglutamyl folate is produced by subsequent addition of glutamate molecules to the glutamyl residue of folate by the activity of polyglutamyl folate synthetase (*folC*, EC 6.3.2.17). **Fig. 1.3 (A)** represents folate biosynthesis pathway and folate gene cluster in *L. lactis* is presented in **Fig.1.3 (B)**. Different folate derivatives are synthesized in a number of enzymatic steps involved in C1 metabolism (de Crécy-Lagard et al., 2007; Rossi et al., 2011; Santos et al., 2008; Sybesma et al., 2003a; Wegkamp et al., 2007).
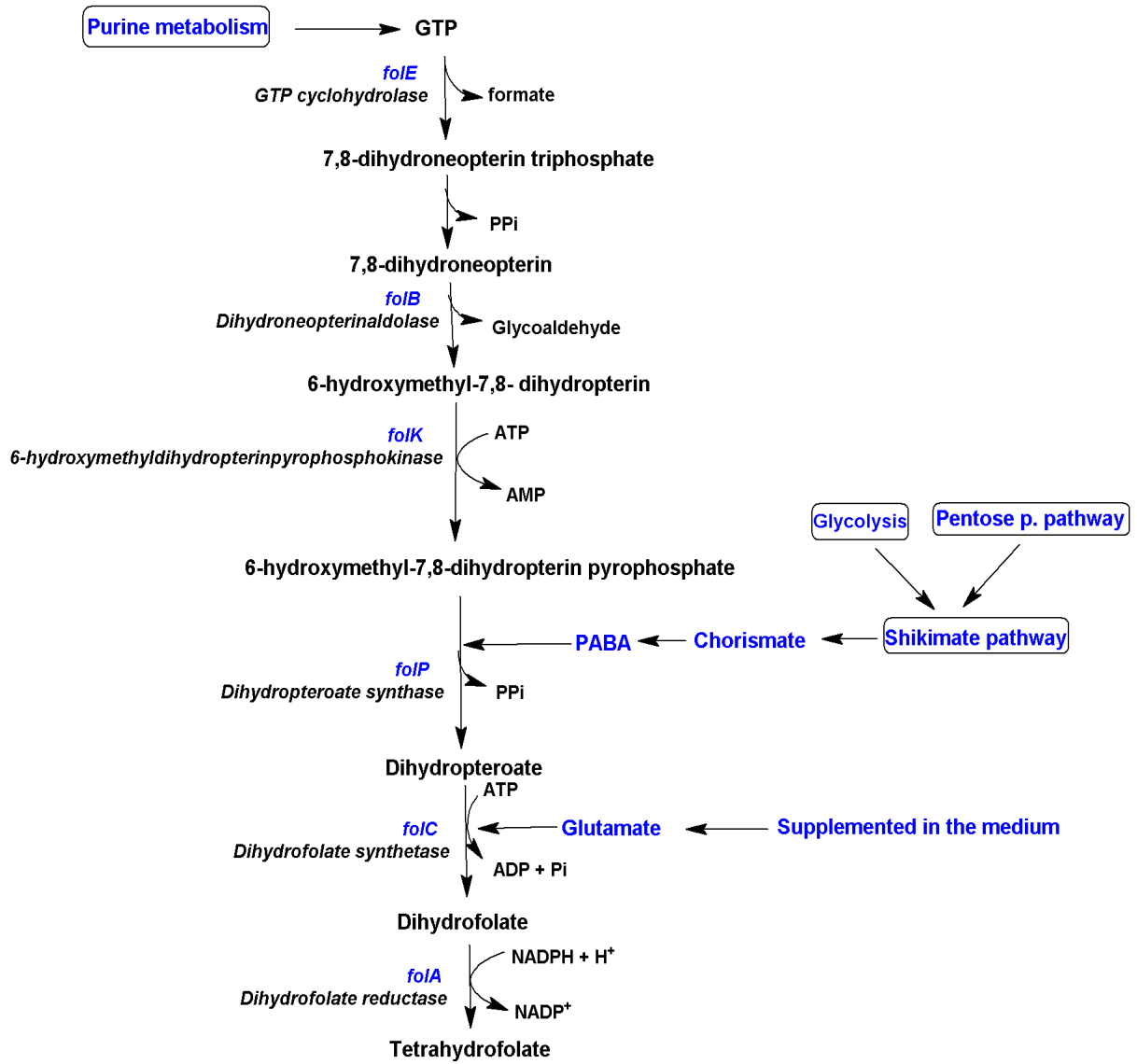
Purine metabolism ──────► GTP

folE
GTP cyclohydrolase ╲►formate

7,8-dihydroneopterin triphosphate

╲► PPi

7,8-dihydroneopterin

folB
Dihydroneopterinaldolase ╲► Glycoaldehyde

6-hydroxymethyl-7,8- dihydropterin

folK ╭ ATP
6-hydroxymethyldihydropterinpyrophosphokinase ╲► AMP

6-hydroxymethyl-7,8-dihydropterin pyrophosphate

Glycolysis   Pentose p. pathway

PABA ◄ Chorismate ◄ Shikimate pathway

folP
Dihydropteroate synthase ╲► PPi

Dihydropteroate
ATP

folC ◄ Glutamate ◄ Supplemented in the medium
Dihydrofolate synthetase ╲► ADP + Pi

Dihydrofolate

folA ╭ NADPH + H⁺
Dihydrofolate reductase ╲► NADP⁺

Tetrahydrofolate

Fig.1.3 (A) Folate biosynthetic pathway in *L. Lactis*

folA *clpX* *ysxC* *folB* *folKE* *folP* *ylgG* *folC* *dukB* *hom*
~10 kb
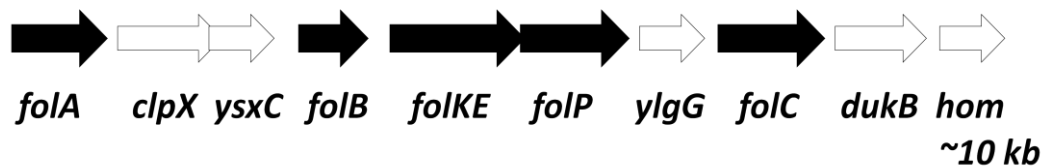
Fig. 1.3 (B) Schematic representation of folate gene cluster in *L. lactis*

Black arrows represent genes involved in folate biosynthesis and white arrows represent genes that are not expected to be involved in folate biosynthesis; Adapted from Sybesma et al (2003a).

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Different forms of folate are produced by LAB including folates with more than three glutamyl residues. Sybesma et al (2003b) have reported the intracellular retention of upto 90% of the produced folate by *L. lactis* as 5,10-methenyl-THF and presumably 10-formyl-THF, both with four, five or six glutamate residues. But in *S. thermophilus* most of the folate extracellular and the intacellular folate was present as 5-formyl-THF and 5,10-methenyl-THF, both with three glutamyl residues. The main function of polyglutamyl tail is believed to be the intracellular retention of folate. This could be due to the negative charge of carboxyl groups (polyglutamyl) of folate. In *S. thermophilus* intra- and extracellular folate was influenced by pH. At low pH most of the folate produced was extracellular. This could possibly due to the protonation of folate at low intracellular pH which makes it electrically neutral thereby enhancing the transport across the membrane. In *L. lactis* pH did not seem to have an effect on intra- and extracellular folate distribution (LeBlanc et al., 2011; Sybesma et al., 2003b). Due to strain level variation in folate production, an adequate combination of strains is essential to develop fermented foods with enhanced folate levels.

1.3.10. Lactic Acid Bacteria

LAB are a clade of Gram-positive, low GC, catalase negative, microaerophilic, acid tolerant, non sporing cocci, coccobacilli or rods with lactic acid as the main product of carbohydrate fermentation. Man has consumed foods fermented with LAB for thousands of years. Because of their long history of safe use in foods, LAB are considered as non toxic, food grade microorganisms and most of them have a GRAS status. LAB have an important role in food production due to their positive contribution to flavour and preservation of the final product. LAB belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae*. LAB are composed of the genera *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus*, *Lactococcus, Leuconostoc*, *Oenococcus, Pediococcus*, *Streptococcus*, *Tetragenococcus, Vagococcus, and Weissella*. Carnobacteria, Lactobacilli and some *Weissella* are rods while the remaining genera are cocci (Axelsson, 2004; Stiles & Holzapfel, 1997; von Wright & Axelsson, 2011). In addition to traditional fermentation, LAB have been used extensively as a preservative, acidulant and flavourant in food processing, as an intermediate in pharmaceutical and cosmetic manufacture (e.g. surgical

dressing), in the manufacture of biodegradable polylactic acid polymers (Liu, 2003) and for the development of probiotics and nutraceuticals.

1.3.11. LAB in Food Fermentation

Food fermentation is regarded as one of the oldest ways of food processing and preservation. Throughout the ancient history health promoting fermented foods have played a role in sustaining thriving civilizations. Fermentation enhances the flavour and nutritional quality of food and increases its shelf life. The products of fermentation depend on the microorganisms involved, substrates used and also on the fermentation conditions. The microorganisms used for food fermentation are non pathogenic and the enzymes such as proteases, amylases and lipases produced by them help in the breakdown of complex food materials into simple non-toxic products with desirable flavour and texture (Steinkraus, 1997). LAB are the most common and dominant microorganisms present in fermented foods and therefore, lactic acid fermentation is considered as the major contributor to the beneficial characteristics observed in those foods (Chelule et al., 2010). Their importance is associated mainly with their safe metabolic activity thereby giving various functional attributes to the food. Based on their fermentation pattern LAB are classified as homofermentative (e.g. *Lactococcus, Streptococcus*) and heterofermentative (e.g. *Weissella, Leuconostoc*). Homofermenters generate two moles of lactate per mole of glucose via EMP pathway whereas heterofermentors utilize pentose phosphate pathway to produce equi molar amounts of lactate, CO_2 and ethanol from glucose (Caplice & Fitzgerald, 1999).

1.3.12. LAB as Starter Cultures

A starter culture consists of a large number of live microorganisms of a single strain or a mixed culture that initiates and speeds up the food fermentation. LAB have a pivotal role in this and have long been used as starters in various food fermentation processes (Caplice & Fitzgerald, 1999; Leroy & De Vuyst, 2004). A starter culture can provide particular characteristics in a more controlled and predictable fermentation. The primary function is the rapid acidification of the food by producing mainly lactic acid as well as small quantities of acetic acid. In addition, LAB produce substances like bacteriocins, exopolysaccharides, vitamins, aroma compounds and enzymes thereby enhancing the flavour, texture and nutritive content of the food (Rattanachaikunsopon & Phumkhachorn, 2010). This paved the way for functional starter cultures (de Vuyst, 2000).

Functional starter cultures possess at least one intrinsic functional property and can add to food safety and/or offer one or more industrial, nutritional or health advantages (Rattanachaikunsopon & Phumkhachorn, 2010). Some of the fermented foods and associated LAB strains are listed in **Table 1.4.**

Table 1.4. LAB in common fermented foods

Fermented foods	Reported LAB
Yoghurt	*Lb. delbrueckii* subsp. *bulgaricus, S. thermophilus*
Cheddar cheese	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, S. thermophilus*
Italian Cheese such as Mozzarella	*Lb. delbrueckii* subsp. *bulgaricus, Lb. helveticus, Lb. lactis, S. thermophilus*
Swiss cheese types	*Lb. delbrueckii* subsp. *bulgaricus, Lb. lactis, L. lactis* subsp. *biovar diacetylactis, Leuc. mesenteroides* subsp. *cremoris, , L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, S. thermophilus*
Goat Cheese and Sheep Cheese	*L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *biovar diacetylactis, Leuc. mesenteroides* subsp. *cremoris*
Butter and buttermilk	*L. lactis* subsp. *lactis, L. lactis* subsp. *lactis biovar. diacetylactis, L. lactis* subsp. *cremoris, Leuc. menesteroides* subsp. *cremoris*
Kefir	*Lb. kefir, Lb. kefiranofacies, Lb. brevis*
Fermented, probiotic milk	*Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii*
Fermented sausages	*Lb. sakei, Lb. curvatus*
Sauerkraut	*Lb. plantarum, Lb. brevis, Leuc. mesenteroides*
Pickles	*Lb. plantarum, Lb. pentosus, Lb. plantarum, Leuc. mesenteroides*
Kimchi (Korea)	*Lb. plantarum, Leuc. mesenteroides, L. brevis*
Idli/dosa (India)	*Leuc. mesenteroides, E. faecalis*
Wine (malolactic fermentation)	*O. oeni*

Rice wine	*Lb. sakei*

E.= Enterococcus, L.= Lactococcus, Lb.= Lactobacillus, Leuc.=Leuconostoc, O.=Oenococcus, P.= Pediococcus, S.= Streptococcus; Source: Divya et al., 2012

1.3.13. LAB as Probiotics

Following the recommendations of FAO/WHO (2002) probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Sanders, 2008). Most of the known probiotics belong to LAB and bifidobacteria. Several LAB species including *Lb. acidophilus, Lb. casei, Lb. gasseri, Lb. plantarum, Lb. paracasei, Lb. rhamnosus, Lb. johnsonii, Lb. reuteri* are considered as probiotics. Probiotics, following ingestion, form part of the colonic microbiota, at least temporarily, and are used with a view to improve the health and well being of the host. The possible mechanisms of action of probiotics include production of antimicrobial compounds, competitive exclusion of pathogen binding, competition for nutrients and immunomodulation (Magnusson et al., 2003). Foods fermented with LAB were found to improve the quantity, availability and digestibility of some dietary nutrients. An increase in folate content was observed in yoghurt, bifidus milk and kefir when fermented with folate producing LAB (LeBlanc et al., 2007; Parvez et al., 2006). In addition to vitamins LAB synthesize and release various enzymes, short chain fatty acids (SCFA), lactic, propionic and butyric acids into the intestinal lumen (Parvez et al., 2006).

Many uses of LAB as probiotics in gastrointestinal diseases have been proposed, including the prevention and treatment of intestinal infections. *Helicobacter pylori* is a Gram negative pathogen responsible for type B gastritis, peptic ulcers and gastric cancer. It was reported that the administration of *Lb. reuteri* had a beneficial effect on *H. pylori* infection in humans (Saggioro et al., 2005). *Lb. rhamnosus* GG and *Bifidobacterium lactis* BB-12 were found effective in the prevention and treatment of acute diarrhoea mainly caused by rotaviruses in children (Guandalini et al., 2000; Isolauri et al., 1991; Saavedra et al., 1994; Szajewska et al., 2001). Several clinical trials have confirmed the beneficial effects of probiotic strains in several human diseases including inflammatory bowel diseases (IBDs). An improvement in the symptoms of IBD, pouchitis and ulcerative colitis was noticed when certain strains of lactobacilli were administered (Ouwehand et al., 2002; Parvez et al., 2006). Probiotic strains alleviate constipation possibly by increasing the

intestinal mobility and by decreasing the gut pH (Parvez et al., 2006). *Lb. plantarum* 299v (DSM 9843) strain was shown in clinical trials to reduce abdominal pain, bloating, flatulence, and constipation associated with irritable bowel syndrome (IBS) (Ducrotté et al., 2012; Parvez et al., 2006). Preliminary evidences show that probiotics can prevent or delay the onset of certain cancers. This possibly could be due to its antimicrobial activities against the carcinogen producing microorganisms, antimutagenic properties, and alteration of tumour differentiation processes (Liong, 2008). *In vivo* studies with *Lb. rhamnosus* strains GG and LC-705 showed a decrease in availability of carcinogenic aflatoxin in the lumen (El-Nezami et al., 2000). Animal studies suggest that a combination of suitable prebiotic with probiotic exerts protective effects against tumour development in the colon (Rafter et al., 2007).

A number of studies conducted *in vitro* and in animals showed that probiotics can modify immune parameters. Studies suggest that probiotics enhance the innate immunity and modulate pathogen-induced inflammation via toll-like receptor regulated signalling pathways (Vanderpool et al., 2008). A series of randomized, double blind, placebo controlled clinical trials demonstrated that dietary consumption of *B. lactis* HN019 and *L. rhamnosus HN001* enhanced immunity in the elderly (Arunachalam et al., 2000; Gill et al., 2001). Oral administration of *Lb. casei* Shirota was found to enhance innate immunity by stimulating the activity of natural killer (NK) cells (Matsuzaki & Chin, 2000). In a double-blind, randomised placebo-controlled trial *Lactobacillus* GG was found to be effective in prevention of early atopic disease in children at high risk (Kalliomäki et al., 2001). The generation anti-inflammatory interleukin 10 and transforming factor β by *Lactobacillus* GG could play a role in reducing the risk of atopic eczema (Kalliomäki et al., 2003).

Preliminary evidences indicate that probiotics or their fermented products play a role in blood pressure control. Reduction in systolic and diastolic pressure in elderly hypertensive patients was observed when they consumed fermented milk containing *Lb. helveticus* and *S. cerevisiae* (Hata et al., 1996). Though the effect of probiotic LAB in lowering the blood cholesterol levels in humans is not conclusive, clinical trials on humans do suggest that they may have a beneficial role in blood lipid levels. It was reported by Taranto and colleagues that administration of low levels of *Lb. reuteri* for 7 days decreased total cholesterol and triglyceride levels by 38% and 40%, respectively, and

increased the high-density lipoprotein (HDL): Low density lipoprotein (LDL) ratio by 20% in hypercholesterolemic mice (Taranto et al., 1998). A mean 32% reduction in total cholesterol and 35% reduction in LDL cholesterol were observed in hyperlipidaemic patients when *Lb. sporogenes* was administered over a period of three months (Mohan et al., 1990). Probiotic LAB are also reported to alleviate symptoms of lactose intolerance by increasing the lactase activity of the small intestine (Parvez et al., 2006). Daily use of *Lactobacillus* GR1 and RC14 as oral capsules restored lactobacilli dominated vaginal flora and lower risk of urinary tract infection (UTI) recurrences (Reid et al., 2001). Besides all these specific health benefits probiotics have a role in maintenance of normal health status of the host. **Fig.1.4** summarizes the health benefits of probiotics.
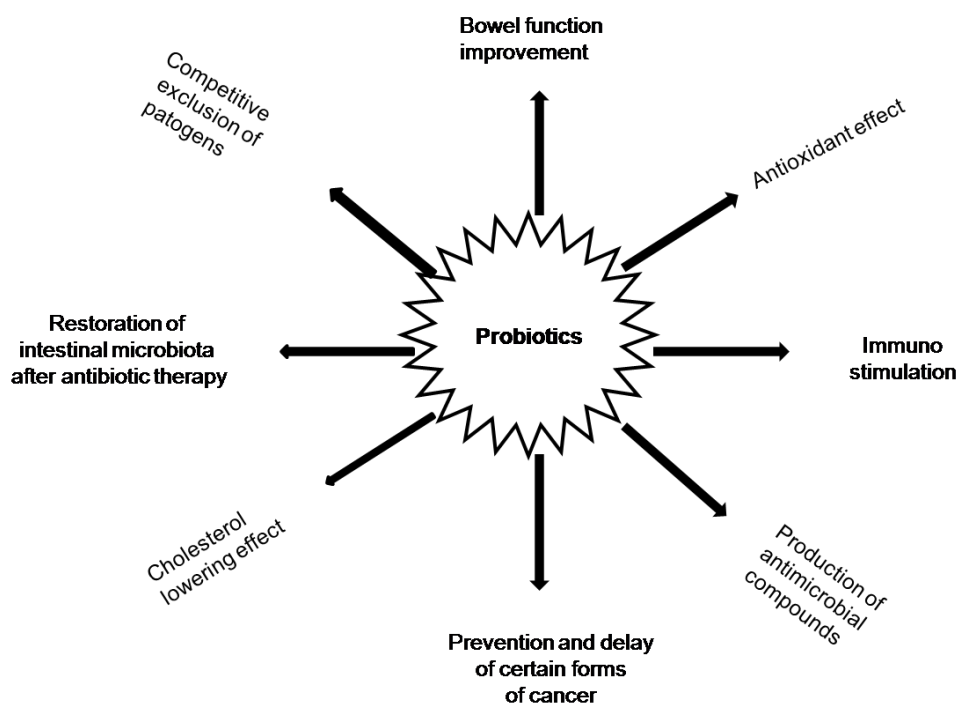

Fig. 1.4. Health benefits of probiotics; Source: (Divya et al., 2012)

1.3.14. Development of Probiotic Foods

Probiotics are incorporated in foods like yoghurt, cheese, ice cream, infant formulas, breakfast cereals, sausages, luncheon meats, chocolates, puddings and also sold as capsules containing freeze-dried cell powders and tablets. While adding probiotics to a food product several factors must be considered that may influence the viability of the culture as well as its activation in the intestine. These factors include 1) the physiological state of the probiotic organisms added (growth phase), 2) storage conditions (e.g.

temperature, humidity), 3) chemical composition of the food matrix (e.g. titratable acidity, available carbohydrate content, nitrogen sources, vitamins, minerals, prebiotics, food additives, water activity, and oxygen content), and 4) possible interaction between the probiotics and the starter cultures (e.g. antagonism, mostly caused by the production of bacteriocins, and synergism) (Heller, 2001).

Probiotics, when used in conjunction with prebiotics an improvement in the survival of the bacteria crossing the upper part of the gastrointestinal tract was observed thereby enhancing their effects in the large bowel. Prebiotics are defined as a non-digestible food ingredient (oligosachharides, inulin etc) that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson GR 1995; Schrezenmeir & de Vrese, 2001). The combination of probiotics and prebiotics is termed as synbiotics. It was reported that early enteral nutrition with synbiotics significantly reduced postoperative bacterial infections in patients following pancreatoduodenectomy with only single-shot antibiotic prophylaxis (Rayes et al., 2007). In another study a synbiotic diet containing *Lb. casei* ASCC 292, fructooligosaccharide (FOS), and maltodextrin beneficially altered cholesterol levels and produced a healthier bowel microbial population without translocation of lactobacilli to other organs (Liong & Shah, 2006).

The major challenge in probiotic food preparation is the retention of viability of the cultures. Typical methods for preserving sensitive biological materials include freeze drying, cryopreservation, and spray drying. These techniques involve the use of extreme temperatures which may initiate structural damages to the cell membranes, protein denaturation, and/or DNA damage and can lead to a decrease in cell viability (Leslie et al., 1995). The minimum of bio value (MBV) index represents the minimum number of probiotic cells (CFU/g) in the product at the moment of consumption that is necessary for the beneficial effects (Mortazavian & Sohrabvandi, 2006). According to the recommendation by the International Dairy Federation (IDF), MBV should be $\geq 10^7$ CFU/g up to the date of minimum durability (Ouwehand & Salminen, 1998). In order to increase the resistance of probiotic bacteria against the detrimental food processing conditions, several approaches like selection of acid and bile tolerant strains, microencapsulation, packaging in oxygen protected materials, double step fermentations, pre adaptation to stress conditions and addition of micronutrients are being employed (Gismondo et al.,

1999). Recently researchers from Technische Universität München developed an environmental friendly low temperature vacuum drying process to increase the rate of cell viability (http://www.sciencedaily.com).

Microencapsulation of the probiotic bacteria using various biopolymers can noticeably increase the viability of the bacteria thereby increasing the shelf life of the probiotic food. Microencapsulated cells are easier to handle and the number of cells in each bead can be quantified thus allowing controlled dosages. It was found that microencapsulation of probiotic along with a prebiotic (co-encapsulation) increases the viability of the probiotic microbiota. Once the matrix beads have been dried, a surface coating by polymers like chitosan, alginate or carrageenan can be applied (double encapsulation) providing extra protection for the cells which may also enhance the sensory properties of the product. According to some studies functionality of a multistrain probiotic could be more effective and more consistent than that of monostrain probiotic provided the strains are compatible and preferably synergistic. In a clinical study conducted it was found that a multistrain probiotic preparation significantly reduced the symptoms of IBS (Williams et al., 2009). The selection of suitable probiotic strains, coating material and prebiotics has a major role in the efficiency of the process.

1.3.15. Probiotic Industry

The increased awareness among consumers and growing scientific evidences on the health benefits of probiotics facilitated the growth of global probiotic market day by day. Probiotic fortified baked foods, ice creams and chocolates are gaining popularity among consumers. The global probiotic market is geographically segmented into North America, Europe, Asia Pacific and the rest of the world. Nestle S.A., Chr. Hansen, Yakult Honsha Co., Ltd., Lifeway Foods, Inc., China-Biotics, Inc., Mother Dairy, Danisco and Groupe Danone S.A are some of the key players in the global probiotic market. According to a report by Markets and Market the global probiotic market is projected to reach USD 46.55 Billion by 2020. Europe forms the largest market for probiotics in 2015 whereas Asia Pacific region is predicted to be the fastest growing during the review period (http://www.marketsandmarkets.com/PressReleases/probiotics.asp).

Indian probiotic industry is in its infancy stage and currently accounts for less than 1% of the total world market turnover in the probiotic industry. According to India Probiotic Market Forecast and Opportunities, 2019, India's probiotic market is projected to

grow at a compound annual growth rate (CAGR) of around 19% till 2019. **Table 1.5** shows some of the probiotics available in the market, manufacturer, probiotic organisms and the health benefits associated with. The major companies in the Indian market are Mother Dairy, Amul, Danone Yakult, Nestle, Tablets India, Dr Reddy Laboratories, Unique Biotech, Zeus Biotech etc

 (http://www.researchandmarkets.com/research/b7c3nq/india_probiotic).

Table 1.5. Probiotics in market

Trade Name	Product	Manufacturer	Microorganism	Health benefits
Culturelle	Probiotic capsule	Amerfit Brands	*Lactobacillus GG*	Restores natural balance of good bacteria in the digestive tract, boost immune system, reduces digestive upset
Yakult	Fermented milk	Yakult Honsha	*Lb. casei Shirota*	Improves intestinal health, builds immunity
Lifeway Kefir	Cultured milk smoothie	Lifeway Foods	*B. lactis, Lb. lactis, S. florentinus, S. diacetylactis, Lb. acidophilus, B. longum, Lb. casei, Lb. reuteri, Lb. plantarum, Lb. rhamnosus, B. bacterium breve, Leuc. cremoris*	Boost immunity, aids in lactose digestion

Activia	Yoghurt	Danone	*B. lactis* CNCM I-2494 , *Lb. bulgaricus, S. thermophilus*	Helps in digestion, vitamin production
Actimel	Yoghurt	Danone	*Lb. casei* DN 114001, *Lb. delbrueckii* subsp. *bulgaricus, S. salivarius* subsp. *thermophilus*	Contributes to healthy gut flora
Attune probiotic chocolate	Chocolate Bar	Attune Foods	*B. lactis* HN019, *Lb. acidophilus* NCFM, *Lb. casei* LC11	Helps digestion
Good Belly SuperShot	Probiotic Oat Drink	GoodBelly	*Lb. plantarum* 299V	Improve digestive health
TruBiotics	Probiotic Capsules	Bayer HealthCare	*Lb. acidophilus* LA5, *B. animalis* BB12	Helps to replenish body's good bacteria, boosts immunity, support digestive health

1.3.16. Nutraceuticals from LAB

The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice, and defined as, "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease"(Brower, 1998; DeFelice, 1995). These products may range from dietary supplements, herbal products, functional foods (yoghurt, cereals and enriched foods) to genetically engineered 'designer' foods (Andlauer & Fürst, 2002). The word 'nutraceutical' in the food industry has no regulatory definition. The terms nutraceuticals, functional or medical foods, or dietary supplements are often used interchangeably. However, according to different perspectives these concepts can be distinguished e.g. functional food is a term to emphasize foods that may have a beneficial effect on the health (Bagchi, 2006). But when a functional food is associated with the prevention and/or treatment of disease(s) other than anaemia, it is called a nutraceutical. In other terms, a functional food for one consumer can be a nutraceutical for the other (Kalra, 2003).

Since nutraceuticals provide nutrition and health benefits they can be considered as food. At the same time they can be used for the prevention, treatment or cure of a disease and hence can be considered as drugs. But actually nutraceuticals occupy a grey area between food and drugs. Food is GRAS whereas nutraceuticals, even though they contain "natural" substances may not be GRAS. In order to be approved, a drug must demonstrate its safety and effectiveness. Nutraceuticals are not drugs simply because they have not gone through an approval process (Merton Boothe, 1998).

Nutraceuticals can be grouped into the following three broad categories (1) Nutrients: substances with nutritional value like vitamins, minerals, amino acids and fatty acids (2) Herbal: plant extracts and concentrates (3) Dietary supplements: nutritional supplements derived from other sources (e.g., pyruvate, chondroitin sulphate, steroid hormone precursors) that can have specific functions, such as sports nutrition, weight-loss supplements and meal replacements (Hathcock, 2001). Nutraceuticals are reported to prevent or treat hypertension, hypercholesterolemia, cancer, atherosclerosis and other CVDs, diabetes, obesity, arthritis, osteoporosis, macular degeneration (leading to irreversible blindness), cataracts, menopausal symptoms, insomnia, diminished memory and lack of concentration, digestive upsets and constipation and headaches (Shahidi & Naczk, 2003; Stauffer, 1999).

Besides lactic acid, LAB can also produce other compounds that contribute to the unique product characteristics like flavour, texture and nutrition. Nutraceuticals produced by LAB include B vitamins (mainly folate, riboflavin and cobalamin), low calorie sugars (mannitol, sorbitol, tagatose), L-alanine, and exopolysaccharides (EPS) (Hugenholtz et al., 2002b). Dairy industry is rapidly evolving in the area of nutraceuticals. Bio yoghurts containing *Lb. acidophilus* and bifidobacteria, other specialist fermented products like Yakult (providing *Lb. casei* Shirota), Nestles LC1 (providing *Lb. johnsonii*) and the Culturelle (providing Lactobacillus GG) are leaders in this sector (Morelli, 2002). **Table 1.6** shows the important nutraceuticals reported from LAB and their health benefits.

Table 1.6. Nutraceuticals from lactic acid bacteria

Nutraceuticals	Health benefits
B vitamins	
Folate	Involved in nucleotide biosynthesis. Prevents neural tube defects in newborns
Riboflavin	Prevents liver and skin-disorders, disturbed metabolism of the red blood cells
Cobalamin	Prevents pernicious anaemia
Low calorie sugars	
Sorbitol	Acts as low calorie sweetener and have anticancer properties
Mannitol	Acts as antioxidant and low calorie sweetener
Tagatose	Acts as low calorie sugar, prebiotic and anti-plaque agent
Exopolysaccharides	Increases flavour and texture of food and used as food additives

1.3.17. Metabolic Engineering of LAB for nutraceutical production

The advancement in the field of metabolic engineering has contributed much in the nutraceutical production in LAB. The biosynthetic capacity, metabolic versatility and relatively simple physiology of LAB make them suitable organisms for metabolic engineering. Modern metabolic engineering approaches mainly focus on more complex,

biosynthetic pathways leading to nutraceuticals (Kleerebezem & Hugenholtz, 2003). Various cloning systems, chromosome modification systems and expression systems have been developed to generate GM-LAB (de Vos, 1999). The most popular transformation system is electroporation with self-replicating vectors. In addition to efficient cloning systems, suitable expression systems have been   developed for the controlled expression of homologous and heterologous genes. Controlled constitutive expression is achieved by using a system of synthetic promoters (Solem & Jensen, 2002), while a nisin induced controlled expression system (NICE) allows the gradual over expression of genes (Kleerebezem et al., 1997). Nisin is the only bacteriocin with GRAS status for use in specific foods and has a history of 25 years of safe use in many European countries. This was supported by the results of various studies proving its nontoxic, nonallergenic nature (Rattanachaikunsopon & Phumkhachorn, 2010). **Fig. 1.5** gives an overview of the NICE system. In NICE system, the product of the expressed gene can either accumulate in the cell or secreted depending on the presence of signal sequence in the construct (Mierau et al., 2005).
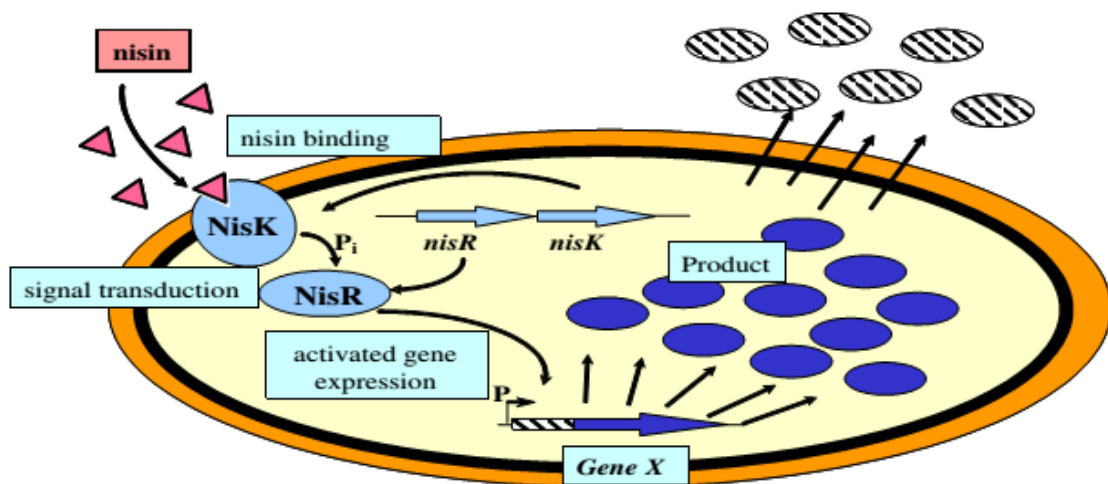

Fig. 1.5. Schematic representation of the NICE system: components and function
Courtesy: (Mierau et al., 2005)

The NICE system has been successfully used in *L. lactis* for the overexpression of folate biosynthetic genes (Sybesma et al., 2003a) and also for the overexpression of genes involved in riboflavin synthesis (Hugenholtz et al., 2002b). Metabolic engineering strategies resulting in the simultaneous overproduction of folate and riboflavin in *L. lactis* could eventually lead to 'multivitamin LAB' (Kleerebezem & Hugenholtz, 2003). Other systems

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are controlled by promoters based on sugar utilisation, e.g., the lactose operon promoter (Payne et al., 1996).

The key challenge in finding an effective nutraceutical is its bioavailability or absorption rate. The bioavailability of these nutrients will be higher in foods in its natural state. Even unprocessed foods are not broken down and digested as effectively. Hence, nutraceuticals with poor absorption rates results in nutrients being eliminated from the body without providing any health benefit (Keservani et al., 2010). Studies have shown that by adapting suitable metabolic engineering strategies it is possible to increase the bioavailability of certain nutraceuticals like folate (Sybesma et al., 2003a). Future analysis of other complex pathways will provide us with valuable knowledge concerning the potential of LAB as a better producer of nutraceuticals. Some of the metabolic engineering landmarks resulted in better yield of selected nutraceuticals were listed in **Table 1.7**.

Table 1.7. Successful metabolic engineering strategies employed in LAB

Nutraceuticals	Host LAB	Modified genes	Expression system/vector	Reference
B vitamins				
Folate (B9)	*L. lactis*	Folate gene cluster and PABA synthesis genes	NICE	(Sybesma et al., 2003a) (Wegkamp et al., 2007)
Riboflavin (B2)	*L. lactis*	GTP cyclohydrolase II (*ribA*)	NICE	(Hugenholtz et al., 2002b)
Low calorie sugars				
Mannitol	*L. lactis*	mannitol-1- phosphatase gene	NICE	(Wisselink et al., 2004)
Sorbitol	*Lb. plantarum*	sorbitol 6-phosphate dehydrogenase genes (*srlD1*, *srlD2*)	pGIZ90 vector	(Ladero et al., 2007)
Exopolysaccharides	*L. lactis*	EPS gene cluster	NICE	(Boels et al., 2003)

Nutraceutical industry is a rapidly evolving billion dollar industry. The growing awareness towards health, nutrition and lifestyle diseases among people resulted in an increased interest towards nutraceuticals. A collective effort by health professionals, nutritionists and regulatory toxicologists is necessary to provide the ultimate health and medicinal benefits of nutraceuticals to the mankind. Similar to drugs, there should be strict regulatory controls for nutraceuticals. Also the effect of different processing methods on the biological availability and effectiveness of nutraceuticals are to be evaluated (Pandey et al., 2010).

1.4. Conclusion

This literature review revealed that certain LAB are endowed with the ability to synthesize folate and this property of LAB could be utilized for the development of fermented foods with natural folates that might eventually substitute the chemical fortification using folic acid. The use of probiotic LAB offers an added advantage as the lactic acid fermentation itself can enhance the flavour and micronutrient profile of the fermented foods. Probiotic bacteria that are being used commercially in India are of foreign origin but a wide variety of indigenous probiotics remain unknown and underutilized. Though several LAB have been reported for folate production there is a continuing need to find new strains or to improve the properties of the existing ones by metabolic engineering. Since folate production is highly strain specific, careful selection of LAB strains and cultivation conditions are important parameters that decide the functionality of the product. Combinations of probiotic strains for folate fortification have enormous potential to be exploited that possibly could enhance the health benefits attributed to them reach the target population. At the same time the viability and stability of probiotic strains is very crucial to express their health benefits and technologies such as microencapsulation have been addressed to enhance their shelf life. Moreover, the recent advances in gene technology opened new possibilities for modulation of folate production by LAB.