Chapter 7

Biosynthesis of Vitamins by Probiotic Bacteria

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Abstract

Vitamins are important micronutrients that are often precursors to enzymes, which all living cells require to perform biochemical reactions. However, humans cannot produce many vitamins, so they have to be externally obtained. Using vitamin-producing microorganisms could be an organic and marketable solution to using pseudo-vitamins that are chemically produced, and could allow for the production of foods with higher levels of vitamins that could reduce unwanted side effects. Probiotic bacteria, as well as commensal bacteria found in the human gut, such as *Lactobacillus* and *Bifidobacterium*, can de novo synthesize and supply vitamins to human body. In humans, members of the gut microbiota are able to synthesize vitamin K, as well as most of the water-soluble B vitamins, such as cobalamin, folates, pyridoxine, riboflavin, and thiamine.

Keywords: probiotic, folate, riboflavin, cobalamin, biosynthesis

1. Introduction

Vitamins are typically categorized as fat-soluble vitamins, which includes vitamins A, D, E, and K, or as water-soluble vitamins, which includes vitamin C, biotin (vitamin H or B7), and a series of B vitamins—thiamin (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6), folic acid (B11), and cobalamin (B12). While fat-soluble vitamins act as important elements of cell membranes, water-soluble vitamins serve as coenzymes that typically transport specific chemical groups [1]. Humans are incapable of synthesizing most vitamins and they consequently have to be obtained exogenously. The use of vitamin-producing microorganisms might represent a more natural and consumer-friendly alternative to fortification using chemically synthesized pseudo-vitamins.

The biochemical pathways involved in B-vitamin biosynthesis by food microorganisms were previously described in detail [2]. Many prokaryotes need water-soluble vitamins for nutritional purposes [3], but also typically need them for biosynthetic processes. The ability of particular microorganisms to produce B vitamins could supplant the expensive chemical production of these vitamins to enrich food or be improved for in situ fortification of fermented foods. Much research has been conducted in recent years to elucidate the biosynthetic pathways of these vitamins in a number of microorganisms.

Probiotic bacteria positively impact the immune system and the composition and functioning of the gut microbiota [4]. Furthermore, the production of vitamins has resulted in many healthy benefits to the host. Probiotic bacteria, mostly belonging to the genera *Lactobacillus* and *Bifidobacterium*, confer a number of health benefits, including vitamin production [5]. Probiotic bacteria, members of the gut microbiota, are able to synthesize vitamin K and most of the water-soluble B vitamins, such as biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin, and thiamine, in humans [6].

The production of B-vitamins, especially folate and riboflavin (B2), by probiotic bacteria has been extensively researched as described in a recent review [7, 8]. Several lactic acid bacteria (LAB) species (e.g., *Lactococcus lactis, Lactobacillus gasseri*, and *Lactobacillus reuteri*) and *Bifidobacterium* (e.g., *B. adolescentis*) produce these vitamins, often in large quantities, and are, therefore, often found in fermented foods [9, 10]. Moreover, increased vitamin biosynthesis has been obtained by metabolic engineering [11, 12]. Folate biosynthetic genes and riboflavin biosynthetic operon have been overexpressed in *L. lactis,* resulting in types that produce folate [12] or riboflavin [12] at higher rates. Sybesma et al. [13] modified the biosynthetic pathways of folate and riboflavin in *L. lactis*, resulting in the simultaneous overproduction of both vitamins, through directed mutagenesis and selection and metabolic engineering.

This review focused on riboflavin, folic acid, and cobalamin, three of the water-soluble B vitamins whose biosynthetic pathways were inextricably linked, briefly covering their physiological functions and dietary sources before concentrating on novel overproduction strategies in probiotics.

2. Riboflavin biosynthesis

In contrast to many plants, fungi, and bacteria, humans cannot produce riboflavin or vitamin B2, and thus require it as a dietary supplement. Riboflavin is available as a dietary source and is also produced by the microflora of the large intestine [6, 14]. Riboflavin (vitamin B2) plays an essential role in cellular metabolism, as it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which both act as hydrogen carriers in many biological redox reactions.

Riboflavin is synthesized by many bacteria and its biosynthetic pathway has been studied extensively in *Bacillus subtilis* and *Escherichia coli*. Bacher et al. [15, 16] found that riboflavin biosynthesis requires the precursor's guanosine 5′-triphosphate (GTP) and ribulose 5-phosphate. The first step of the GTP-dependent branch of the biosynthetic pathway is encoded by

ribA in *E. coli*. In *B. subtilis* it is also encoded by *rib*A but in this case RibA acts as a bifunctional enzyme that also catalyzes the configuration of 3,4-dihydroxy-2-butanone 4-phosphate from ribulose 5- phosphate [17]. The overexpression of RibA in *B. subtilis* produces 25% more riboflavin, indicating that this enzyme is rate-limiting in riboflavin biosynthesis [18]. However, in *Lactococcus lactis*, the overexpression of *ribA* did not lead to increased riboflavin production [12].

The ability of some bacteria and fungi to overproduce riboflavin has been harnessed for industrial production. Such commercial producers include the ascomycetes *Eremothecium ashbyii* and *Ashbya gossypii*. However, advantages were perceived in developing bacterial and yeast fermentations to avail of their high growth rates, and less costly and complex growth media. Currently, *A. gossypii*, *Candida famata*, and *B. subtilis* are exploited for riboflavin production, with riboflavin production levels reaching 15 g/L, 20 g/L, and 14 g/L, respectively [19–21]. In *A. gossypii*, metabolic engineering increased riboflavin production almost 10-fold [22]. *A. gossypii* has also been targeted as a microorganism to overproduce riboflavin using oil waste [23]. In the case of *B. subtilis*, high levels of riboflavin production were achieved as a result of exposure to purine analogues and the toxic riboflavin analogue roseoflavin, or by genetic engineering [19, 24].

It has been reported that fermentation of cow milk with *L. lactis* and *Propionibacterium freudenreichii* ssp. *shermanii* as starter cultures significantly increased the riboflavin content of milk. Since the riboflavin produced by starter cultures is largely in the free form, the bio-availability is expected to be better than the bio-availability of riboflavin in unprocessed milk [12, 25]. The food-grade fermentative LAB *L. lactis* also grows in the absence of riboflavin. On the basis of the genome sequence of *L. lactis* IL1403 [26], it seemed that all genes involved in riboflavin biosynthesis (*rib* genes) were present in this organism.

Species and/or strain-specific traits in LAB provided genetic information for riboflavin biosynthesis. Several of the sequenced members of LAB possessed similar abilities to produce riboflavin, as suggested by comparative genome analysis, but an interrupted *rib* operon was sometimes seen in certain strains. Deficient genetic information was usually related to the inability to produce riboflavin in LAB. For instance, the sequenced genome of *Lactobacillus plantarum* strain WCFS1 had an incomplete *rib* operon, which lacked the entire *rib*G and part of the *rib*B genes [27]. Further, this strain could not grow unless riboflavin was present [28]. However, several selected strains of *L. plantarum* contained the whole rib operon and could produce vitamin B2. The *L. plantarum* strain NCDO 1752, and the recently sequenced *L. plantarum* strain JDMI and *L. plantarum* strains, for example, were isolated from cereals-derived products [28, 29]. Furthermore, even in LAB strains that contained all *rib* genes, riboflavin production had to be confirmed by chemical analysis.

3. Folate biosynthesis by human gut commensals

Folic acid, also known as vitamin B11, is a dietary necessity for humans, because it is used in several metabolic reactions, such as the biosynthesis of the building blocks of DNA and RNA,

the nucleotides. It is recommended that adults take 200 μg daily, but pregnant women are encouraged to take a double dose daily, as folic acid could thwart neural-tube defects in newborns [30]. Low folic acid has been linked to high homocysteine levels in the blood, which could lead to coronary diseases [31, 32]. It has also been shown to protect against some forms of cancer [33]. Folate is conspicuously absent in many food products and is considered an essential additive to the general diet.

Folates are comprised of a mono- or polyglutamyl conjugate and these compounds were named after the number of glutamyl residues (PteGlu*n*), where *n* denoted the total number of glutamyl residues. The folates act as enzyme co-substrates in one-carbon (C1) metabolism of amino acids and nucleotides, in which the fully reduced (tetrahydro-) form functions as an acceptor or donor of a single carbon unit [34]. Folic acid has played a significant role in the production of purines and pyrimidines, and, therefore, in DNA synthesis. Methionine synthase uses 5-methyltetrahydrofolate in the conversion of l-homocysteine to l-methionine [35]. A majority of the methionine formed is converted to S-adenosylmethionine, which is a common donor of methyl groups for DNA, RNA, hormones, neurotransmitters, membrane lipids, and proteins [36]. The folate molecule contains one pterin moiety, created from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), bound to para-aminobenzoic acid (pABA, vitamin B10). As such, de novo biosynthesis called for both the precursors, DHPPP and pABA. Plants and bacteria could make the latter from the pentose phosphate pathway. Erythrose 4-phosphate and phosphoenolpyruvate go through the shikimate pathway to become chorismate, which acts as a branching point toward the biosynthesis of aromatic amino acids and pABA. Chorismate is transformed via aminodeoxychorismate synthase into 4-amino-4-deoxychorismate. Subsequently, pyruvate is cleaved by 4-amino-4-deoxychorismate lyase to give pABA, which is ultimately necessary for folate biosynthesis. The biosynthesis of DHPPP proceeds via the conversion of GTP in four consecutive steps. The first step is catalyzed by GTP cyclohydrolase I and involves an extensive transformation of GTP, through Amadori rearrangement, to form a pterin ring structure. Following dephosphorylation, the pterin molecule undergoes aldolase and pyrophosphokinase reactions, which produce the activated pyrophosphorylated DHPPP.

Folate biosynthesis continues with the formation of a C–N bond joining DHPPP to pABA. This condensation reaction, catalyzed by dihydropteroate synthase, yields 7,8-dihydropteroate (DHP). DHP is glutamylated by dihydrofolate synthase, resulting in dihydrofolate (DHF). It is then reduced by DHF reductase to the biologically active cofactor tetrahydrofolate (THF) and subjected to the addition of multiple glutamate moieties by folylpolyglutamate synthase to yield THF-polyglutamate. Polyglutamilation may also take place before the occurrence of the reduction step, being catalyzed by DHF synthase or, in many bacteria, by a bifunctional enzyme that is responsible for both DHF synthase and folylpolyglutamate synthase activities [37].

However, although all available complete bifidobacterial genomes are expected to specify aminodeoxychorismate synthase, a gene specifying a putative 4-amino-4-deoxychorismate lyase can only be found on the genome of *B. adolescentis* ATCC15703 and *B. dentium* Bd1 [9], which are, thus, expected to accomplish de novo biosynthesis of pABA. In contrast, *B.*

animalis subsp. *lactis* does not appear to possess the entire pathway for DHPPP biosynthesis or the gene encoding dihydropteroate synthase. Thus, *B. animalis* subsp. *lactis* was predicted to be auxotrophic for folates or DHP, and would, therefore, be unable to complete folate biosynthesis, even if pABA was present.

Lactobacilli are also typical human gut commensals and were recently investigated to discover if they could serve as possible folate producers [38]. Lactobacilli from various fermented foods have been investigated as starter cultures for the manufacturing of folate-fortified dairy products, while lactobacilli isolated from the human gut have been explored as folate-producing probiotics [39–42]. The availability of genome sequences of various lactobacilli provided an important contribution to the genetics underlying folate biosynthesis in this group of microorganisms [38]. For example, lactobacilli did not appear to harbor the genetic determinants for de novo pABA synthesis, with the exception of *L. plantarum* WCFS1 [27], suggesting that the vast majority of lactobacilli were unable to synthesize folate in the absence of pABA.

Currently, the strains of *Lactobacillus* with the greatest relevance for the manufacturing of probiotics and functional foods belong to the species *L. acidophilus*, *L. casei*, *L. paracasei*, *L. plantarum*, *L. reuteri*, and *L. salivarius* [43]. Like *L. lactis*, these species harbor a folate biosynthesis cluster that includes the gene encoding dihydropteroate synthase and all of the genes for the biosynthesis of DHPPP, with the exception of alkaline phosphatase. In *L. lactis*, the dephosphorylation of dihydroneopterin triphosphate into the monophosphate was demonstrated to occur through an alternative route, involving a Nudix pyrophosphohydrolase [44]. Many lactobacilli contain various genes encoding putative Nudix enzymes, such as *mut*T genes for DNA repair. However, *Lactobacillus sakei*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* have a homologue of the *L. lactis* gene in the *fol* cluster. In contrast, in *Lactobacillus fermentum*, *L. plantarum*, and *L. reuteri*, the *fol* cluster held the gene of a putative non-Nudix purine NTP pyrophosphatase, which could be responsible for hydrolyzing dihydroneopterin triphosphate in these species. As such, *L. plantarum*, *L. sakei*, *L. delbrueckii*, *L. reuteri*, *L. helveticus*, and *L. fermentum* were predicted to generate DHPPP and could also be folate producers if cultured with pABA present [37, 44].

4. Vitamin B12 biosynthesis

Vitamin B12, otherwise known as cobalamin, is the biggest and most intricate vitamin. Cobalamin describes a cluster of cobalt-containing compounds (corrinoids) that have a lower axial ligand, which holds the cobalt-coordinated nucleotide (5, 6-dimethylbenzimidazole) as a base. Although humans only use vitamin B12 for two enzymatic activities, it is still an important dietary supplement. (R)-methyl-malonyl-CoA mutase assists in the metabolism of propionyl-CoA, which compounds such as valine, thymine, methionine, and odd-chain fatty acids produce when broken down. This ado-cobalamin-dependent enzyme catalyzes the rearrangement of propionyl-CoA following its carboxylation and epimerization to succinyl-CoA, which then goes through the citric acid cycle. Methionine synthase needs vitamin B12 in

the form of methylcobalamin. Using 5-methyltetrahydrofolate as a methyl donor, this enzyme methylates homocysteine to form methionine [45].

Humans cannot synthesize vitamin B12, and, thus must obtain it from organisms that can. Only a limited number of bacteria are known to produce vitamin B12, three of which—*Pseudomonas denitrificans*, *Bacillus megaterium*, and *Propionibacterium freudenreichii*—are used for commercial production [46–48].

Cobalamin has the most complex structure of all the vitamins synthesized by bacteria requiring about 30 genes for its biosynthesis. Most of the work in characterizing cobalamin biosynthesis has been performed in *Salmonella typhimurium* and *P. denitrificans*. Two different pathways exist for adenosylcobalamin (ado-cobalamin) biosynthesis: (1) an oxygen-dependent pathway, which is found in *P. denitrificans*, and (2) an anaerobic pathway, which has been identified in, among others, *S. typhimurium*, *P. freudenreichii* subsp. *Shermanii*, and *B. megaterium*. Every gene required in the anaerobic cobalamin biosynthesis was found on the genome of *S. sanguinis* [49].

Genes encoding enzymes contributing to the oxygen-dependent pathway have been given the prefix *cob*, while those involved in the oxygen-independent pathway have the prefix *cbi* [50]. Due to the early insertion of cobalt in the anaerobic pathway, the remaining intermediates are cobalto-complexes and therefore require enzymes with different substrate specificities than the intermediates in the aerobic pathway although many of the reactions catalyzed are similar. CobZ was identified in *Rhodobacter capsulatus*, which catalyzes a reaction similar to that advanced by CobG, but in a different way, as the two proteins did not display any primary sequence resemblance. CobZ was also found to have a flavin in the form of a non-covalently bound FAD, two Fe-S centers, and a b-type heme, which was not similar to CobG [51]. It was thought that the final step in the cobalamin biosynthetic pathway in *S. typhimurium* involved the dephosphorylation of adenosylcobalamin-5′-phosphate, which is catalyzed by CobC and challenges the pathway indicated where CobS catalyzes the condensation of a-ribazole and an Ado-GDP-cobinamide [52]. The gene that reduces cobalt in the aerobic pathway has yet to be identified, but two candidate genes were identified to encode this enzyme, named CobR [53].

LAB are traditionally known as auxotrophic for cobalamin and are generally used for the biological analysis of this vitamin. Recently, however, cobalamins were identified in *L. reuteri* as were some of the genes encoding enzymes for the biosynthesis of this vitamin [54]. The presence of a B12-dependent metabolic pathway that converts glycerol into propanediol most likely allowed this LAB to synthesize B12. The discovery of the biosynthetic genes could increase the production of B12 through metabolic engineering, and facilitate the transfer of the production pathway to other LAB.

L. reuteri CRL1098 was also found to metabolize glycerol in a B12-free medium, indicating that a LAB might also be able to make cobalamin [55]. Chromatographic analysis of the intracellular bacterial extract of *L. reuteri* CRL 1098 proved that this strain was able to produce a cobalamin-like compound with an absorption spectrum that was similar to that of standard cobalamin but had a distinct elution time, while cobalamin production was proved with different bioassays [55]. Genetic evidence of cobalamin biosynthesis by *L. reuteri* CRL 1098 was then achieved by using different molecular biology techniques, and it was found that at least 30

genes assisted the de novo synthesis of the vitamin. The genetic organization (*cob* and *cbi* genes) resembled that of *Salmonella enterica* and *Listeria innocua* [56].

The complete genome of *Lactobacillus sanfranciscensis* TMW 1.1304, isolated from industrial sourdough fermentation, was also recently sequenced [57]. The data showed that only one gene necessary to the cobalamine synthesis was encoded by the sequenced strain *L. sanfranciscensis* TMW1.1304. Conversely, growth experiments revealed that several *L. sanfranciscensis* strains grew on vitamin B12-free media, which implied that these strains could synthesize cobalamine de novo [57].

Other strains of genus *Lactobacilli* such as *Lactobacillus coryniformis* isolated from goat milk [58], *L. plantarum* isolated from *kanjika* or Japanese pickles [59, 60], *Lactobacillus rossiae* isolated from sourdough [61], and *Lactobacillus fermentum* CFR 2195 isolated from breast-fed healthy infants' fecal matter [62] were shown to produce cobalamin-type compounds. Moreover, the genetic and biochemical data suggested that cobalamin biosynthesis genes would be spread to *Lactobacillus buchneri*, *Lactobacillus hilgardii*, and *Lactobacillus brevis*, and also contain genes of the *cob-pdu* gene cluster [63]. Therefore, the possibility of various cobalamin-producing strains and species of LAB would benefit not only from future basic studies on cobalamin production, but also from its application in the development of vitamin B_{12}-contained fermented products.

5. Biosynthesis of other B-group vitamins

Thiamine (vitamin B1) is a coenzyme in the pentose phosphate pathway that is required to synthesize fatty acids, steroids, nucleic acids, and the aromatic amino acid precursors into various neurotransmitters and other bioactive compounds essential for brain function [64]. Beyond its role as a necessary cofactor in the folate cycle, vitamin B6 (pyridoxine) also plays an important role in amino acid metabolism, which makes it a rate-limiting cofactor in the synthesis of neurotransmitters such as dopamine, serotonin, gamma-aminobutyric acid (GABA), noradrenaline, and the hormone melatonin [64].

LAB fermentation in yogurt, cheese, and other fermented products was shown to result in increased levels of riboflavin, folate, vitamin B12, niacin, and pyridoxine [65, 66]. Soy fermentation with *Streptococcus thermophilus* ST5 and *Lactobacillus helveticus* R0052 or *Bifidobacterium longum* R0175 also caused a small increase in thiamine and pyridoxine concentration that was not statistically significant [67].

6. Biosynthesis of vitamin K

Vitamin K serves as a cofactor for the enzyme that converts specific glutamyl residues in a few proteins to g-carboxyglutamyl (Gla) residues, aiding in the process. Humans obtain the daily nutritional requirement for vitamin K through the dietary phylloquinone that exists in plants, and, to some extent, through bacterially produced polyisoprenyl-containing compounds

called menaquinones created in the human gut [68]. LAB were examined for their ability to produce quinone compounds, as vitamin K occurred naturally in two forms, namely, K1 (phylloquinone) in green plants, and K2 (menaquinones) in animals and some bacteria [69].

7. Conclusions

The use of vitamin-producing strains provided a new perspective on the specific uses of probiotics. Many food-grade bacteria overproduce B vitamins, including riboflavin (vitamin B2), folate (vitamin B11), and cyanocobalamine (vitamin B12), which could allow them to organically enrich raw food materials like soy, milk, meat, and vegetables with B vitamins, preventing the need for additives. Thus, the food industry could take advantage of these novel and efficient vitamin-producing strains to add nutritional value to fermented products and save money in the process. Notably, vitamin metabolism pathways were shown in genes that specified the biosynthetic enzymes for riboflavin, cobalamin, and folate production. It is increasingly possible to identify potential vitamin-producing strains and interpret the intertwined mechanisms for their biosynthesis, because of the expanding availability of genome sequences, which could be used to expand the vitamin-producing capacities of the human gut.

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