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Current advances in the bioactivity, biosynthesis, and production enhancement strategies of 5-methyltetrahydrofolate from *Moringa oleracea* Lam.: A comprehensive review

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ABSTRACT: Folate, primarily derived from plants, plays a crucial role in plant growth and metabolism, and is closely associated with human health, including the prevention of birth defects, maintenance of neurological and fetal health, and treatment of anemia. Owing to its diverse biological activities, folate has been widely applied in the fields of medicine and food. Moringa (*Moringa oleifera* Lam.) is currently recognized as the plant with the highest known folate content and is extensively utilized in folate-related research. 5-Methyltetrahydrofolate (5-MTHF) is the only folate derivative that can directly participate in the methylation cycle, addressing the unmet needs of populations for whom synthetic folate is ineffective. However, 5-MTHF is present in plants at low levels and is challenging to extract. Consequently, with advancements in synthetic biology, novel approaches have emerged to provide greener, more efficient, and stable production pathways for 5-MTHF. This article systematically reviews the classification, biological activities, and biosynthetic pathways of folate, with a focus on biological strategies to enhance 5-MTHF production, aiming to deepen the understanding of its functional value. The review seeks to offer innovative perspectives for food science, nutrition, and related industries, while also exploring solutions for populations with special needs.

Keywords: *moringa*, *5-methyltetrahydrofolate*, *bioactivity*, *biosynthesis, yield*

1. Introduction

Folate, a group of water-soluble B vitamins, has the molecular formula $C_{19}H_{19}N_7O_6$. It is composed of pteridine, *p*-aminobenzoic acid, and *L*-glutamic acid conjugated together[1]. Folate plays a crucial role in the human body, and its deficiency can lead to various diseases, particularly during embryonic development when cellular activity is exceptionally high. These diseases include neonatal neural tube defects[2], megaloblastic anemia[3], cleft lip and palate[4], depression[5], tumors[6], and others, as shown in Figure 1. The human body cannot synthesize folate independently and primarily relies on dietary intake and folic acid supplements.

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Disorders of folate metabolism enzymes are a significant cause of folate deficiency, as supplemented folic acid cannot be effectively converted and utilized[7]. Currently, the main component of nearly all folate products on the market is synthetic folic acid (FA). The synthesis of FA requires the body's enzyme system to convert it into 5-methyltetrahydrofolate (5-MTHF) for absorption and utilization. However, long-term intake of FA may increase the burden on the liver, and excessive intake may even increase the risk of diseases such as cancer[8].

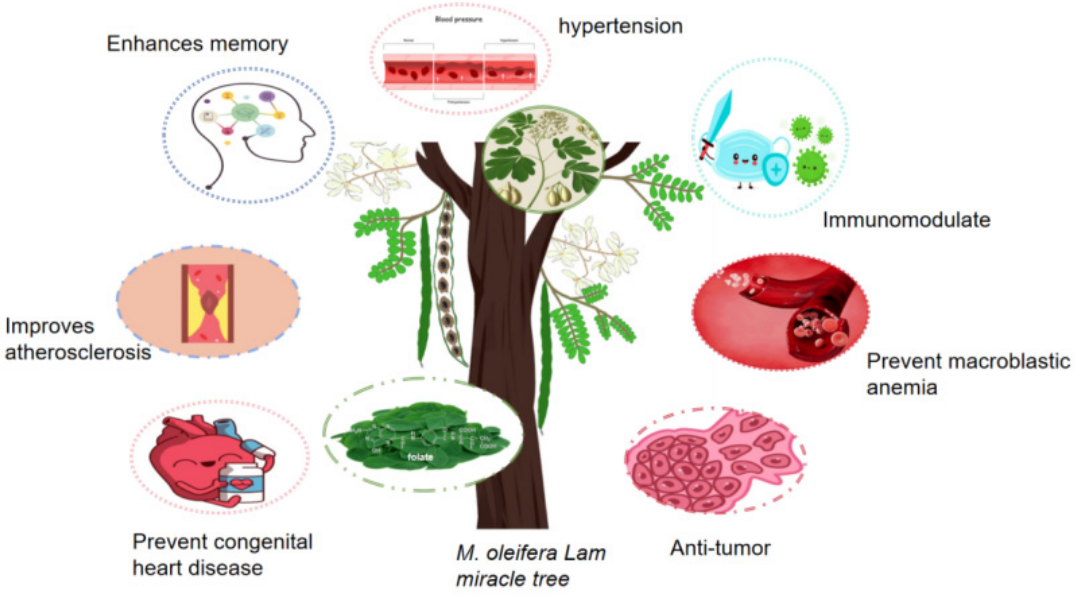


Figure 1. Folate-Related Biological Activities of *M. oleifera* Lam.

Natural folate (folate, folacin) is the reduced form of synthetic FA, and its structural characteristics determine that natural folate exhibits better bioavailability and safety compared to synthetic FA[9]. Among them, 5-Methyltetrahydrofolate (5-MTHF), one of the most active components of natural folate, is the only form of folate that can directly participate in the methylation cycle. It is also a fundamental substance essential for maintaining human life activities, cell functions, and DNA and protein synthesis[10]. China has classified 5-MTHF as a food nutrient enhancer within the scope of food additives and has established corresponding regulations regarding its addition and intake[11]. Therefore, supplementing with 5-MTHF, which can be directly absorbed and utilized by the body, can avoid the metabolic issues associated with synthetic FA. This ensures that there are no non-responsive individuals in the folate supplementation program, which is of great significance for disease prevention and the promotion of physical and mental health.

Moringa oleifera Lam. (commonly known as the drumstick tree) is a perennial deciduous tree native to tropical and subtropical regions. It belongs to the genus Moringa within the family Brassicaceae (formerly classified as Cruciferae)[12]. It is native to countries[13] such as Pakistan, India, Bangladesh and Afghanistan and is one of the ancient species of trees used as a source of food and medicinal resources. Due to its unique geographical and climatic conditions, Yunnan began to introduce moringa for cultivation as early as the 1960s. After 2015, its planting area accounted for 70% of the country's total, making it an economically advantageous species in Yunnan[14]. The roots, stems, leaves, flowers, and fruits of moringa all possess high

nutritional value and unique medicinal properties[15]. Widely used in food, medicine, and other fields, it is known as the "diamond of plants" and the "miracle tree" in some tropical countries[16]. Research indicates that Moringa oleifera is an excellent natural source of folate, with its leaves containing remarkably high folate concentrations of 1.11-1.24 mg/100 g. This represents a 13-fold higher concentration compared to spinach (88 μg/100 g) and significantly exceeds levels found in other conventional folate-rich vegetables including kale (1.00 $mg \cdot kg^{-1}$ fresh weight) and cooked asparagus (146 μg/100 g)[17-21]. Specific data reveal that the total folate content in Moringa leaves reaches 899.9 μg/100 g dry weight, with 5-MTHF accounting for 16.1% of the total[22]. Furthermore, there is a notable disparity in the distribution of folate (including 5-MTHF) among different tissues of *Moringa oleifera*. A study by Guo *et al*.[23] on the *Moringa oleifera* cultivar PKM-1 revealed that the pods exhibited the highest folate content, reaching 46.76 μg/100g (fresh weight), which was statistically significant compared to other parts ($P < 0.05$). The leaves contained 24.64 μg/100g, while the stems and roots had relatively lower levels, at 23.03 μg/100g and 21.18 μg/100g, respectively. Compared to the control variety "Northern Moringa Leaves" (52.90 μg/100g), the fresh leaves of PKM-1 contained slightly less folate; however, its pods demonstrated a significantly higher accumulation capacity, indicating that pods serve as a key reservoir for folate in *Moringa*. On the other hand, analysis of Moringa leaf powder (dry product) by Liu *et al*.[24] further underscores its exceptionally high folate value: the folate content in the leaf powder was as high as 1184.57 μg/100g, significantly surpassing that of traditional functional foods such as spirulina (only 60 μg/100g). Based on a daily intake of 25 g of leaf powder, approximately 296 μg of folate can be consumed, fulfilling 74% of the Recommended Nutrient Intake (RNI) for adults and 49.4% for populations with higher folate requirements, such as pregnant and lactating women.

However, despite the relatively high content of 5-MTHF from natural sources, large-scale isolation and preparation remain considerably challenging[25]. In contrast, synthetic folic acid is significantly different from 5-MTHF in terms of metabolism and toxic side effects *in vivo*[26], making it difficult to meet the current societal demands for folate supplementation[27]. Therefore, finding new ways to obtain 5-MTHF more efficiently and conveniently has become a key challenge for researchers.

In recent years, with the discovery of key enzymes involved in the biosynthesis of active ingredients, it has been increasingly recognized that synthetic biology can be used to design and engineer microbial strains for the fermentation-based production of natural medicines. Therefore, synthetic biology has become a novel and efficient approach to obtain 5-MTHF in an eco-friendly manner. The paper provides an overview of the categorization of folate, its biological activity, and the 5-MTHF biosynthetic pathway. Additionally, it discusses molecular biological methods to enhance 5-MTHF content, aiming to offer insights for the high-yield production, modernisation, and application of 5-MTHF.

2. Folate, FA, and active folate (active folate supplements)

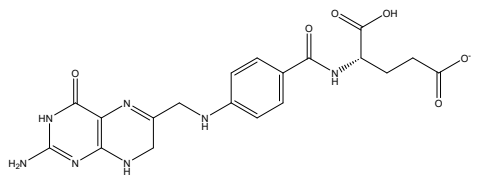
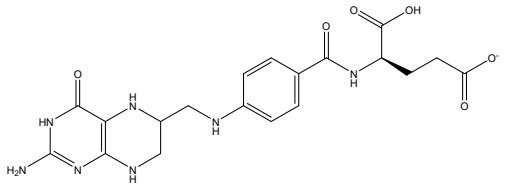
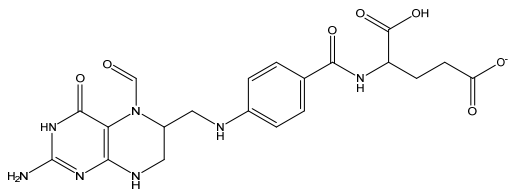
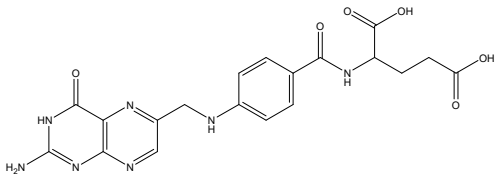
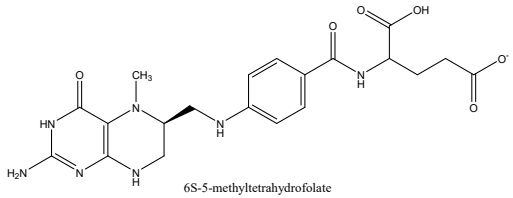
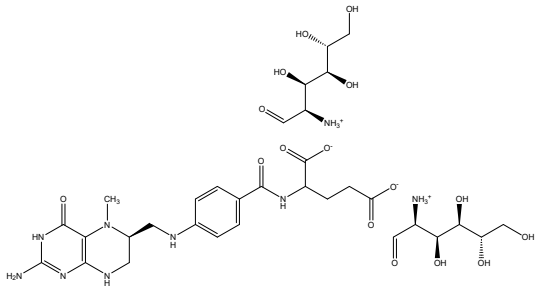
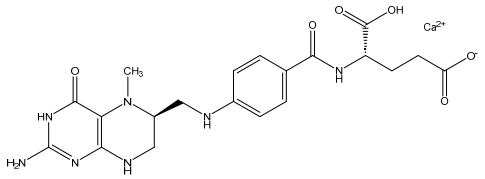
Folate molecules exist in many forms, theoretically as many as 150, but fewer than 50 are found in plant and animal tissues[28]. Naturally occurring folate refers to a group of biologically active compounds primarily obtained from dietary sources. Various forms of folate are generated through different substitutions on the

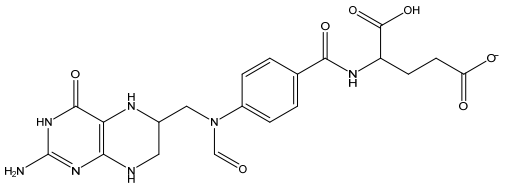
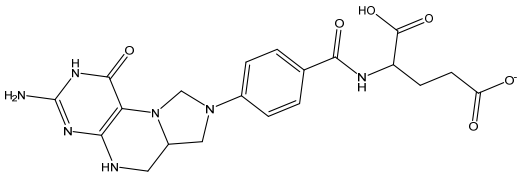
pyrazine ring and variations in the number of glutamate residues attached to aminobenzoylglutamate[29]. FA is an artificially synthesized oxidized form of monosodium glutamate, mainly used in fortified foods and dietary supplements. 5-MTHF is the active form of folate, which does not require complex metabolic conversion and can be directly absorbed by the intestine and enter the bloodstream for human utilization. Its bioavailability is close to 100%, and the peak plasma concentration is usually 2-3 times higher than taking an equivalent dose of synthetic FA. Table 1 summarizes the comparisons among natural folate, synthetic FA, and 5-MTHF (active folate supplements) in terms of sources, chemical forms, stereochemical configurations, etc., providing a clearer distinction of their advantages and disadvantages[7, 30].

Among them, after entering the human body, the synthesis of FA requires a complex multi-step enzymatic reduction process to be converted into biologically active 5-MTHF. This process is first absorbed in the intestine, and then gradually converted into dihydrofolate, tetrahydrofolate, 5,10-methylenetetrahydrofolate under the action of various enzymes such as liver dependent dihydrofolate reductase (DHFR). Finally, 5-MTHF is generated under the catalysis of methylenetetrahydrofolate reductase (MTHFR). 5-MTHF is particularly important due to its unique biological properties, it is able to bypass the limitations of DHFR and MTHFR, it is not a substrate for DHFR or MTHFR and can directly participate in one-carbon unit metabolism. The effects of MTHFR enzyme kinetics and gene polymorphism have been studied. MTHFR is a key enzyme in the folate metabolism pathway, catalyzing the reduction of 5,10-methylenetetrahydrofolate to 5-MTHF. The efficiency of this process is crucial for the effective utilization of folate. The polymorphism of MTHFR gene (especially C677T locus) significantly affects the activity of the enzyme: *CC* genotype (wild type): It was found that polymorphisms at 677 of the MTHFR gene would lead to a decrease in MTHFR activity and affect the body's ability to metabolize folic acid. The 677 locus of the wild-type MTHFR gene was CC, while the folic acid metabolism capacity of 677TT homozygous mutant patients was only 30% of that of 677CC individuals, and the decrease in folate metabolism could increase the risk of URPL[31-33]. For these people with folate metabolism disorders, taking synthetic folic acid that requires MTHFR enzyme to participate in transformation will have a greatly reduced effect, which may lead to insufficient active folic acid actually available, and may be accompanied by an increase in homocysteine (Hcy) level[34, 35]. 5-MTHF completely bypasses the dependence on MTHFR enzyme activity. Therefore, for people with MTHFR gene mutations, supplementing 5-MTHF is a more efficient and reliable choice.

Folate plays a crucial role in the synthesis of nucleic acids, thymidylate, neurotransmitters, and phospholipids. As a carrier of one-carbon units, it participates in various metabolic reactions[36], including the methylation of homocysteine to methionine and the methylation of coding or non-coding genes[37]. Of all these natural forms, 5-MTHF is the main form present in the body's blood circulation and the active form that directly performs physiological functions

Table 1 Comparison of nature folate, synthetic folic acid, and active folate (active folate supplements)

Forms	Folate/ Folacin	Folic Acid, FA	Active folate (active folate supplements)
Source	Deep green vegetables, citrus, beans, animal liver	Dietary supplements, fortified foods (flour, grains, etc.)	1. Produced by the body's own metabolism 2. Active folate supplements (such as 5-methyltetrahydrofolate calcium, 5-methyltetrahydrofolate glucosamine)
Chemical name/form	Multiple forms of folate and polyglutamic acid, encompassing: • Dihydrofolate (DHF) • Tetrahydrofolate (THF) • 5-Formyl-THF • 10-Formyl-THF • 5,10-Methenyl-THF	Butterfly acid monosodium glutamate (oxidized)	L-5-methyltetrahydrofolate (reduced form, active form), encompassing: • 6S-5-methyltetrahydrofolate (6S-5-MTHF) 6S-5-methyltetrahydrofolate calcium, 5MTHF-Ca) (6S)-5-methyltetrahydrofolic acid, glucosamine salt
Stereochemical Configuration (6S/6R)	 Dihydrofolate CAS :4033-27-6 Molecular weight:443.41  Tetrahydrofolate CAS :135-16-0 Molecular weight:445.43  5-Formyltetrahydrofolate CAS :200-361-6 Molecular weight:473.44	 Folic Acid CAS: 59-30-3 Molecular weight: 441.40	 6S-5-methyltetrahydrofolate CAS: 31690-09-2 Molecular weight: 459.46  (6S)-5-methyltetrahydrofolic acid, glucosamine salt CAS:1181972-37-1 Molecular weight: 817.79800  6S-5-methyltetrahydrofolate calcium CAS: 26560-38-3 Molecular weight: 497.52

Forms	Folate/ Folacin	Folic Acid, FA	Active folate (active folate supplements)
	 10-Formyltetrahydrofolate CAS:2800-34-2 Molecular weight: 473.44  5,10-Methylenetetrahydrofolate CAS :2800-34-2 Molecular weight:473.44		
Absorption and metabolic pathways	After being enzymatically hydrolyzed into the form of monosodium glutamate in the intestine, it is absorbed into the liver and also needs to be converted into 5-MTHF by DHFR, MTHFR enzyme. The process is easily influenced by food and health status.	It needs to be reduced and methylated in two steps by DHFR, MTHFR through the liver, and converted into 5-MTHF before it can enter the bloodstream. The process is slow and the ability is limited.	No need for liver metabolism. It can be directly absorbed into the bloodstream by the small intestine and utilized directly by cells. It is the main form of existence in blood circulation.
Bioavailability	Low (about 50%)[38]	High (about 85% -100%)[38, 39]	Highest (equivalent to or higher than synthetic folate)[30, 38]
stability	Low (susceptible to light, heat, pH， and oxygen degradation)	High (solid-state stable for 1~2 years)	6S-5-MTHF: Low (susceptible to light, heat, pH，and oxygen degradation) Crystalline product of 6S-5-MTHF：high (stable for 4 years at room temperature)
Effects on *MTHFR* gene polymorphisms	Affected	Affected	Not Affected
Whether it masks vitamin B_{12} deficiency	No	Yes, it can cause nerve damage	No, high security
Whether it can pass through the blood-brain barrier	Yes	No	Yes, support nerve health
Absorption site	Intestinal absorption, dose is not limited	Liver absorption, limited dose	Intestinal absorption, dose is not limited
Acute toxicity (rat LD_{50})	-	500 mg/kg	> 15,000 mg/kg (actually non-toxic)
Whether unmetabolized folic acid (UMFA) is produced	No	Yes, there is a risk of accumulation	No, there is no residue risk
Allergies and side reactions	-	Decreased immunity, itchy skin, allergies, miscarriage,	-

Forms	Folate/ Folacin	Folic Acid, FA	Active folate (active folate supplements)
		leukemia, arthritis, etc	
Applicable people	general population	General population (non-metabolic disorders, people with normal MTHFR enzyme activity)	Everyone, especially pregnant women, infants and young children, and people with metabolic disorders
Regulatory approvals	Naturally occurring, without specific approval	Widely approved for food fortification	Approved by the Chinese Health Commission for special diets, dairy products, etc. (2020–2021)
Advantages	Widely present in food and easily obtainable	Mature technology, low cost, stable crystal	6S-5-MTHF is an active folate with high bioavailability; Not producing unmetabolized folate; The human body can directly absorb it; Not concealing vitamin B_{12} deficiency; Avoiding MTHFR enzyme polymorphism issues; It is the main form of folate in human blood, and its crystals are stable; No tolerance limit.
Disadvantages	Poor stability, with a loss rate of up to 50%~90% during food storage and cooking, resulting in very little folate that can truly be obtained from food	Multiple metabolic steps are required in the body, which poses a risk of metabolic residues and increases the risk of developing neurological diseases, tumors, and other conditions, resulting in a blind spot population.	At present, the price is higher than that of synthetic folic acid

In recent years, *Moringa oleifera* has garnered considerable attention due to its rich nutritional profile. Studies have revealed that folates in moringa leaves predominantly exist as 5-formyl-5,6,7,8-tetrahydrofolate (5-CHO-H_4-folate), accounting for 55.8% of the total folate content, followed by tetrahydrofolate (H_4-folate, 24.9%), 5-methyltetrahydrofolate (16.1%), and 10-formylfolate[22]. The total folate content, as determined by high-performance liquid chromatography (HPLC), was 899.9 μg/100 g dry weight, which was 10.2% lower than that obtained by the microbiological assay. This discrepancy may be attributed to the absence of certain folate standards and losses during purification, consistent with earlier reports by Ruggeri *et al*.[40] and Konings *et al*.[41], where HPLC-derived values were typically 20%–52% lower than those from microbiological assays. Furthermore, in a comparative study of common vegetables (including moringa leaves, taro leaves, and amaranth leaves), Maharaj *et al*.[42] reported that moringa exhibits significantly higher folate levels than most other plants and demonstrates a dual-value characteristic, primarily reflected in its exceptional nutritional benefits and broad pharmacological applications.

3. Biological Activities and Applications

Folate, a derivative of pteridine, was first extracted from yeast and liver by a British doctor. In 1935, it was demonstrated that this substance could treat anemia in monkeys. Subsequently, in 1942, an American researcher identified the same compound in spinach leaves and discovered that it promoted the growth of Streptococcus lactis. Due to its discovery in green plants, it was named folate[8]. Three years later, Angel successfully synthesised pteroylglutamic acid and identified it as the chemical formula for folic acid. Since then, folate has garnered widespread attention from researchers across various fields due to its unique biochemical properties, particularly its roles in disease resistance, plant growth, and metabolism[43]. To date, numerous studies have explored the relationship between folate and various diseases, providing a significant foundation for advancements in human medicine. The biological activities and functional applications of folate are primarily manifested in the following two aspects.

3.1 Medical Field

3.1.1 Neural Tube Defects (NTDs)

Neural tube defects (NTDs) are severe birth defects caused by the failure of neural tube closure during early embryonic development, and their occurrence has a clear causal relationship with inadequate maternal folate levels. Recent randomized controlled trials (RCTs) and molecular mechanism studies have further validated the intervention effect of folic acid supplementation. In recent years, multiple high-quality RCTs have provided supporting evidence. An intervention study (Pan Lijuan, 2024) on women before and during early pregnancy showed that enhanced folic acid management (daily supplementation of 400 μg folic acid) significantly reduced the incidence of NTDs from 0.26% in the control group to 0.09% ($P < 0.05$). Furthermore, participants who received folic acid education showed significantly better medication adherence compared to the control group (good adherence rate: 94.61% vs. 84.78%, respectively)[44]. Sixiang Niu *et al.*[45] demonstrated through a PC12 cell model that folic acid downregulates the expression of endoplasmic

reticulum stress-mediated apoptotic genes (e.g., caspase-4, caspase-12, Em1, CD44), thereby inhibiting neuronal apoptosis (apoptosis rate reduction, $P < 0.01$). This effect was closely associated with the KEGG-enriched "endoplasmic reticulum protein processing" pathway and DNA repair mechanisms. Lin Lin *et al.*[46] pointed out that single nucleotide polymorphisms (SNPs) in key folate metabolism enzymes (e.g., MTHFR C677T, MTRR A66G) can increase the risk of NTDs by interfering with DNA methylation (reduced SAM/SAH ratio) and homocysteine (Hcy) accumulation. For instance, the offspring of pregnant women with the *MTHFR 677TT* genotype had a significantly higher risk of NTDs compared to those with the *CC* genotype (elevated Hcy levels and abnormal methylation). Combining genetic testing (e.g., *MTHFR C677T*, *MTRR A66G* polymorphisms) with personalized folic acid dosage adjustment (high-risk groups supplemented with 4 mg/d) can further reduce the incidence of NTDs[47]. The critical period for neural tube closure is 15-27 days of gestation. It is recommended to supplement with folic acid (400 μg/d) from 3 months before pregnancy through the first trimester. High-risk populations (e.g., those carrying the *MTHFR TT* genotype) should consult a physician to adjust the dosage accordingly[48]. In summary, folate prevents NTDs through multiple pathways, including epigenetic regulation (DNA methylation), apoptosis inhibition, and metabolic homeostasis maintenance. Clinical RCTs and molecular mechanism studies collectively support the necessity of folate supplementation during pregnancy, and intervention strategies should be optimized based on genetic background.

3.1.2 Cleft Lip and Palate（CLP）

CLP is one of the most common congenital facial malformations, with a prevalence rate of 1 to 2 per 1,000 live births[49]. Based on the presence or absence of other organ malformations, CLP can be classified into syndromic and non-syndromic cleft lip and palate (NSCL/P). The pathogenesis of NSCL/P is closely associated with the interaction between genetic and environmental factors[50]. Recent clinical studies have provided direct evidence linking folate metabolism to CLP. Xingxian Yang *et al*.[51] observed 3,272 pregnant women and found that those with high-risk genotypes (*MTHFR C677T TT* and *MTRR A66G AG/GG*) who did not adhere to standardized folic acid supplementation had significantly higher incidences of complex congenital heart disease and cleft lip with cleft palate in their offspring (ORs of 6.87 and 6.37, respectively). Ruimin Liu *et al.*[52] conducted a case-control study in Gansu Province and discovered that the TT genotype at the *MTHFR C677T* locus increased the risk of NSCL/P by 6.36-fold (95% CI: 3.03–8.21), with a synergistic pathogenic effect observed in cases of insufficient folic acid supplementation. Xiufen Bu[53] further confirmed that inadequate folic acid intake during pregnancy is an independent risk factor for NSCL/P (OR = 5.27, $P < 0.05$), while also demonstrating a significant correlation between red blood cell folate levels and the occurrence of CLP ($P < 0.05$). Notably, genetic polymorphisms exhibit regional variations. Data from Gansu Province indicated a significant association between the MTHFR C677T mutation and NSCL/P, while no such association was observed for the A1298C and MTRR A66G loci. In contrast, studies in Hunan Province found no significant correlation between these three loci and CLP, which may be attributed to differences in sample size or folic acid supplementation practices. Collectively, these three studies underscore the importance of

standardized folic acid supplementation during pregnancy for preventing CLP, particularly for pregnant women carrying high-risk genetic variants. Personalized folic acid supplementation regimens (e.g., 0.8 mg/day for high-risk individuals) can significantly reduce the risk of congenital malformations in offspring[51-53].

3.1.3 Cognitive Impairment

Alzheimer's disease (AD), the most common type of dementia, accounts for 60%-80% of all dementia cases[54]. According to available epidemiological data, 65.2% (9.83/150.07 million) of dementia patients aged ≥60 years are diagnosed with AD[55]. Recent studies have demonstrated a close association between abnormal folate metabolism and cognitive dysfunction. Qingqing Chen *et al.'*s [56] study of 60 AD patients revealed that serum homocysteine (Hcy) levels were significantly higher in AD patients compared to controls ($P < 0.01$), while folate and vitamin B12 levels were significantly lower ($P < 0.01$). MMSE scores showed a negative correlation with Hcy ($P < 0.01$) and positive correlations with folate and vitamin B12 ($P < 0.01$), suggesting that folate deficiency may exacerbate cognitive impairment by elevating Hcy levels. The animal experiments conducted by Zewei Ma 's team further confirmed that folic acid supplementation (8 mg/kg) significantly reduced β-amyloid ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$) deposition in the brains of *APP/PS1* inflammatory model mice ($P < 0.05$) and decreased the expression of the lipid raft marker protein Flotillin-1 ($P < 0.05$). Immunofluorescence analysis revealed that folic acid intervention significantly reduced the co-localization of Flotillin-1 with Aβ1-42/APP/PS1/BACE1 ($P < 0.05$), indicating that folic acid may exert neuroprotective effects by modulating the Flotillin-1-mediated Aβ metabolic pathway[57]. Siting Pei 's[58] meta-analysis (including 13 RCTs) demonstrated that folic acid supplementation reduced Hcy levels in patients with vascular cognitive impairment by 7.62 μmol/L (95% CI: -8.67 to -6.56) and significantly improved MMSE (MD=0.54), MoCA (MD=2.01), and ADAS-cog (MD=-4.36) scores (all $P < 0.01$). Subgroup analysis showed more pronounced effects with intervention durations ≥6 months and folic acid doses ≥2mg. These clinical and basic research findings collectively support the role of folic acid in improving cognitive function through multiple pathways, including reducing Hcy levels and decreasing Aβ deposition, providing evidence-based insights for the prevention and treatment of AD.

3.1.4 Cervical Cancer

Asparagus, spinach, cruciferous vegetables, and beets have been extensively studied for their potential cancer-preventive properties , with their bioactive effects primarily attributed to 5-methyltetrahydrofolate (5-MTHF)[59]. Epidemiological studies have demonstrated that 5-MTHF[60], primarily derived from green leafy vegetables and legumes, has been significantly associated with the risk reduction of several cancers, including breast, colon, cervical and pancreatic cancers[59, 61, 62]. Current clinical evidence remains inconclusive regarding the association between folate and reduced cervical cancer risk. However, a recent U.S.-based randomized controlled trial demonstrated that long-term (24-month) folate supplementation at optimal dosage (400-800 μg/day), combined with adequate vitamin B_{12} levels, may significantly reduce the risk of cervical cancer and its precancerous lesions (cervical intraepithelial neoplasia, CIN)[63]. Tong *et al.*[64]

demonstrated significantly reduced serum vitamin B_{12} and folate levels in patients with cervical lesions compared to healthy controls ($P < 0.01$). In addition, Pathak et al. conducted a comparative study by investigating serum folate levels in three groups: healthy controls, patients with low-grade squamous intraepithelial lesions (LSIL), and patients with cervical cancer. Their findings suggest that folate status may influence the risk of cervical cancer development and HPV infection susceptibility[65]. The study reported a dose-dependent relationship, with each 10 nmol/L increase in serum folate associated with a 15% reduction in cervical cancer risk (95% CI: 0.78-0.92), potentially through enhanced DNA methylation and repair mechanisms. MBarchitta *et al.*[66] demonstrated that low folate status was significantly associated with elevated risk of cervical intraepithelial neoplasia using multivariate regression analysis. Their findings suggest that adequate folate supplementation may reduce the incidence of HPV infection and cervical intraepithelial neoplasia, particularly among younger women and tobacco users. Indeed, the academic controversy surrounding the anti-cancer mechanisms of 5-MTHF, including but not limited to the pro-cancer risks associated with high doses, has not been resolved.

3.1.5 colorectal cancer

Colorectal cancer is currently the third largest cancer in the world and seriously endangers human health[67]. According to statistics, there were about 1.8 million new cases worldwide in 2018, and by 2022, the number of new cases has increased to 2 million, making it the second leading cause of cancer death worldwide, with about 880,000 deaths from the disease. Therefore, many researchers have studied it[68]. Transwell invasion assays demonstrated that FA reduced the invasion ability of colorectal cancer cell lines, COLO-205, LoVo and HT-29. Using COLO-205 as a cell model, we further delineated the molecular mechanism underlying FA-inhibited colorectal cancer cell invasion[69, 70]. *In vitro* MTT experiments showed that the IC_{50} of folic acid-targeted PLGA-1,3-diaminopropane-5-FU nanoparticles prepared in this study was 5.69 μg/mL, which was significantly lower than that of free 5-FU (22.9 μg/mL) and non-targeted PLGA-5-FU nanoparticles (14.17 μg/mL), confirming that the delivery system can significantly improve the anti-proliferative activity of drugs against HT-29 cells[71]. *In vitro* MTT experiments showed that the anti-proliferation IC_{50} value of COL-PPCF-NPs against HT-29 cells was 118.5 μg/mL[72]. The above studies shown that folic acid-targeted nanodrug delivery systems can significantly enhance the inhibitory effect of drugs on tumor cells and improve metabolic abnormalities, providing an important experimental basis for targeted therapy of colorectal cancer.

3.2 Food Area

3.2.1 Nutritional Fortification

In 2023, the European Food Safety Authority (EFSA), through its Nutrition and Novel Foods Unit and the Panel on Food Allergens (NDA), granted approval for 5-MTHF as a novel food ingredient[73]. 5-MTHF is a key nutritional supplement that was officially approved by the National Health Commission of the People's Republic of China in 2017. The approval included 5-MTHF glucosamine salt as a novel nutritional fortifier and its incorporation into the National Food Safety Standard - Standards for the Use of Nutritional

Fortification Substances (GB 14880). This authorization permits its application in various food categories, including formulated milk powder and powdered beverages. (6S)-5-Methyltetrahydrofolic acid, calcium salt, the predominant calcium-based folate formulation commercially available, is approved as a folic acid source for general formula milk powder (excluding infant formula and maternal milk powder, CN food category 01.03.02) and powdered beverages (CN food category 14.06). Its usage levels strictly comply with the specifications outlined in GB 14880[74]. Food fortification with specific nutritional fortificants serves as a crucial public health strategy, enhancing both the nutritional quality of food products and population health outcomes, ultimately contributing to national physical fitness improvement. This strategy resonates strongly with the concept of "food and medicine originating from the same source"—a perspective which recognizes that many natural bioactive components possess dual functions of nutrition and disease prevention, thereby providing an interdisciplinary theoretical basis for the fortification application of 5-MTHF in food categories such as infant formula milk powder and solid beverages[75]. Nutrient supplementation must be implemented prudently, strictly adhering to national regulations and based on comprehensive nutritional surveys[76]. This targeted approach, addressing specific regional nutrient deficiencies, is crucial for effectively resolving local population health issues.

3.2.2 Functional Foods and Beverages

Folate, an essential component of B-complex vitamins, is commonly incorporated into functional foods and dietary supplements. This micronutrient plays crucial roles in various physiological processes, including supporting cellular metabolism, facilitating new cell production, preventing megaloblastic anemia[77], promoting fetal development, and potentially reducing cardiovascular disease risk[78], and functional foods and beverages are mainly produced by adjusting the composition and content ratio of natural nutrients in beverages, and through the fermentation process, *Lactobacillus plantarum* can produce L-5-Methyltetrahydrofolate, which is used to prepare functional dairy products, functional beverages, etc., in order to meet the nutritional needs of some special populations. Therefore, it holds broad application prospects.

3.3 Industrialization and policy support

3.3.1 Industrialization Potential and Market Application Prospects

In recent years, continuous optimization of domestic folic acid production processes has enabled Chinese enterprises to achieve large-scale production capacity for calcium 5-methyltetrahydrofolate (5-MTHF). Improvements in the production system have significantly reduced costs, enhanced raw material self-sufficiency, and strongly supported innovation and development in downstream industries such as functional foods and pharmaceuticals. From the consumption perspective, the global 5-MTHF market continues to expand steadily, with the industry expected to maintain considerable growth rates through 2026. The Asia-Pacific region stands out as a particularly dynamic market, driven largely by increasing health awareness among women of childbearing age and the aging population. In the pharmaceutical sector, the applications of 5-MTHF have expanded beyond traditional prenatal nutrition to multiple clinical areas,

including intervention in hyperhomocysteinemia[79], adjunctive treatment of depressive disorders[63], and nutritional support for cancer patients. It is noteworthy that approximately 40%–60% of the global population carries MTHFR gene mutations, which impair the ability to metabolize synthetic folic acid efficiently, making 5-MTHF the only bioavailable form necessary for this subgroup. This creates a sustained and stable demand for the product.

3.3.2 Policy Support

China's National Nutrition Plan explicitly includes folic acid deficiency intervention as a key task and regulates the application scope of 5-MTHF through National Food Safety Standard for the Use of Food Nutrient Fortifiers (GB 14880)[80, 81]. Furthermore, the Dietary Guidelines for Pregnant Women released by the National Health Commission of China in 2024 specifically recommend that high-risk populations should prioritize the supplementation of active folic acid. The European Food Safety Authority (EFSA) and the United States Food and Drug Administration (FDA) have approved 5-MTHF as a dietary supplement ingredient and permitted health claims such as “supports fetal neural tube development,” thereby providing policy endorsement for market access in international markets[82].

3.3.3 Consumer Acceptance

The global application of 5-MTHF is significantly influenced by varying regulatory policies on health claims across different countries and regions. For instance, the European Union mandates that all health claims must be supported by robust scientific evidence, approved by the European Food Safety Authority (EFSA), and must exclusively employ officially authorized wording. In contrast, the U.S. Food and Drug Administration (FDA) categorizes health claims into three types: authorized claims based on significant scientific agreement, Food and Drug Administration Modernization Act (FDAMA) claims derived from authoritative statements, and qualified health claims permitted with limited supporting evidence. Japan implements a Foods with Specified Health Uses (FOSHU) system, which approves products with specific health benefits. These regulatory differences necessitate distinct labeling strategies for 5-MTHF products in different markets: companies operating in the EU must strictly adhere to approved claim wordings; those in the U.S. can flexibly select claim categories depending on available scientific evidence; while in Japan, the FOSHU certification mark substantially enhances consumer trust. Consequently, enterprises must tailor their labeling strategies according to the regulatory requirements of target markets to improve product appeal[83].

On the other hand, clinical studies also indicate that consumer recognition of 5-MTHF is notably higher than that of synthetic folic acid. A study involving 500 women of reproductive age demonstrated that among individuals with the *MTHFR C677T TT* genotype, supplementation with an equivalent dose of active folate (5-MTHF) resulted in a significantly greater increase in red blood cell folate levels—a key indicator of tissue folate reserves—compared to supplementation with synthetic folic acid[84]. This finding underscores the distinct advantage of 5-MTHF in terms of bioavailability, indicating its superior efficacy in enhancing the body’s folate status. Furthermore, innovation in the functional food sector continues to drive market development. For example, Mengniu Group’s“Active Folate Yogurt” achieved 200% sales growth in 2023,

reflecting strong market demand for highly bioavailable nutrients. This phenomenon illustrates that consumer acceptance of functional foods is not only shaped by the regulatory framework governing health claims but is also closely tied to the scientific substantiation and practical efficacy of the products. In summary, when formulating global market strategies for 5-MTHF, companies must not only adapt to regional regulatory requirements but also integrate solid scientific evidence with product innovation to effectively enhance consumer acceptance and ensure market success.

4. Biosynthetic Pathways and Key Enzymes of 5-MTHF

The biosynthetic pathway of 5-MTHF involves multiple enzymatic steps[85]. Initially, folate is reduced to dihydrofolate (DHF) by dihydrofolate reductase (DHFR, EC 1.5.1.3), followed by its conversion to tetrahydrofolate (THF). Subsequently, serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) catalyzes the formation of 5,10-methylenetetrahydrofolate (5,10-MTHF) from THF. As the direct precursor of 5-MTHF, 5,10-MTHF is then converted by methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20), which serves as the rate-limiting enzyme in this crucial metabolic pathway. Folate and 5-MTHF biosynthesis in higher plants occurs through coordinated activities across three distinct subcellular compartments: plastids, mitochondria, and cytoplasm, each contributing specific enzymatic steps to the pathway[43]. Pterin and p-aminobenzoic acid precursors are synthesized in the cytoplasm and chloroplasts, respectively. Subsequently, these precursors are transported to the mitochondria, where pterin and p-aminobenzoic acid combine to form the complete folate structure. Since pteridines consist of pyrimidine and pyrazine rings, the methyl group at the 6th position can react with p-aminobenzoic acid and glutamic acid to produce pteroylglutamic acid. This process allows the attachment of one or more glutamate tails.

4.1 PABA Biosynthesis

Folate is distributed in various compartments of plant cells, including mitochondria, cytoplasm, vesicles, and plastids. Its synthesis follows similar pathways in both plants and microorganisms. The pteridine ring and p-aminobenzoic acid are first synthesized in the cytoplasm and plastids, respectively. Subsequently, these precursors enter the mitochondria, where they combine with glutamate to form a complete folate molecule[86]. The specific folate synthesis pathway is illustrated in Figure 4. As a key precursor for folate biosynthesis, the production of p-aminobenzoic acid (pABA) specifically occurs in plastids. This biosynthetic pathway utilizes chorismate as the initial substrate, which itself is an important intermediate metabolite in the shikimate pathway and is strictly synthesized in plastids[87]. Notably, significant differences exist in pABA synthesis mechanisms among different biological groups: In Escherichia coli, pABA biosynthesis requires two enzymatic steps—first, aminodeoxychorismate synthase (ADCS, composed of PabA and PabB subunits) catalyzes the transfer of an amino group from glutamine to chorismate, forming 4-amino-4-deoxychorismate (ADC); subsequently, ADC undergoes aromatization via aminodeoxychorismate lyase (PabC) to yield pABA[88]. In plants, however, ADCS exists as a single-chain fusion protein that is homologous to the bacterial PabA/PabB proteins but does not form a heterodimeric structure. Furthermore, organisms such as fungi, actinomycetes, and Plasmodium have evolved a unique bifunctional "PABA synthase" catalytic system. The

promoter region of this enzyme shares homology with pabA/pabB genes and is capable of directly encoding a fully functional PABA synthase[89].

4.2 Pterin Biosynthesis

The biosynthesis of pteridines primarily occurs in the cytoplasm, involving the coordinated catalytic actions of multiple key enzymes, including GTP cyclohydrolase I (GCHI), dihydroneopterin aldolase (DHNA), dihydroneopterin triphosphate pyrophosphatase (DHNTP), and 6-hydroxymethyl-dihydropterin pyrophosphokinase (HPPK). Through the sequential actions of these enzymes, the synthesized pteridine molecules ultimately combine with p-aminobenzoic acid (pABA) under the catalysis of dihydropteroate synthase (DHPS) to form dihydropteroate, the direct precursor of folate. The initial step of this biosynthetic pathway involves the conversion of guanosine triphosphate (GTP) to dihydroneopterin triphosphate (DHN-P3). This reaction not only represents the first step in the entire pteridine biosynthesis pathway but also serves as the rate-limiting step. GCHI, the first key enzyme in this pathway, is encoded by the folE gene and has been successfully cloned in various organisms, including humans, Escherichia coli, yeast, Arabidopsis thaliana, and tomato[87]. Notably, the regulatory mechanisms of GCHI exhibit significant differences among species: in mammals, GCHI activity is subject to feedback inhibition by folate levels, and relieving this inhibition can significantly enhance folate production; whereas in plants and microorganisms, such feedback regulation is absent, but overexpression of the folE gene can increase folate levels. DHN-P3 undergoes a two-step dephosphorylation to yield dihydroneopterin (DHN), which is subsequently converted to 6-hydroxymethyldihydropterin (HMDHP) and dihydromonapterin (DHM) under the catalysis of dihydroneopterin aldolase (DHNA, encoded by the *folQ* gene)[90]. Among these products, HMDHP is transported into the mitochondria to participate in subsequent folate biosynthesis. This series of reactions exemplifies the sophisticated regulatory mechanisms by which different cellular compartments collaborate to accomplish complex metabolic processes.

4.3 Folate Synthesis

Within mitochondria, 6-hydroxymethyldihydropterin (HMDHP) first undergoes pyrophosphorylation catalyzed by Hydroxymethyldihydropterin Pyrophosphokinase, followed by conjugation with p-aminobenzoic acid (pABA) mediated by dihydropteroate synthase (DHPS) to form dihydropteroate. This product subsequently combines with glutamate through the coordinated action of dihydropteroate synthase (DHPS) and folylpolyglutamate synthetase (FPGS), yielding dihydrofolate (DHF). Ultimately, dihydrofolate reductase (DHFR) reduces DHF to biologically active tetrahydrofolate (THF). The biosynthesis of folate is shown in Figure 2. Distinct enzymatic variations and regulatory characteristics in folate biosynthesis pathways are observed across different species: "Dihydrofolate Reductase (DHFR) exhibits" in bacterial systems (exemplified by Escherichia coli), DHFR is encoded by the *folA gene*. The protein forms a fusion with thymidylate synthase and exhibits high sensitivity to trimethoprim-class inhibitors. In plant systems (using Arabidopsis thaliana as a model), the AtDFR-encoded DHFR demonstrates a unique light-regulated expression pattern, reflecting plant adaptation to photoperiodic environments[91, 92].

Methylenetetrahydrofolate Reductase (MTHFR): As the rate-limiting enzyme in folate metabolism, MTHFR displays significant functional divergence across species. Overexpression of the AtMTHFR1 gene in Arabidopsis thaliana can increase 5-methyltetrahydrofolate (5-MTHF) levels by up to 3-fold. In humans, the C677T polymorphism in the *MTHFR* gene reduces enzyme activity by approximately 50%, thereby compromising overall folate metabolic efficiency[92]. Dihydrofolate Synthase (DHFS): This plant-specific folate synthase has been shown through functional loss studies to be essential for development. Arabidopsis DHFS gene mutations lead to severe folate deficiency and embryonic lethality, unequivocally demonstrating the enzyme's indispensability in plant development. These findings not only elucidate the conservation and species-specificity of folate biosynthesis pathways, but also provide critical molecular targets for metabolic engineering approaches across different biological systems[93].

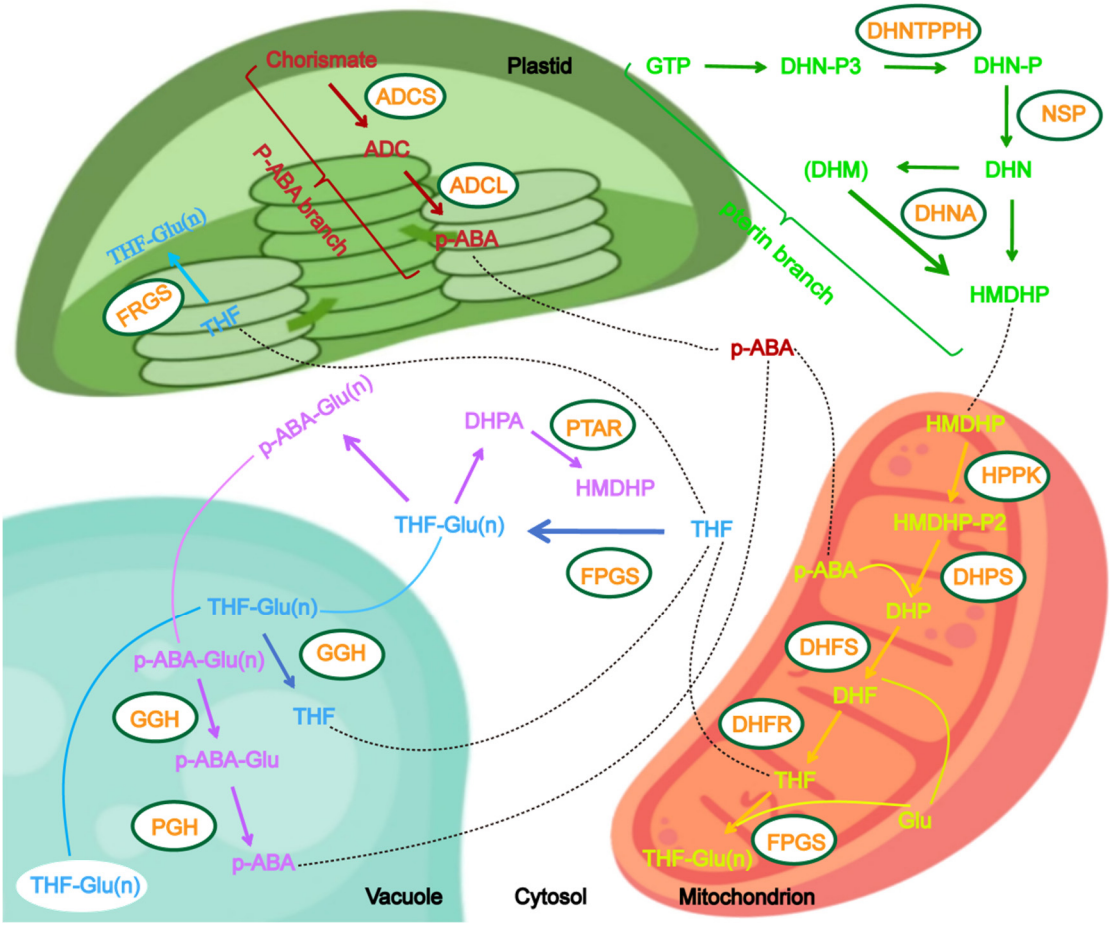


Figure 2. Tetrahydrofolate Biosynthetic Pathway

4.4 Biosynthesis of 5-MTHF

In eukaryotes, serine is an important one-carbon unit donor for the synthesis of 5,10-methylenelessertrahydrofolate (5,10-CH_2-THF). This reaction is mainly catalyzed by serine hydroxymethyltransferase (SHMT), which transfers the hydroxymethyl group on its β-carbon to tetrahydrofolate (THF) under the action of SHMT to produce glycine and 5,10-CH_2-THF. In addition, another core synthesis pathway of 5,10-CH_2-THF in mitochondria is dependent on the photorespiration process. The T protein (aminomethyltransferase) in the glycine decarboxylase complex (GDC) catalyzes the decarboxylation

and deamination of glycine and transfers the resulting methylene unit to THF, resulting in the formation of 5,10-CH_2-THF. This reaction is a key step in the carbon cycle of photorespiration in plants and has a crucial impact on nitrogen metabolism and energy balance in plants[90, 94, 95]. Another source of folate metabolizing one carbon unit is formate. Catalyzed by 10-formyltetrahydrofolate synthase (FTHFS), formate reacts with THF under ATP-powered conditions to produce 10-formyltetrahydrofolate (10-CHO-THF). Subsequently, 10-carmoyltetrahydrofolate can be used for purine biosynthesis and catalyzed by cytosolic formyltetrahydrofolate dehydrogenase (FormylTHF dehydrogenase) to 5,10-methyltetrahydrofolate (5,10-CH^+-THF), which is then oxidized to 5,10-submethyltetrahydrofolate cyclohydrolase (commonly abbreviated as MTHFC) to produce 5,10-methylenelessetrahydrofolate (5,10-CH_2-THF). In peas and Arabidopsis, catalyzing the above two-step reactions (i.e., formyltetrahydrofolate dehydrogenase activity and cyclic hydrolase activity) were found to be conjugated to the same bifunctional enzyme. This enzyme is known as methylene tetrahydrofolate dehydrogenase-cyclic hydrolase bifunctional enzyme in Arabidopsis.

10-formyltetrahydrofolate deformylase (10-FDF) catalyzes the hydrolysis of 10-formyltetrahydrofolate (10-CHO-THF) to produce tetrahydrofolate (THF) and formate. This reaction is a critical step in the folate-mediated formate cycle and is essential for the recovery of THF cofactors and the regeneration of one-carbon units. In Arabidopsis, there are two genes encoding 10-FDF (their phylogenetic names are *AtFTHFDH1*/At4g17360 and *AtFTHFDH2*/At5g47435). Studies have shown that knocking out these two genes at the same time will lead to severe stunting and pale leaves, which confirms that 10-FDF plays an indispensable role in removing excess 10-CHO-THF produced by mitochondria and maintaining one-carbon metabolic homeostasis during photorespiration[96]. In addition, the intermediate for the formation of 5-methyltetrahydrofolate 5,10-methylenelessertrahydrofolate (5,10-CH_2-THF) involves two pathways, the first of which is catalyzed by 5-formyltetrahydrofolate cyclase (EC 6.3.4.3, formerly known as 5-FCL). The enzyme uses ATP as an energy source to catalyze the cyclic dehydration of 5-formyltetrahydrofolate (5-CHO-THF) to produce 5,10-hypomethyltetrahydrofolate (5,10-CH^+-THF). The second pathway is mainly related to histidine degradation, which is common in mammals and bacteria. This pathway is catalyzed by a bifunctional enzyme, methylaminotransferase-cyclic deaminase (FTCD). Its first reaction (transferase activity) transfers methyliminoamino acid from N-methyleniminoglutamic acid (FIGLU) to THF to produce glutamic acid and 5-methyliminotetrahydrofolate (5-formimino-THF). Immediately afterwards, cyclic deaminase activity on the same enzyme catalyzes the dehydration and cyclization of 5-methyliminotetrahydrofolate to produce 5,10-hypomethyltetrahydrofolate (5,10-CH^+-THF) and ammonium ions[97, 98]. Finally, the production of 5-methyltetrahydrofolate (5-MTHF) is obtained by the irreversible reduction of 5,10-methylenelessetrahydrofolate reductase (MTHFR) catalyzed by 5,10-methylenelessertrahydrofolate (5,10-CH_2-THF). 5-MTHF is the main circulating form and one-carbon unit donor in the folate cycle in the body, which can be catalyzed by methionine synthase (MS, or MTR) under the condition that vitamin B_{12} (cobalamin) is used as a cofactor to provide methyl groups to homocysteine (Hcy) and convert it to methionine (Met); At the same time, it regenerates tetrahydrofolate (THF) to complete

the folic acid cycle. Methionine synthase reductase (MTRR) is responsible for maintaining the active state of vitamin B_{12} cofactor in methionine synthase to ensure that the methylation reaction can continue.. The 5-methyltetrahydrofolate biosynthetic pathway is shown in Figure 3.

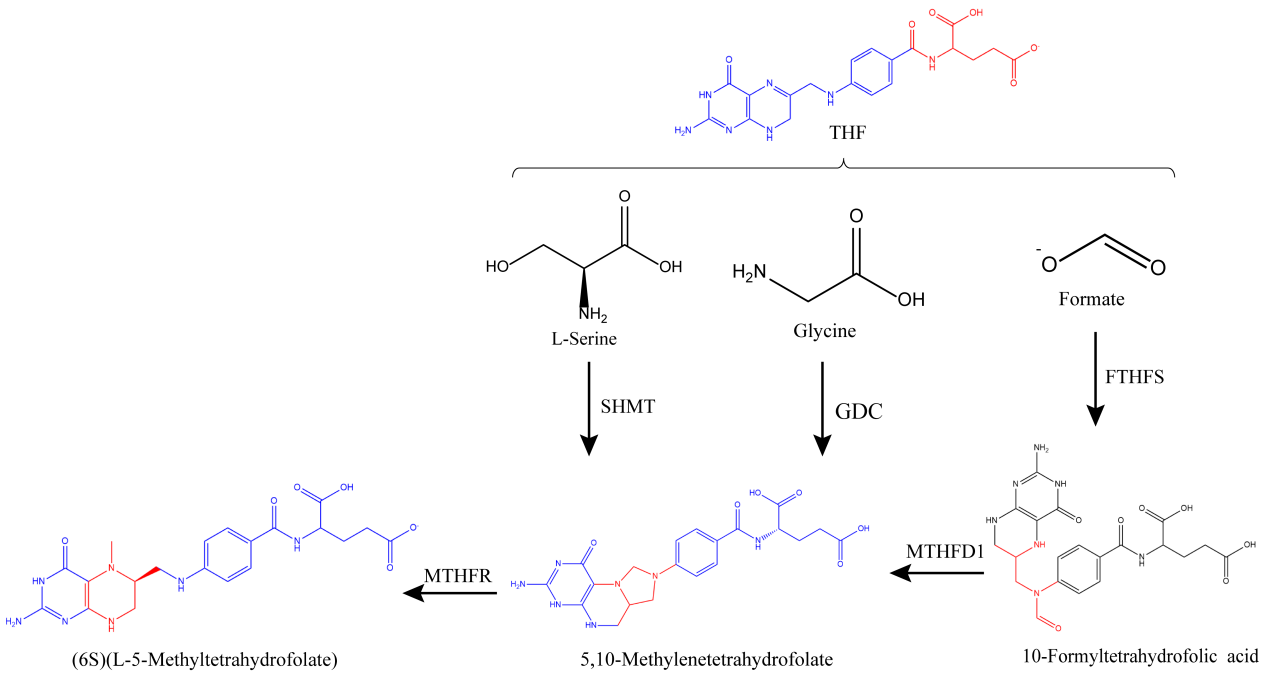


Figure 3 5-MTHF Biosynthetic Pathway

5-MTHF is closely associated with the coordinated action of multiple key enzymes (e.g., MTHFR, SHMT, GDC) in the folate metabolic pathway, and its regulatory mechanism plays a crucial role in biological methylation reactions and amino acid metabolism. However, the efficiency of natural biosynthetic pathways is often suboptimal due to constraints such as enzyme activity, substrate availability, or feedback inhibition (e.g., GTP cyclohydrolase I (GCHI) being regulated by folate levels). Consequently, strategies for enhancing 5-MTHF production through artificial interventions, such as metabolic engineering, enzymatic catalysis, and nanodelivery—have become a major focus in current research. The subsequent sections will provide a detailed discussion on the advancements in these technologies (see Section 5 for specifics).

5. Strategies to Enhance 5-MTHF Production

In contemporary scientific research, the methods for increasing the yield of 5-methyltetrahydrofolate (5-MTHF) not only include traditional chemical synthesis, extraction optimization, and synthetic biology, but also involve cutting-edge technologies such as microbial fermentation, enzyme engineering, metabolic regulation, and nanodelivery systems. Wenshu Jiao *et al.*[99] employed Response Surface Methodology (RSM) to optimize the culture medium composition and fermentation conditions of Lactococcus lactis KLD54.0325. As a result, the folate yield increased from 0.260 μg/mL to 0.814 μg/mL, representing a 3.13-fold enhancement in production. Qi Liu *et al.* [100] successfully constructed Escherichia coli expression systems for the enzymes maltotriose synthase (MTSase), maltotriose hydrolase (MTHase), and 4-α-glucosyltransferase (4αGT). These systems were used to establish a three-enzyme catalytic system, which was further optimized to significantly enhance the conversion rate of trehalose. Ultimately, the optimized recombinant *E. coli* was

applied to the conversion of trehalose, increasing the conversion rate from 78.65% to 83.33%. Han Yang *et al*.[27] enhanced the production of 5-MTHF by replacing the bifunctional enzyme YitJ with MetF from Escherichia coli through heterologous expression, and by strengthening the expression of DHFR, DHFS/FPGS, and GCHI. As a result, the yield of 5-MTHF increased from 3.93 μg/L to 960.27 μg/L, representing a 244-fold increase. Haimei Wu *et al*.[101] investigated the delivery effects of nanoplastics (NPs) on nucleic acids (siRNA) using plant gene silencing technology (RNAi). They elucidated the mechanism by which positively charged nanoplastics (20 nm) efficiently deliver siRNA into plant cells through electrostatic adsorption, thereby significantly reducing the expression levels of target genes (GFP mRNA transcription decreased by 97.6%, and the expression of the anthocyanin synthesis gene CHI was reduced by 69.35%). This study provides direct evidence that nanoplastics, as a novel type of pollutant, can interfere with plant gene functions. It also clarifies the regulatory patterns of size dependency (with optimal effects at 20 nm) and temporal effects (the action persists for 3 days before gradually recovering). These findings lay the foundation for the synthesis of 5-MTHF. Yating Yu *et al*.[102] optimized the prebiotic effects of four plant polyphenols (tea polyphenols, apple polyphenols, grape seed polyphenols, and peanut skin polyphenols) using an in vitro fermentation model. The results showed that tea polyphenols (1.25 mg/mL) and apple polyphenols (2.5 mg/mL) exhibited the best antibacterial effects. Additionally, apple polyphenols increased the production of propionic acid and butyric acid by 25% and 23%, respectively, while tea polyphenols enhanced the DPPH free radical scavenging activity by 7%. The study confirmed that different plant polyphenols can specifically modulate the structure of the gut microbiota, with tea polyphenols demonstrating the most optimal overall prebiotic characteristics. These findings provide important evidence for the development of functional prebiotic products. Han Yang *et al*.[27] employed the CRISPRi system to repress the genes of competing pathways (panB, thyA, trpE, and pheA), thereby increasing the yield of 5-MTHF from 960.27 μg/L to 1.58 mg/L, representing a 1.6-fold improvement. Ma *et al*.[103] obtained acid-resistant 6-phosphogluconate dehydrogenase (6PGDH) and diaphorase (DI) through directed evolution. The catalytic efficiency of these enzymes was enhanced by 42-fold and 4-fold, respectively, compared to the wild-type enzymes at *pH* 5.4, with their stability also being improved.

Traditional chemical synthesis methods typically have higher costs for raw materials and energy and may generate harmful waste. In contrast, synthetic biology strategies, such as microbial fermentation, can reduce production costs and enhance sustainability[104]. For example, the biosynthesis of artemisinin is approximately 30% less costly than chemical synthesis and is more environmentally friendly[105]. The advantages and disadvantages of specific strategies and their core technologies are shown in Table 2. In addition, the study of bioactive compound biosynthesis requires an integrated approach encompassing the entire pipeline from screening to validation and reconstruction. Complementary technologies can accelerate the translation from gene discovery to industrial applications. Therefore, this paper also systematically summarizes and compares three key aspects: functional gene screening methods, functional gene validation techniques, and biosynthetic pathway elucidation strategies, see details in Table 3.

Table 2. Methods for increasing the yield of 5-MTHF

Method	Core technology	Advantage	Disadvantage
Microbial fermentation[106]	Genetically engineered bacteria and metabolic pathway optimization	Low cost and sustainable, Improve food quality and ensure food safety[107]	High technical requirements, high contamination risk，Microorganisms are inconsistent and of uneven quality[108]
Enzyme catalysis[100]	Multi-enzyme cascade, immobilized enzymes	High purity, green chemistry, Technology convergence potential[109], Efficient catalysis[110]	High cost[111], poor stability, low versatility
Metabolic regulation[112]	Epigenetic editing, methylation support	Individualized interventions, Increase production efficiency[111]， Enhance cell adaptability and drug stability	Poor controllability, high regulatory costs, response delay.
Nano delivery[113]	Liposomes, targeted nanoparticles	Improves bioavailability, Targeted delivery to enhance drug stability[114], Reduces immune response[115]	Safety concerns, immune risks, high costs, technically complex, Biocompatibility issues[116].
Gut microbiota[102]	Probiotics, FMT	Naturally synthesized and regulates systemic metabolism[117, 118].	Significant individual differences, complex mechanisms of action, lack of standardization[118].
Plant biofortification[119]	CRISPR crop breeding	Food-grade source, Strong sustainability and economic benefits[120]	Low social acceptance, influence of environmental factors, ecological impact.
Photo/electrocatalysis[121]	photosensitizer, electrochemical reduction	Pollution-free and efficient[122].	High energy consumption, low efficiency, insufficient stability[122, 123].

Table 3. Comparison and summary of research methods for bioactive compound biosynthesis

Methods	Core methods	**Advantages**	**Limitations**
Module 1. Functional Gene Screening Methods			
Differential Expression Screening[124]	-Representative Display Analysis，RDA -mRNA Differential Display，DD -Serial Analysis of Gene Expression，SAGE -DNA Microarray	-Enables high-throughput genome-wide expression analysis[125] -Uses well-established protocols[125]	-Requires biological replicates -Needs complementary functional annotation[125]
Gene Co-expression Network Analysis[126]	-Weighted Correlation Network Analysis, WGCNA	-Systematically analyzes gene regulatory relationships -Discovers genes with unknown functions[127] -Modular analysis	-Computationally intensive -Requires high-quality expression data -Network construction is parameter-dependent
Bioinformatics Analysis[128]	-FASTA -BLAST	-Rapid and cost-effective -Particularly suitable for data-deficient species	-Database-dependent -Potential functional divergence of homologs -Requires experimental validation
Module 2. Functional Gene Validation Technologies			

Methods	Core methods	**Advantages**	**Limitations**
Gene Transduction[129]	-Physical Mediation: electroporation, microinjection, gene gun, and heat shock methods. -Chemical Mediation: iposome-mediated transfection -Biological Mediation: Viral vector-mediated transfection	- Broad applicability (prokaryotic/eukaryotic systems) - Technically mature methodology	- Low transformation efficiency - Host limitations (e.g., E. coli cannot modify eukaryotic proteins)
Gene Editing Technology[130]	-Zinc finger nuclease, zfn -Transcription activator-like effector nuclease, talen -Clustered regularly interspaced short palindromic repeats, crisprs -Pentatricopeptide repeat protein, ppr -Rna interference, rnai	- High efficiency and precision - Enables multiplex genome editing - Cost-effective	- Potential off-target effects - Delivery constraints (e.g., low transfection efficiency in plants)
Antisense Technology[131]	-Antisense oligonucleotides, ASON -Ribozyme -Small interfering, siRNA	- High specificity - Flexible design - Reversible regulation	- Low stability - Poor delivery efficiency - Off-target effects
miRNA Technology[132]	-MiRNAs (~19-25 nt) are endogenous small RNAs that regulate genes by binding to mRNA 3'UTRs	- Highly efficient regulation - Strong conservation - Tissue specificity	- Off-target risks - Delivery challenges - Functional complexity
Microarray analysis[133]	-Microarray (gene chip) is a high-throughput detection technology, whose core principle is nucleic acid hybridization	- High-throughput - Highly standardized - Technologically mature	- Requires known sequences - Narrow dynamic range - High sample input demand
Module 3. Biosynthetic Pathway Elucidation Strategies			
Multi-omics Integrative Analysis[134]	-Transcriptomics -Metabonomics -Proteomics	- Systematically uncover gene-metabolite associations - Identify novel pathway nodes	- Large-scale datasets requiring multidisciplinary integrated analysis
Classical enzymology validation[135]	-Oxido-Reductases -Transferases -Hydrolases -Lyases -Isomerases -Ligases	- Direct evidence of enzymatic function - Enables kinetic characterization	- Protein purification challenges - Some enzymes require membrane systems
Pathway genes and regulatory genes studied via plant genetic transformation technology[136]	-Agrobacterium-mediated method -Gene overexpression -Gene suppression	- Directly links phenotypes to genes - Combinatorial techniques (CRISPR/RNAi/VIGS)	- Low transformation efficiency - Metabolic redundancy interference - Vector limitations
Synthetic Biology[15]	-Design artificial pathways → assemble in chassis cells (e.g., yeast) → optimize production yield	- Enable heterologous production - Overcome plant extraction limitations -Interdisciplinary collaboration	- Pathway complexity requires balancing (e.g., precursor competition) - High demand for component compatibility

5.1 Enhancing 5-MTHF Production Through Molecular Biotechnology

The synthetic biology strategy involves elucidating and simulating the fundamental principles of bioactive compound biosynthesis in organisms, acquiring genetic elements, and artificially designing novel biological systems (plant or microbial systems) with specific physiological functions. This approach enables the efficient and targeted production of bioactive ingredients. Selecting appropriate biological components based on synthetic biology modular strategies can enhance the efficiency of biosynthetic pathways and increase target product yields, while also serving as an effective approach to validate the accuracy of bioactive compound pathway elucidation. Synthetic biology builds upon the preliminary elucidation of biosynthetic pathways for bioactive compounds. To further verify pathway reliability and identify key enzymatic genes and rate-limiting steps in the target compound's biosynthesis, research can be conducted using a modular synthetic biology framework through the following approaches: Mining, characterization, engineering, and standardization of biological parts; design and construction of chassis cells (In synthetic biology, a chassis cell refers to an engineered, standardized host cell system that serves as a foundational platform for modular biosynthesis. It provides essential genetic background, energy, and metabolic support for heterologous pathways to enable efficient production of target compounds); assembly and integration of metabolic pathways, followed by synthesis and structural characterization of target compounds; systematic optimization and balancing of metabolic pathways. In addition, transcriptomic and genomic etc. studies have been conducted on a variety of important medicinal plants such as *moringa, Panax notoginseng, Dendrobium, Salvia miltiorrhiza, Lepidium meyenii* and walnut, leading to the establishment of the first comprehensive multi-omics integrated database for medicinal plants, MPOD[137], as shown in Figure 4. Table 4 compares the advantages and disadvantages of expression systems of different hosts (e.g., *Escherichia coli, Bacillus subtilis*, yeast, and plant cells), echoing Figure 4. Based on the screening of genes and the study of their functions, the artificial design and construction of new biological systems with specific physiological functions can elucidate and simulate the fundamental principles of the biosynthesis of bioactive compounds. This approach also represents a highly promising method for obtaining such resources.

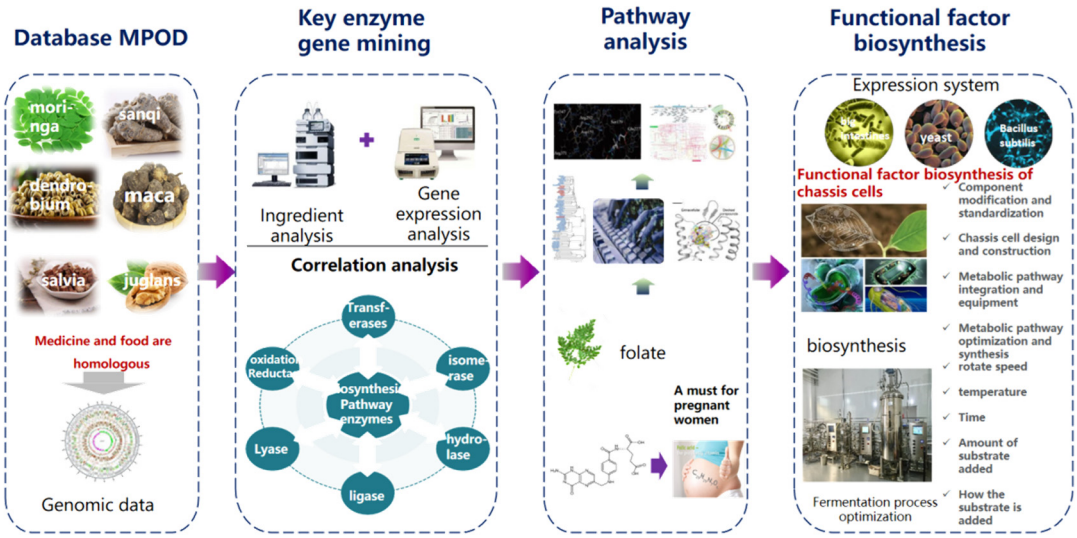


Figure4 Biosynthesis strategy for bioactive molecules from medicinal and food sources

Table 4 Advantages and disadvantages of different host expression systems

Expression System	Host	Advantages	Disadvantages
Prokaryotic System	*Escherichia coli*	- Well-characterized genetics, rapid growth, short culture cycle - Low cost, easy manipulation[138, 139] - High efficiency for most proteins	- Lacks post-translational modifications (e.g., glycosylation)[140] - Absence of endoplasmic reticulum and cytochrome P450 reductase limits applications in certain metabolic pathways[138, 141]
	Bacillus subtilis	- Strong secretory protein capacity, suitable for industrial use[142] - No endotoxin, high safety[143]	- Lower expression efficiency[144] - Limited ability to modify complex proteins[145]
Bacillus subtilis	Yeast (e.g., *Saccharomyces cerevisiae*)	- Capable of eukaryotic post-translational modifications (e.g., glycosylation)[146] - Fast growth, easy manipulation[147] - Suitable for functional studies of cytochrome P450 enzymes	- Glycosylation patterns differ from higher eukaryotes - Low expression efficiency for complex proteins[148]
	Mammalian cells	- Post-translational modifications closest to native proteins[149] - Ideal for functional studies of complex proteins	- High culture cost, long cycle[149] - Complex operation, low expression efficiency
Plant System	*Arabidopsis thaliana*, *Nicotiana tabacum*	- Secondary metabolic pathways and post-translational modifications are more similar to medicinal plants[150] - Suitable for functional validation of plant-derived genes[150]	- Long genetic transformation cycle[151] - Potentially low expression levels[152]
Viral System	Insect cells (Baculovirus system)	- Complete post-translational modification system[153] - Large cloning capacity, high expression efficiency - High safety	- Limited application in medicinal plants, immature technology[153] - High culture cost

5.1.1 Mining, Identification, Modification and Standardization of Biomolecular Components

Biological sequences, such as amino acids and nucleotides, are molecules with specific functions and represent the most basic building blocks in the genetic system. As an emerging discipline, the research on synthetic biology has just begun. In this field, the analysis of biosynthetic pathways related to the active ingredients, as well as the mining and identification of cross-species biological components, are urgent tasks that need to be addressed[154]. The Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, conducted whole-genome sequencing, assembly, gene prediction, and annotation of Ganoderma lucidum, a medicinal model species. Through these efforts, the Institute discovered all genes involved in the synthesis of the ganoderic acid backbone and identified 197 *CYP450* genes that may be involved in the modification of the ganoderic acid framework. Furthermore, 16 genes were screened from these candidates for further functional identification through co-expression analysis and phylogenetic studies[155]. In addition, the Institute of Medicinal Plants has conducted large-scale transcriptome studies on more than 10 important medicinal plants, including ginseng, American ginseng, notoginseng, Panax notoginseng, ginkgo, campytree, as shown in Figure 5. These studies identified over 2,000 candidate genes involved in the biosynthesis of ginsenoside, tanshinone, salvianolic acid, glycyrrhizin, camptothecin, and other related compounds. Gao *et al*.[156] used DNA microarray technology to identify two diterpene synthases involved in the tanshinone biosynthesis pathway: SmCPS and SmKSL. Zhou *et al*.[157] synthesized the tanshinone precursor (subtanshinone diene) by expressing the fusion protein of SmCPS and SmKSL in yeast, and the yield of the precursor reached 365 mg/L. These explorations have laid a good foundation for the research of synthetic biology of traditional Chinese medicine. This forms the foundation of the entire project. First, it was essential to identify and mine genes, enzymes, and regulatory elements involved in folic acid synthesis. Subsequently, these components need to be engineered to optimize their efficiency and compatibility. Standardization ensures the availability of reliable 'parts' for subsequent genetic and metabolic engineering applications.

5.1.2 Microbial Chassis Cell Modification

Lexin Li *et al*.[158] used *Escherichia coli* Nissle 1917 (EcN), a probiotic that can colonize the gut, as a chassis cell for the production of 5-MTHF. They further improved 5-MTHF production by replacing key enzymes, screening promoters, optimizing the RBS sequence, and regulating the expression of exogenous genes. The results showed that the genes metF and folA were overexpressed from the original pACYCDuet-1 and integrated into the genome for expression. The promoters of these two genes were replaced with the strong constitutive promoter Ptip using Red homologous recombination to reduce the growth pressure caused by the overexpression of multiple plasmids. Additionally, the optimized 5-MTHF synthetic pathway was transferred into EcN. After induced fermentation, the target probiotic EcN was obtained with a 5-MTHF yield of 316.27 μg/L. During the construction of the EcN engineered bacteria, promoters were not screened and compared. However, this lack of screening may have limited the optimization of gene expression. Although the selected promoters help to improve gene expression levels, they should be analyzed in conjunction with the genome

and transcriptome data to identify stronger promoters and select elements that are more suitable for use in EcN. Following the introduction of exogenous genes, chassis cells require metabolic optimization to enhance their suitability for folic acid synthesis and accumulation. This can be achieved by, for instance, increasing the supply of cofactors or minimizing interference with competing metabolic pathways.

5.1.3 Construction, Assembly, and Integration of Metabolic Pathways

At present, constructing biosynthetic pathways for bioactive compounds in different chassis cells is mainly achieved through two approaches:

The first approach is to transfer, reconstruct and engineer the inherent pathways of bioactive compounds based on a thorough understanding of the metabolic pathways, which is the primary construction method in synthetic biology research. The gene elements used in this approach are modular and universal, which not only facilitates optimization but also allows for their convenient application in the construction of other pathways. This transforms many complex biosynthetic pathways into engineered biological systems that can be easily disassembled and reassembled at any time. The second construction strategy is the design, screening, assembly, and programming of entirely new synthetic pathways for bioactive compounds. This strategy involves designing a synthetic route based on the chemical structure of the bioactive compound. Based on this synthetic route, enzyme genes that can participate in the designed synthetic pathway are screened from the gene database, and these enzyme genes are then introduced into the chassis cells to assemble a new biosynthetic pathway for the bioactive pharmaceutical compound within the chassis cells. By reconstructing the biosynthetic pathways and metabolic networks of bioactive compounds in chassis cells, the heterologous transformation of these bioactive compounds can be achieved under suitable conditions. The structures of the resulting products are then identified using techniques such as nuclear magnetic resonance (NMR) spectroscopy, chromatography, and mass spectrometry.

5.1.4 Systematic Optimization and Balance of Metabolic Pathways

The optimization and balance of metabolic pathways are essential for increasing the biosynthesis of target products and verifying the accuracy of biosynthetic pathway analysis. This includes optimizing exogenous pathways, enhancing endogenous metabolism (by providing sufficient precursors and cofactors), and balancing upstream and downstream pathways. The synthesis efficiency of the target product depends on multiple factors at multiple levels—from the gene level to the protein level, and from the biosynthetic pathway to the metabolic flux (Metabolic flux refers to the flow rate of metabolites (substrates, intermediates, and products) through biochemical pathways in living cells, reflecting the kinetics of metabolic reactions under specific physiological conditions). Local horizontal optimization usually refers to adjusting and optimizing limiting factors within the synthesis system. The optimization of the gene encoding the rate-limiting enzyme can be achieved by increasing the gene copy number, modifying promoters, optimizing codons, replacing genes, and regulating transcriptional activity. Following the completion of gene knockout and modular engineering, systematic optimization of the entire metabolic pathway is required to ensure balanced metabolic

flow at each step, prevent the accumulation or bottleneck of intermediate products, and ultimately maximize the yield of 5-MTHF.

6. Conclusions and perspectives

Since the discovery of the unique physiological properties of 5-MTHF, researchers both domestically and internationally have been dedicated to its practical application in the fields of food and human medicine. However, natural sources of 5-MTHF are low in content, and its chemical synthesis is complex. And it has its own instability problem, which needs to be converted into stable crystal products to achieve application. In order to solve these problems, finding new ways to obtain 5-MTHF in a green, efficient, stable and controlled way has become an urgent challenge for researchers. In this review, we present the first systematic summary of the bioactivity of 5-methyltetrahydrofolate (5-MTHF) in Moringa, its potential biosynthetic pathways and key enzyme genes involved, as well as strategies and approaches to enhance 5-MTHF production. It is hoped that in the future, by identifying key enzyme genes involved in 5-MTHF biosynthesis from *M. Oleracea* Lam. and exploring its metabolic pathways, the original biosynthetic pathways of plant active ingredients can be transferred, reconstructed, and engineered. By obtaining amino acid or nucleotide sequences with specific functions, constructing recombinant plasmids for the target genes, and selecting host cells such as *E. coli*, we can engineer bacteria capable of producing the target products. Through further optimization of fermentation conditions and production processes, we expect to obtain engineered strains with high yields of 5-MTHF. This breakthrough establishes a novel synthetic biology approach for high-yield 5-MTHF production, provides theoretical foundations for its complete biosynthetic pathway and scale-up manufacturing, and paves the way for industrial-scale production of 5-MTHF.

Nevertheless, several critical challenges remain in this technological route: (1) Biosafety concerns, particularly microbial contamination risks during fermentation: large-scale fermentation processes may encounter phage contamination, microbial competition, or plasmid instability in engineered strains (e.g., *E. coli*), potentially leading to reduced product yield or batch failure, as well as genetic stability issues[159-161]; (2) Metabolic pathway complexity: The biosynthesis of 5-MTHF involves multiple enzymatic reactions, where insufficient activity of key enzymes (e.g., dihydrofolate reductase) or limited cofactor supply may become production bottlenecks, necessitating dynamic regulation or cofactor engineering to optimize metabolic flux[162-164].

To address these challenges, future research should prioritize the following optimization strategies: First, efforts should be devoted to applying gene-editing technologies such as CRISPR-Cas9 to construct genomically integrated strains, thereby fundamentally resolving plasmid instability issues. Second, exploring the integration of automated fermentation monitoring with AI-driven metabolic modeling could enable intelligent optimization and precise control of the bioprocess. In addition, there should be a strong development of next-generation production technologies, such as cell-free synthesis systems and in vitro biotransformation, to integrate AI design, using its high flexibility and controllability to overcome the limitations of traditional biomanufacturing. These innovative strategies are not only expected to

systematically overcome current technological bottlenecks but also promote the wider application of 5-MTHF in precision nutrition and targeted therapy.

Conflicts of interest

The authors have no conflict of interest.

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