Chapter 3
Lactic Acid Bacteria and B Vitamins

Wanqiang Wu and Baixi Zhang

Vitamin is one of the most significant micronutrients in all the biological metabolism progress. The 13 vitamins which are necessary to human bodies can be divided into lipid-soluble (vitamins A, D, E, K) and water-soluble (vitamin C and eight kinds of B vitamins). B vitamins contain thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pantothenic acid (vitamin B5), vitamin B6, biotin (vitamin B7 or vitamin H), folic acid (vitamin B9), and cobalamin (vitamin B12). Each B-type vitamin has different chemical properties, and its derivatives often participate in metabolism (such as energy production, red blood cell synthesis, etc.) as specific co-enzymes in physiological activities and play an important role in maintaining homeostasis (Table 3.1). B vitamins exist in various foods (Table 3.2) and easy to be destroyed by cooking and processing. Thus, the deficiency of B vitamins is the common problem influencing human health.

Lactic acid bacteria (LAB) are a kind of microorganism widely used in fermented foods, which are able to extend the shelf life of foods, improve the nutritional value, and develop the flavor. Lactic acid bacteria can synthesize and release some beneficial substances in food. The vitamin is one of the functional components of LAB synthesize. With the rapid development of genomics, humans can not only identify potential vitamin synthesis strains through genetic information but also improve their vitamin production by investigating their genetically anabolic networks. Therefore, as food microorganisms, the LAB is a kind of ideal microorganism for solving the problem of insufficient vitamin intake in humans in the future.

W. Wu (✉) · B. Zhang
Jiangnan University, Wuxi, China
e-mail: wuwanqiang@jiangnan.edu.cn; zbx@jiangnan.edu.cn

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Table 3.1 Coenzyme form and function of vitamin B

Vitamin B	Coenzyme forms	Function
B_1	Thiamine phosphate (TPP)	Oxidative decarboxylation, aldehyde group transfer
B_2	Flavin-5-phosphoric acid (FMN), flavin-5′-adenosine diphosphate (FAD)	Transfer H^+/electron
B_6	Phosphopyridoxal	Transamination, decarboxylation
B_9	Tetrahydrofolic acid	Carbon carrier
B_{12}	Cobamide	Transmethylase

Table 3.2 Food sources of vitamins B

Vitamin B	Food sources
B_1	Seed epidermis, animal viscera and lean meat, vegetables, and fruits are not abundant
B_2	Animal liver, milk, eggs, beans, and green leafy vegetables
B_6	Widely available, especially in liver, milk, egg yolks, vegetables, fish, whole grains, beans
B_9	Animal livers, kidneys, eggs, beans, yeast, nuts, green leafy vegetables, and fruits
B_{12}	Animal food, liver, egg yolk, meat, shellfish; milk and dairy products contain small amounts

3.1 Folic Acid

3.1.1 Chemical Structure and Demand

Folate, also known as folic acid, is the vital nutrient in the human body, participating in important cell metabolism, such as the copying, repairing, and methylation of DNA and the formation of nucleotide, other vitamins and amino acids, and so on. In addition to the essential role in repairing and copying of DNA, it also has antioxidant effects and protects DNA from free radicals (Duthie et al. 2002).

The folate deficiency presents in plenty of diseases, like Alzheimer's disease (Hugenholtz et al. 2002; Luchsinger et al. 2007), coronary heart disease (Danesh and Lewington 1998; Daniel and Bobik 1998), osteoporosis (Baines et al. 2007), neural tube defects (Group 1991; Czeizel and Dudas 1992), and so forth. Due to the prevalence of folic acid deficiency, the researchers currently focus on developing the food with high folate content. In this section, the term "folate" includes all folate derivatives, such as polyglutamic acid commonly found in foods and synthetic folic acid, often used as a food fortifier.

Folic acid (or pteroyl-L-glutamic acid) is attached to the pteridine and L-glutamic acid by P-aminobenzoic acid (Fig. 3.1). The form of natural folate differs depending on the nature of the pteroyl and acridine substituents as well as the number of glutamic acid residues attached to the pteroyl group. Natural folic acid consists of

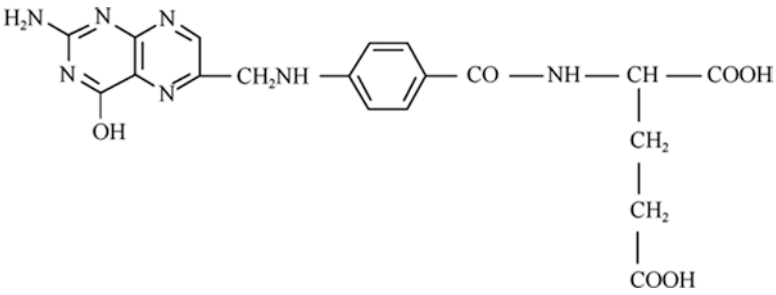

Fig. 3.1 Chemical structure of folic acid (pteroyl-L-glutamic acid)

5-methyltetrahydrofolate (5-MTHF), 5-formyltetrahydrofolate (5-formyl-THF), 10-formyltetrahydrofolate (10-formyl-THF), 5, 10-methylenetetrahydrofolate (5, 10-methylene-THF), 5-iminomethyltetrahydrofolate (5-formimino-THF), 5,6,7,8-tetrahydrofolate (THF), and dihydrofolate (DHF). Most of the natural folic acid is in the form of pteropolyglutamic acid, which is formed by the attachment of 2–7 glutamic acids on the amide (peptide) to γ-carboxyglutamic acid. The main folate in the cell is pteroglutamate, and the main extracellular folic acid is pteroyl monoglutamate. Natural pteropolyglutamic acid has up to 11 glutamic acid residues.

At present, the research on the nutrition of folic acid has been furthered deeply. The body cannot synthesize folic acid itself; therefore absorbing from food is necessary. Folic acid is found in many foods such as beans (soybeans, nuts, peas, etc.), leafy vegetables, citrus fruits, liver, and fermented or unfermented dairy products. Although soy and green leafy vegetables are good sources of folic acid, this does not meet the needs of the human body. The recommended daily intake (RDI) of folate from the US Food and Drug Administration (FDA) is 200–400 μg for adults and 400–600 μg for pregnant women. Despite the abundant sources of folic acid from food, lacking folic acid is still common, even in developed countries (Konings 2001; O'Brien et al. 2001). Based on these studies, some countries require mandatory folic acid supplementation. For example, in Canada and the United States, since 1998, folate has been mandatorily added in flour to reduce the incidence of neonatal neural tube defects.

3.1.2 Folate in Fermented Food

3.1.2.1 Folate in Fermented Dairy Products

Many important industrial LAB are able to synthesize folic acid. Commonly, there are *Streptococcus thermophilus*, *Bifidobacterium*, *Lactobacillus delbrueckii*, *Bulgarian subspecies*, etc. *Lactobacillus reuteri* CRL1098 is a well-known vitamin B12-producing strain that can also synthesize large amounts of folic acid (Santos et al. 2008). Therefore, the utilization of LAB to ferment the diary product can

improve the level of folate and, to some extent, alleviate the deficiency of folic acid. However, the ability of microbial strains to produce or utilize folic acid is also related to the type and characteristics of the strain. Most researchers believe that *Streptococcus thermophilus* usually produces folic acid, while *Lactobacillus delbrueckii* subsp. *bulgaricus* consumes folic acid during growth, so proper combination of fermenting microbes is essential for the development of fermented foods and increasing vitamin content. The study shows that the combination of *Streptococcus thermophilus* and *Bifidobacterium animalis* increases the amount of folic acid in fermented dairy products by six times (Crittenden et al. 2003).

3.1.2.2 Folate in Other Fermented Foods

Studies also show that the use of certain LAB as a starter can produce large amounts of folic acid in vegetable fermentation. These starters are combinations of two or three following bacteria, *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc*, *Lactobacillus flexeri*, *Lactobacillus pentosus*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *lactis*, and *Propionibacterium freudenreichii*. Finally, the highest yield of folic acid, usually 5-MTHF which can be directly utilized by the body, is twice that of the unfermented vegetables. Thus, with this research, new foods for vegetables can be developed to supplement the body's required folic acid (Jägerstad et al. 2004).

Another example of using LAB to increase the folic acid content in fermented foods is the fermentation of corn flour. After fermenting corn flour at 30 °C for 4 days with *Streptococcus faecalis*, the level of folic acid increases by nearly three times (Murdock and Fields 1984).

3.1.2.3 Folate-Producing LAB

Many studies have shown that industrially fermenting bacteria such as *Lactobacillus* and *Streptococcus thermophilus* have the ability to synthesize folic acid. Therefore, some fermented dairy products have higher levels of folic acid than unfermented dairy products. However, the ability of different industrially fermenting bacteria to produce or utilize folic acid varies widely. Most LAB cannot synthesize this vitamin (Hugenholtz and Kleerebezem 1999; Crittenden et al. 2003), but experiments have shown that *Lactobacillus plantarum* can produce folic acid in chemical synthesis media without folic acid. The folic acid content in milk is 20–60 g/L, while the folate content in yogurt may increase to above 200 g/L, depending on the fermentation and storage conditions (Wouters et al. 2002). The folate content also depends on the type of strain of *Streptococcus thermophilus* and the *Lactobacillus delbrueckii* subsp. *bulgaricus* which has been proved to reduce folate production during growth. Recent studies demonstrate that some probiotics (such as *Bifidobacteria*) can also synthesize folic acid (Table 3.3).

Table 3.3 The LAB in fermented dairy products which produces folate

LAB	Output	Reference
S. thermophilus CSCC2000	36.5 ng/g	Crittenden et al. (2003)
S. thermophilus CSCC2002	36.2 ng/g	
S. thermophilus CSCC2010	36.5 ng/g	
S. thermophilus CSCC2012	36.3 ng/g	
S. thermophilus CSCC2013	28.6 ng/g	
S. thermophilus CSCC2016	30 ng/g	
S. thermophilus CSCC2018	32 ng/g	
B. animalis lactate subspecies CSCC5127	13.5 ng/g	
B. animalis lactate subspecies CSCC5123	13 ng/g	
B. animalis CSCC1941	8.5 ng/g	
B. Bifidobacterium CSCC5128	9 ng/g	
B. longum subspecies *infantis* CSCC5187	20.5 ng/g	
B. breve CSCC5187	33.5 ng/g	
B. animalis lactate subspecies *Lafti* B94CSCC5127	10.5 ng/g	
E. Faecium CSCC5140	11.5 ng/g	
L. acidophilus ATCC4356	13.3 ng/mL	Rao et al. (1984)
Lactococcus lactis cremoris CM22	12.5 μg/L	Gangadharan et al. (2010)
Lactococcus lactis cremoris CM28	14.2 μg/L	
L. acidophilus N1	63.9 ± 5.2 μg/L	Lin and Young (2000)
L. acidophilus 4356	53.9 ± 4.6 μg/L	
L. bulgaricus 449	62.8 ± 2.1 μg/L	
L. bulgaricus 448	46.7 ± 5.0 μg/L	
S. thermophilus 573	59.6 ± 2.3 μg/L	
S. thermophilus MC	75.8 ± 6.5 μg/L	
L. Bifidobacterium ATCC15708	99.2 ± 3.8 μg/L	
B. longum B6	397 ± 60 μg/L	Hugenschmidt et al. (2010)
L. plantarum SM39	131 ± 196 μg/L	
L. brevis SM34	125 ± 28 μg/L	
L. reuteri ATCC55730	84 ± 32 μg/L	Pompei et al. (2007)
L. fermentum SM81	44 μg/L	
B. adolescentis MB114	65 μg/L	
B. adolescentis MB115	54 μg/L	
B. adolescentis MB227	54 μg/L	
B. adolescentis MB239	27 μg/L	
B. longum subspecies *infantis* ATCC15697	82 μg/L	
B. pseudocatenulatum MB116	41 μg/L	
B. pseudocatenulatum MB237	12 μg/L	

(continued)

Table 3.3 (continued)

LAB	Output	Reference
B. pseudocatenulatum MB264	95 μg/L	Sybesma (2003)
L. lactis cremoris B42	92 μg/L	
L. lactis cremoris B64	116 μg/L	
L. lactis cremoris B697	90 μg/L	
L. lactis cremoris B628	116 μg/L	
L. lactis cremoris NZ9000(6)	291 μg/L	
L. lactis cremoris NZ9010(6) *Ldh negative*	91 μg/L	
L. lactis cremoris B26	69 μg/L	
L. lactis cremoris B27	62 μg/L	
L. lactis cremoris B1172	63 μg/L	
L. lactis cremoris B1173	57 μg/L	
L. lactis cremoris B621	98 μg/L	
L. lactis cremoris B86	100 μg/L	
L. lactis cremoris B87	79 μg/L	
L. lactis cremoris B103	29 μg/L	
S. thermophilus B108	202 μg/L	
S. thermophilus B119	120 μg/L	
S. thermophilus B911	45 μg/L	
Leuconostoc B629	44 μg/L	
Leuconostoc WCFS-1	90 μg/L	
L. helveticus B219	89 μg/L	
L. helveticus B230	1 μg/L	
L. bulgaricus B194	41 μg/L	

3.1.2.4 Biosynthesis and Regulation Mechanism of Folic Acid in LAB

In LAB, plants and fungi, the de novo synthesis of folic acid includes the pteridine metabolic branch and the para-aminobenzoic acid (pABA) metabolic branch, both of which are indispensable.

The pteridine metabolic branch of lactic acid bacteria is as follows. Firstly, an important intermediate-6-hydroxymethyl-7,8-dihydropteridine pyrophosphate (DHPPP) is gradually synthesized through the participation of guanosine triphosphate (GTP) cyclized hydrolase I (GCHY-I), neopterin aldolase (DHNA), dihydroneopterin triphosphate pyrophosphatase (DHNTP), and 6-hydroxymethyldihydropterin pyrophosphate kinase (DHPPK). DHPPP then binds to pABA with the action of dihydropteroate synthase (DHPS) to form dihydropteroate, which is a direct precursor of folic acid synthesis. Finally, it is catalyzed into polyglutamic acid tetrahydrofolate by dihydrofolate reductase (DHFR) and folate glutamate synthase (FDGS).

The pABA metabolic branch of lactic acid bacteria is the formation of pABA by the branched acid with the action of 4-amino-4-deoxylated acid synthase and lyase and then the same as the pteridine metabolic branch. pABA will bind to DHPPP

with the participation of DHPS, forming the direct precursor dihydropteroic acid; thus folic acid is synthesized.

Genes for folate biosynthesis have been identified in *Lactococcus lactis* (Sybesma et al. 2003), *Lactobacillus plantarum* (Kleerebezem et al., 2003), and *Lactobacillus bulgaricus* (van de Guchte et al., 2006). These genes encode enzymes that regulate the de novo synthesis of folic acid, including the *follB* gene encoding DHNA, the *folK* gene encoding DHPPPK, the *folE* gene encoding GCHI, the *folP* gene encoding the DHPS and the *folC* gene, the *folQ* (ylgG) gene encoding DHNTPase, the *folA* gene encoding DHFR, and so on. Not every kind of LAB produces folic acid because some LAB lack genes involved in folate biosynthesis, such as *Lactobacillus gargle* (Wegkamp et al., 2004) and *Lactobacillus saliva* (Claesson et al., 2006).

Metabolic engineering can increase folate levels in *Lactococcus* and *L. reuteri* (Sybesma et al. 2003; Wegkamp et al., 2007). By controlling the overexpression of the *folKE* gene encoding 6-hydroxymethyl-dihydropyrophosphate kinase and GTP cyclohydrolase in *Lactococcus*, the production of extracellular folate can be increased by ten times, and the total folate content of the product is increased three times more than the former. At the same time, overexpression of the *folA* gene encoding dihydrofolate reductase reduces total folic acid production by about 50%. In addition, simultaneous overexpression of *folKE* and *folC* facilitates the accumulation of intracellular folate. Overexpression of GTP cyclase I is also beneficial for increasing the flux of folate biosynthesis. Therefore, the appropriate combination of overexpressed *folKE* with other genes that overexpress or inhibit expression can significantly increase the yield of folic acid. Transgenic LAB are as safe as natural LAB (Leblanc et al. 2010).

In fact, we can not only increase folic acid production yield by overexpressing genes involved in folate biosynthesis but also increase folate production by overexpressing other genes involved in related metabolism. For example, an excess of pABA does not result in an increase in the active pool of folate itself. However, overexpression of pABA and folate biosynthesis gene clusters yields high levels of folate (Wegkamp et al. 2007). This is a process that is not affected by pABA supplementation.

After transferring the plasmid of *Lactococcus* MG1363 containing the complete folate synthesis genes (*folA*, *folB*, *folKE*, *folP*, *ylgG*, and *folC*) to *Lactobacillus gasseri* ATCC33323, the recombinant strain is thus transformed into a folate-producing strain (Wegkamp et al. 2004).

3.2 Riboflavin

3.2.1 Chemical Structure and Demand

The term "riboflavin" refers to all biologically active vitamin B_2, including riboflavin, riboflavin-5-phosphate (FMN), and riboflavin-5′-adenosine diphosphate (FAD). The chemical name of riboflavin is 7,8-dimethyl-10-(1′-D-ribosyl)-isorazine, and its molecular formula is $C_{17}H_2ON_4O_6$. The molecular structure

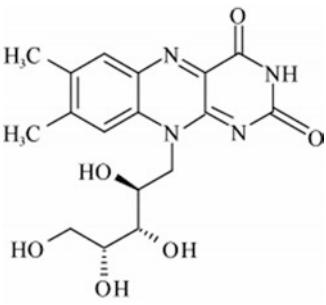

Fig. 3.2 Chemical structure of riboflavin

includes a ribitol side-chain isorazine group (Fig. 3.2), which is the basis of all riboflavin derivatives. Riboflavin is an orange-yellow odorless needlelike crystal that is easily destroyed by sunlight and ultraviolet light, but it is stable to the thermal environment and is soluble in neutral liquid. Heating in the liquid at 120 ° C for 6 h was less damaged.

Riboflavin (vitamin B_2) was initially considered as a growth factor in rabbits. It plays a key role in cell metabolism as a precursor of riboflavin-5-phosphate (FMN) and 5′-adenosine diphosphate (FAD). Riboflavin is directly involved in the biological oxidation of carbohydrates, proteins, and fats in vivo and plays a variety of physiological functions.

The symptoms of riboflavin deficiency in the human body include throat congestion, sore throat, edema of mouth, cleft lip, glossitis, etc. (Wilson 1983). Severe cases are relatively rare. Human demand for riboflavin is closely related to gender, age, physiological status (pregnancy, lactation), and so on. Due to the inadequate storage of riboflavin, normal adults need to take 0.3–1.8 mg riboflavin daily to meet their metabolic needs. Although riboflavin is found in many foods such as dairy products, meat, eggs, and green vegetables, riboflavin deficiency is still widespread in both developed and developing countries (O'Brien et al. 2001; Blanck et al. 2002).

3.2.2 Riboflavin in Fermented Food

3.2.2.1 Riboflavin in Fermented Dairy Products

Dairy products contain riboflavin, but this is not the best source of such essential vitamins. Drinking milk contains 1.2 mg riboflavin per liter. In the case of low daily milk intake, increasing the content of riboflavin in milk has become an important method to prevent riboflavin deficiency. Clinical trial has shown intake of 200 g probiotics or traditional yogurt daily for 2 consecutive weeks can promote absorption of vitamin B_2 (Fabian et al. 2008). In addition, the content of riboflavin in dairy products varies with processing technology and microbial activity. For example, the contents of riboflavin and folic acid in cheese were 1.7 mg/L and 90 μg/L, respectively, and the contents of riboflavin and folic acid in yoghurt were 2.0 mg/L and

80 μg/L, respectively. The contents of riboflavin and folic acid in kefir were 1.3 mg/L and 50 μg/L, respectively, which were significantly higher than that in unfermented milk (1.2 mg/L) and folic acid (40 μg/L). The increase in riboflavin content in fermented dairy products is due to the use of a starter that can produce riboflavin during processing.

3.2.2.2 Riboflavin in Other Fermented Foods

Indonesian cardamom, also known as Tianbei, is a naturally fermented soybean product with very rich nutrition. It is known as "alternative food for meat" and is an indispensable food for Indonesians and a representative food for healthy food. Indonesian soybean sauce contains high concentration of B vitamins, including thiamine, riboflavin, vitamin B_6, folic acid, vitamin B_{12}, and so on. These vitamins are produced by a variety of microorganisms during the fermentation process. It was found that *Streptococcus*, *Lactobacillus casei*, *Lactobacillus plantarum*, and other LAB could be isolated from Indonesian soybean sauce and its soaking water (Keuth and Bisping 1993).

3.2.3 Riboflavin-Producing LAB

Recent studies have shown that many lactic acid bacteria can produce riboflavin (Table 3.4).

3.2.4 Biosynthesis and Regulation Mechanism of Riboflavin in LAB

Riboflavin biosynthesis begins with GTP and D-ribose-5-phosphoric acid and undergoes seven-step enzymatic reactions to synthesize riboflavin (Bacher et al. 2000). The imidazole ring of GTP is hydrolyzed to produce 4,5-diaminopyrimidine, which is converted to 5-amino-6-ribitol amino-2,4(1H, 3H)-pyrimidinedione by deamination, side-chain reduction, and dephosphorylation. Condensed 5-amino-6-ribitol amino-2,4(1H,3H)-pyrimidinedione from 6,7-dimethyl-8-ribitol group provided by ribulose-5-phosphate.The riboflavin and 5-amino-6-ribitol amino-2,4(1H, 3H)-pyrimidinedione were produced by the disproportionation with 5-amino-6-ribitol amino-2,4(1H, 3H)-pyrimidinedione,3,4-dihydroxy-2-butanone-4-phosphate and pyridine dioxide derivatives. This process requires two bifunctional enzymes, RibA and RibG, and riboflavin synthases, RibH and RibB. Among them, II/3,4-dihydroxybutyrone phosphate synthase hydrolyzed by GTP imidazole ring is a rate-limiting enzyme in riboflavin synthesis, which is encoded by *ribA* gene (Hu mbelin et al. 1999).

Table 3.4 Riboflavin-producing lactic acid bacteria

lactic acid bacteria	Yield	Reference
Lactobacillus fermentum MTCC8711	2.8 mg/L	Jayashree et al. (2010)
Lactobacillus fermentum GKJFE[a]	3.49 mg/L	
Lactobacillus plantarum NCDO1752	600 μg/L	Burgess et al. (2006)
Leuconostoc mesenteroides CB200	150 μg/L	
Leuconostoc mesenteroides CB201	160 μg/L	
Leuconostoc mesenteroides CB202	400 μg/L	
Leuconostoc mesenteroides CB203	300 μg/L	
Leuconostoc mesenteroides CB204	160 μg/L	
Leuconostoc mesenteroides CB205	150 μg/L	
Leuconostoc mesenteroides CB206	180 μg/L	
Leuconostoc mesenteroides CB207	500 μg/L	
Leuconostoc mesenteroides CB208	260 μg/L	
Leuconostoc mesenteroides CB209	120 μg/L	
Leuconostoc mesenteroides CB210	130 μg/L	
Lactococcus lactis subsp. *cremoris* NZ9000	700 μg/L	Burgess et al. (2004)
Lactococcus lactis subsp. *cremoris* NZ9000[a]	24 mg/L	Sybesma et al. (2004)
		Leblanc et.al. (2005)
Lactobacillus plantarum CRL725	(91 ± 11)ng/mL	Valle et al. (2014)
Lactobacillus plantarum CRL725 variant G[a]	(1100 ± 20)ng/L	

[a]Genetic engineering bacteria

Although LAB strains have the ability to synthesize riboflavin, most probiotic strains also consume riboflavin while synthesizing, thereby reducing riboflavin levels in the fermented product (Elmadfa et al. 2001). Therefore, how to improve the riboflavin production of LAB is our concern. Screening fermentation strains is a traditional method to improve riboflavin production of LAB. Screening of resistant strains with rosin is an effective method to obtain riboflavin-producing strains. Rose yellow pigment is a toxic riboflavin analogue that is often used to mutagenize food-grade riboflavin-producing microorganisms, such as *Lactococcus lactis* NZ9000 (riboflavin yield after mutation screening is 700 μg/L) (Burgess et al. 2004), *Lactobacillus plantarum* NCDO1752 (riboflavin yield after mutation screening is 600 μg/L), *Leuconostoc mesenteroides* spp. (riboflavin yield after mutation screening is 500 μg/L), and *Propionibacterium freudenreichi* spp. (riboflavin yield after mutation screening is 3000 μg/L) (Burgess et al. 2006). The high-yield riboflavin strain, *Lactococcus* CB010, screened by rosin resistance has similar bioavailability to pure riboflavin, can treat most riboflavin deficiency, and improve growth shrinkage, high activation coefficient values, and hepatomegaly in animal experiments (Leblanc et al. 2005). Other research proved that the strain of *Propionibacterium freudenreichii* B2336 screened by rosin resistance can produce riboflavin with high yield and is beneficial for the recovery of animal models of riboflavin deficiency. Foods fermented with *Propionibacterium freudenreichii* B2336 have higher levels

of riboflavin and alleviate the clinical symptoms of most riboflavin deficiency (Leblanc et al. 2006).

In addition to traditional strain screening methods, another method to increase riboflavin production in fermented foods is genetic engineering, which can improve the production of riboflavin in lactic acid bacteria by genetic engineering. This method is currently a hot topic of research, and the food-grade fermentation strain after transformation has great potential in the future food field.

Hydrated ammonium oxalate-corynebacterium sulfate contains all the genes for riboflavin biosynthesis, and high-yield strains can be obtained by recombinant DNA technology. The recombinant strain produced and accumulated 17-fold more riboflavin than the original strain that was not genetically engineered (Koizumi et al. 2000). In the *Lactococcus* NZ9000 containing the pNZGBAH sequence, simultaneous overexpression of four biosynthetic genes *ribG*, *ribH*, *ribB*, and *ribA* can greatly increase riboflavin production, eventually reaching 24 mg/L. Yogurt fermented with this strain as a feed for animals in animal experiments can alleviate the symptoms of riboflavin deficiency in mice (Leblanc et al. 2005).

Genetic engineering methods have significantly reduced production costs compared with current methods of vitamin production. Another advantage of this method is that the modified host microorganism can produce not only one vitamin. Sybesma et al. (2004) altered the biosynthesis pathway of folic acid and riboflavin in *Lactococcus* by site-directed mutagenesis and metabolic engineering and then screened for a mutant *Lactococcus lactis* NZ9000 by leucine-resistant resistance screening. The upstream regulatory region of the riboflavin biosynthesis gene of the mutant has changed, resulting in an increase in riboflavin production. At the same time, the GTP cyclohydrolase I of the mutant has overexpressed and increased the yield of folic acid (Sybesma et al. 2004).

3.3 Cobalamin

3.3.1 Chemical Structure and Demand

Vitamin B_{12}, also known as cobalamin, is a general term for a class of porphyrin compounds containing cobalt. Vitamin B_{12} is obtained in industrial production and is not naturally occurring (Rucker et al. 2001). The main forms of natural vitamin B_{12} are deoxyadenosylcobalamin (coenzyme B_{12}), methylcobalamin, and pseudocobalamin. Structurally, the cobalamin molecule can be divided into three parts (Fig. 3.3): central glucolin ring, Coβ ligand attached to the adenosine methyl group, and a Co alpha ligand containing nucleotide rings (usually dimethylbenzimidazoles). However, in some anaerobic bacteria, other forms of ligands such as adenine also exist, forming pseudocobalamin (pseudo B_{12}) and other active factors (Martens et al. 2002). The crystal structure of vitamin B_{12} is very complex. Hodgkin firstly analyzed it in 1956 by X-ray method (Hodgkin et al. 1956).

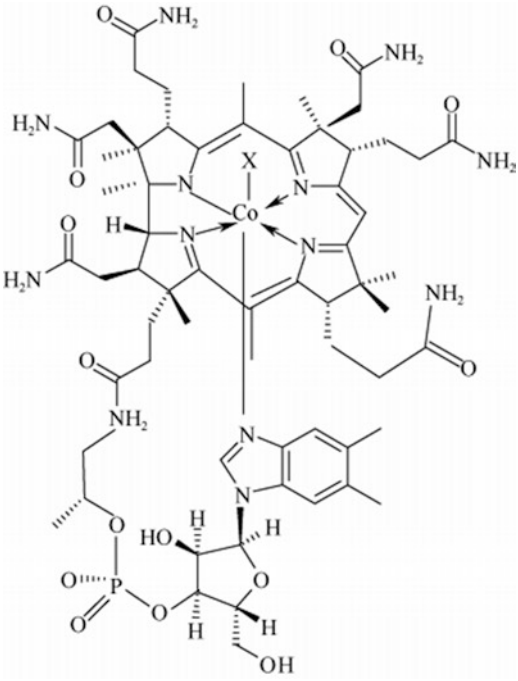

Fig. 3.3 Chemical structure of vitamin B_{12}

Animals and plants cannot produce vitamin B_{12}, which is the only vitamin produced by microorganisms. Actinomycetes and bacteria are the main microorganisms that synthesize vitamin B_{12} (Roth et al. 1996; Smith et al. 2007). Among them, *Streptomyces* in actinomycetes (such as *Streptomyces antibiotics*, *Streptomyces faecalis*, *Streptomyces griseus*, *Streptomyces roseolus*, etc.) and *Flavobacterium*, *Bacillus megaterium*, *Bacillus subtilis*, *Lactobacillus*, and *Lactobacillus reuteri* in bacteria are the most commonly used fermentation strain in production. Bacteria in the rumen of adult ruminants and vegetarians can produce vitamin B_{12} in large quantities, which is the main source of the vitamin. There is no such microorganism in the small intestine of humans. Therefore, in order to obtain vitamin B_{12}, humans must take it from the outside. Good sources of vitamin B_{12} are animal meat (especially liver and kidney), eggs, dairy products, and functional foods. The daily demand for vitamin B_{12} in adults is 2–3 μg. The deficiency of vitamin B_{12} can affect hematopoietic function and damage the nervous system and cardiovascular system to varying degrees. Severe patients may suffer from malignant anemia.

3.3.2 Cobalamin in Fermented Food

In fermented food, cobalamin content in fermented dairy products is higher. After fermentation, the cobalamin content of milk increased. Especially in cheese and yogurt, the cobalamin content can reach 1.5 times than before fermentation (Wouters et al.

2002). Sufu is a traditional folk food of the Han nationality, which has been circulating for thousands of years in China. It is very popular in China and the Southeast Asia because of its good taste, high nutritional value, and unique flavor. A study showed that the content of cobalamin in sufu increased by two times during fermentation.

3.3.3 Cobalamin-Producing LAB

Lactobacillus reuteri is a representative strain of LAB for the fermentation of vitamin B_{12}. The *cob* cluster (methyltransferase gene cluster) of *Lactobacillus reuteri* strains has a distinct feature. There is a *hem* gene in the center of *cob* cluster of *Lactobacillus reuteri*, which can only be found in some *Clostridium* strains (Rodionov et al. 2003). Bioassays have confirmed that *Lactobacillus reuteri* can produce 50 μg/L of vitamin B12, and the production of *Lactobacillus reuteri* can meet the daily needs of adults. However, the cobalamin produced by the anaerobic pathway of *Lactobacillus reuteri* is pseudocobalamin (Santos et al. 2007). Although pseudocobalamin is a coenzyme of many anaerobic and facultative anaerobes, it does not play a role in animals and humans (Rucker et al. 2001). Recent data indicate that *Lactobacillus reuteri* strain CRL1098 can produce not only pseudocobalamin but also a small amount of other Guerin-like compounds, including coenzyme B_{12} under microaerobic conditions (Santos et al. 2007).

3.3.4 Biosynthesis and Regulation Mechanism of Cobalamin in LAB

The first biological model for studying the biosynthesis of vitamin B12 is *Propionibacterium freudenreichii*, which is an industrial strain of vitamin B12 (Battersby 1994). Researchers at Battersby and Scott Labs elucidated the aerobic synthesis pathway of vitamin B12 by studying *Pseudomonas aeruginosa* (Blanche et al. 1995). Escalante-Semerena (2007) found that the anaerobic synthesis of vitamin B_{12} is carried out by *Propionibacterium freudenreichii*, *Salmonella*, and *Bacillus megaterium*. The conclusions of these studies indicate that the synthesis of cobalamin can be carried out under both aerobic and anaerobic conditions, and the two synthetic pathways are slightly different. In the anaerobic pathway, the central Co^{2+} will be inserted at an earlier step. The porphyrin ring produces an unstable, difficult-to-separate intermediate, whereas under aerobic conditions, Co^{2+} inserts a porphyrin ring at a later step, resulting in a relatively stable intermediate.

The synthesis of cobalamin can be divided into three steps. The first step is the condensation of the precursor 5-aminolevulinic acid (ALA) to form uroporphyrin III. The second step is to catalyze the synthesis of adenosylcobalamin acid from uroporphyrinogen III. The third step is to catalyze the synthesis of adenosylcobalamin from adenosylcobalaminic acid.

The common methods to increase vitamin B_{12} production are random mutation and genetic engineering technology (Martens et al. 2002; Burgess et al. 2009). At present, different metabolic engineering methods can increase the production of vitamin B_{12} of *Propionibacterium freudenreichii*. Through metabolic engineering, *Propionibacterium freudenreichii* containing *cobA*, *cbiLF*, and *cbiEGH* increased the production of vitamin B_{12} by 1.7, 1.9, and 1.5 times, respectively. After overexpression of *hemA* gene and homologous *hemB* AND *cobA* genes from *Rhodopseudomonas* spp., the vitamin B_{12} yield of *Propionibacterium freudenreichii* was 2.2 times higher than that of wild-type strains (Piao et al. 2004).

In order to effectively utilize vitamin B_{12} biosynthetic genes, researchers have established a genetic transformation system of *Propionibacterium freudenreichii*. pRGO1 is a cryptic plasmid of *Propionibacterium* E214; Kiatpapan et al. (2000) determined the complete nucleotide sequence of pRGO1, which has a length of 6868 bp and a GC content of 65% and contains six open reading frames (orf1 to orf6). Kiatpapan et al. (2000) also constructed a shuttle vector pPK705, which shuttles between *Escherichia coli* and *Propionibacterium* containing orf1, orf2, orf5, and orf6. Finally, the vector successfully transformed *Propionibacterium freudenreichii* IFO12426, while high conversion efficiencies are also found in other species of *Propionibacterium*, and the pPK705 vector is also stably present in *Propionibacterium freudenreichii*. The successful construction of this host-vector system facilitates genetic research and factory production of vitamin B_{12}.

3.4 Other B Vitamins

3.4.1 Vitamin B1

Vitamin B1, also known as thiamine or anti-neuritis, is a combination of a pyrimidine ring and a thiazole ring (Fig. 3.4). It is a white crystalline or crystalline powder and the first vitamin to be found. It can be synthesized by fungi, microorganisms, and plants, while animals and humans can only be obtained from food.

Vitamin B1 is widely found in foods (especially in the epidermis and germ of grains). But the peel during food processing leads to a large loss of VB_1, resulting in insufficient intake of VB_1. The study found that vitamin B1 daily intake below

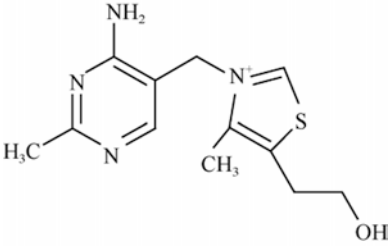

Fig. 3.4 Chemical structure of vitamin B1 and thiamine pyrophosphate

0.2 mg will result in vitamin deficiency. VB_1 deficiency leads to diabetic microangiopathy (Stirban et al. 2006), neurodegenerative diseases (Zhao et al. 2008), and so on.

Vitamin B1 is involved in glucose metabolism in the body (Haas 1988). After being phosphorylated in the body, it mainly exists in three forms, respectively. It is thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate (TTP), the most important biologically active form of which is TPP. TPP is an important cofactor in the transketolase (TK) reaction of pyruvate dehydrogenase complex (PDHC), α-ketoglutarate dehydrogenase complex (KGDHC), and pentose phosphate pathway, among which PDHC and KGDHC are the important components of the cell's use of glucose to produce ATP pathways; TK is involved in gluconeogenesis (Gibson and Blass 2007). Therefore, VB_1 plays an important role in glucose metabolism. It also participates in numerous redox reactions in the body and improves energy metabolism in cells.

Owing to the rich sources of VB_1, there are few studies using biosynthesis for VB_1 production. Research have shown that the concentration of VB_1 in milk increased by 11% after 48 h of fermentation with *B. longum* (Hou et al. 2000), indicating that LAB have the ability to produce VB_1.

3.4.2 Vitamin B6

The activity of vitamin B_6 is reflected by three kinds of vitamin B_6 and their 5′-phosphate esters. The active coenzyme form of vitamin B_6 is pyridine 5′-phosphate (PLP). Vitamin B_6 is a coenzyme of about 140 enzymes in the human body, including transaminase, decarboxylase, racemase, and dehydratase. PLP is also involved in the metabolism of carbon groups, folic acid, and vitamin B12 and is involved in more than 80 biochemical reactions. It has an irreplaceable role in human protein metabolism, glycogen decomposition into glucose and lipid metabolism, hormone regulation, niacin formation, nucleic acid, and immune metabolism. Vitamin B_6 is a general term for pyridoxine, pyridoxal, and pyridoxamine (Fig. 3.5).

Vitamin B_6 is widely found in various foods, including animal liver, eggs, brown rice, sunflower seeds, walnuts, soybeans, carrots, and bananas. In addition to the body's intake of vitamin B_6 from the food, the intestinal bacteria in the body can also synthesize a part of vitamin B_6, so the primary deficiency of vitamin B6 is not

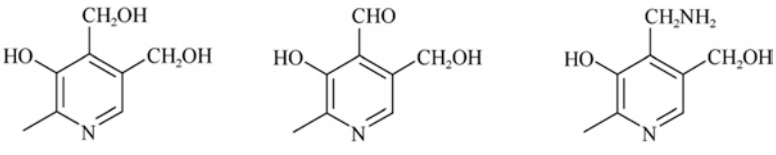

Fig. 3.5 Chemical structure of vitamin B_6 and its derivatives

widespread. The Chinese Nutrition Society recommends that vitamin B_6 can tolerate a maximum intake of 50 mg/day for children and 100 mg/day for adults.

The LAB starter of yoghurt, cheese, and other fermented foods can increase the production of vitamin B_6 (Shahani and Chandan 1979; Alm 1982). Recent studies have found that co-fermentation of *Streptococcus thermophilus* ST5, *Lactobacillus helveticus* R0052 ST5, or *Bifidobacterium longum* R0175 can increase the content of vitamins B1 and B6 in soybean products (Champagne et al. 2010).

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