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Improved methyl supply for 5-methyltetrahydrofolate production in *E. coli* through a novel C1 transfer pathway

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Abstract

Background *L*-5-Methyltetrahydrofolate (5-MTHF) is the sole biologically active form of folate present in human blood and serves as an essential nutritional supplement. While microbial biosynthesis of 5-MTHF offers a sustainable alternative to chemical synthesis, its low yield limits industrial potential.

Results In this study, strategies for improving the methyl supply combined with engineering the tetrahydrofolate (THF) synthetic pathway were employed in *E. coli* to increase 5-MTHF production. First, a new exogenous C1 pathway was introduced to improve the intracellular methyl supply through acetyl-CoA breakdown. High expression of key rate-limiting genes *folE*, *folP* and *purU* enhanced metabolic flux of THF pathway, resulting in a 5-MTHF titer of 1.075 mg/L during shake-flask fermentation. A subsequent increase in 5-MTHF production was achieved by knocking out the *metE* gene, which is involved in the consumption of 5-MTHF. The best engineered strain, M3012, produced 8.2 mg/L 5-MTHF in a 5 L bioreactor via fed-batch fermentation, which presented the highest 5-MTHF titer to date.

Conclusion We successfully engineered *E. coli* by introducing a novel exogenous C1 metabolic pathway to augment the methyl donor pool essential for the biosynthesis of 5-MTHF. Further metabolic optimizations, including the enhancement of the THF precursor flux and the elimination of competing degradation pathways, developed a recombinant strain with significantly increased yield, which paves the way for industrial production of 5-MTHF.

Keywords Methyl supply, Metabolic engineering, 5-MTHF, *Escherichia coli*

Background

Folate (also known as vitamin B9) is a collective term for various structurally similar compounds involved in the methylation processes of nucleic acid and amino acid synthesis [1, 2]. Humans cannot synthesize folate endogenously, and folate deficiency is associated with the occurrence of various diseases, such as cancer, anemia, cardiovascular diseases, and neural tube defects [3–8]. *L*-5-Methyltetrahydrofolate (5-MTHF) is the primary physiologically active form of folate in the human body [9, 10].

Chemical synthesis remains the predominant method for the industrial production of 5-MTHF; however, it is

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associated with significant environmental concerns and necessitates chiral separation to yield the physiologically active form [8, 11, 12]. Many microorganisms and plants can synthesize folate and its derivatives de novo [13–16]. To enhance the 5-MTHF synthesis pathway in microorganisms, researchers have promoted the intracellular accumulation and secretion of folate, screened for high-yield strains, optimized culture conditions, and developed a series of improved strains (Table S1). Serrano-Amatriain et al. enhanced the production of folate and 5-MTHF in the industrial fungus *Ashbya gossypii* by identifying GTP cyclohydrolase I as the rate-limiting step in folate biosynthesis [1]. The production of 5-MTHF was significantly increased in *Lactobacillus* NZ9000 by increasing 5,10-methylenetetrahydrofolate reduction, NADPH supply, and folate synthesis [17]. Yang et al. conducted in-depth research on the synthesis of 5-MTHF using *Bacillus subtilis* as the host and achieved 5-MTHF production at level of 3.41 mg/L through systematic metabolic engineering [18, 19].

In *E. coli*, 5-MTHF synthesis was achieved through multistep enzyme catalysis, starting from guanosine triphosphate (GTP) and chorismite (Fig. 1) [16, 20]. In addition to facilitating the folate backbone synthesis of 5-MTHF, the availability of methyl group sources serves as a significant limiting factor in the biosynthesis of 5-MTHF. In *E. coli*, the methyl group of 5-MTHF is derived from 5,10-methylenetetrahydrofolate which is formed through the degradation pathways of serine and glycine, involving both amino acid synthesis and degradation metabolism (Fig. 1). Overexpression of *glyA* and *gcvPHT* genes in *E. coli* was conducted to enhance the synthesis of 5,10-methylenetetrahydrofolate and increase C1 supply. However, this approach yielded limited outcomes [21]. Introducing an exogenous C1 supply pathway to bypass intricate metabolic regulation presents a viable approach. By introducing a C1 metabolic pathway to utilize formate as the methyl source for 5-MTHF synthesis, engineered *E. coli* achieved a production of 1.24 mg/L 5-MTHF [22]. In addition, formate dehydrogenase (FDH), which converts formate into carbon dioxide, was further introduced to increase the NADPH required for 5-MTHF synthesis, and the concentration of 5-MTHF reached 3.01 mg/L [23]. However, enhancing the methyl supply pathway in *E. coli* requires intricate metabolic regulation, which demands precise control over the expression of associated genes and the alleviation of inherent feedback inhibition, thereby significantly limiting the efficient synthesis of 5-MTHF. In conclusion, although some attempts have been made to increase the methyl supply required for 5-MTHF production in *E. coli*, the low efficiency of relevant metabolic pathways still impedes the complete fulfillment of a sufficient methyl supply.

In this study, we proposed enhancing the methyl supply during the 5-MTHF synthesis process in *E. coli* through the utilization of the Wood–Ljungdahl pathway

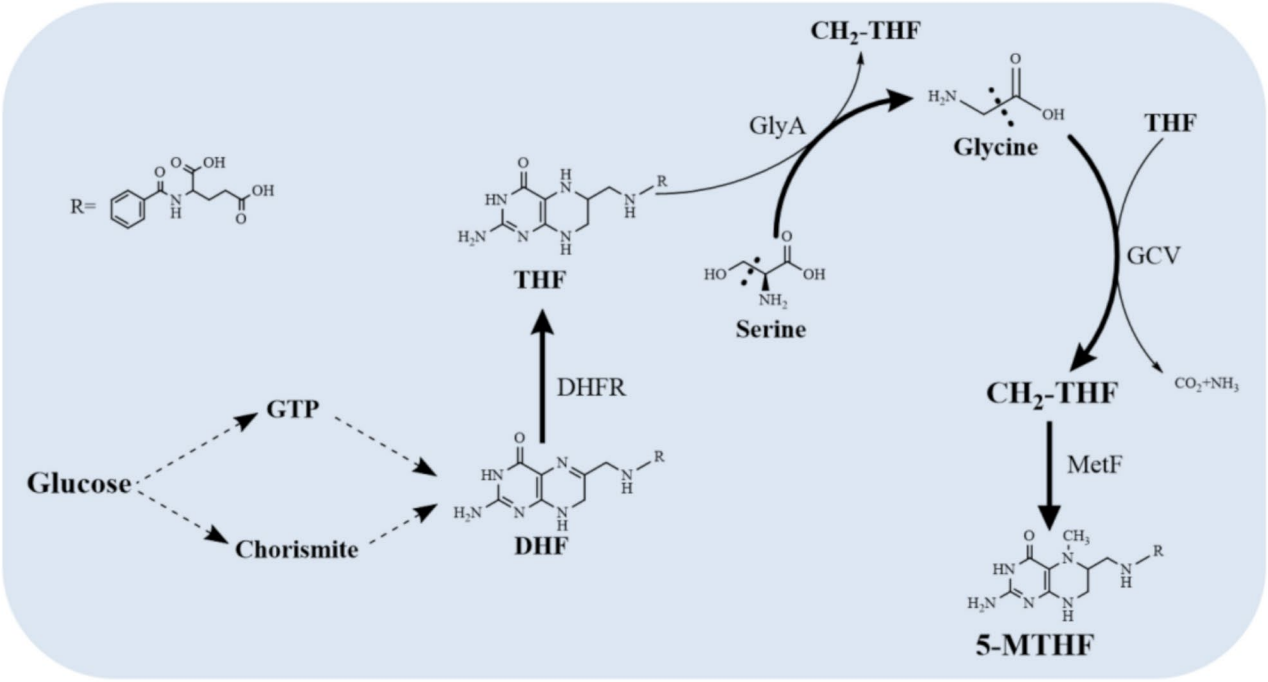

Fig. 1 Biosynthetic pathway of 5-MTHF in *E. coli*. DHF, dihydrofolate. THF, tetrahydrofolate. CH_2-THF, 5,10-methylenetetrahydrofolate. 5-MTHF, 5-methyltetrahydrofolate. DHFR, dihydrofolate reductase. GlyA, serine hydroxymethyltransferase. GCV, glycine cleavage system. MetF, 5,10-methylenetetrahydrofolate reductase

(C1 transfer pathway) [24]. Through the introduction of related enzymes from the C1 transfer pathway, we aimed to establish a novel methyl supply pathway from acetyl-CoA cleavage for 5-MTHF synthesis (Fig. 2). This pathway integrates the acetyl-CoA decarbonylase/synthase β subunit CdhC2 from *Methanosarcina thermophila*, the iron–sulfur protein AcsCD, and the methyltransferase AcsE from *Moorella thermoacetica* to facilitate the transfer of the methyl group generated from acetyl-CoA breakdown to 5-MTHF via the intermediate CH_3–CoFeSP.

Materials and methods

Strains, genes, and reagents

E. coli DH5α was used for plasmid construction and amplification. *E. coli* BL21(DE3) was used as the host strain for gene expression and 5-MTHF production. The genes *cdhC2* encoding acetyl-CoA decarbonylase/synthase β subunit from *M. thermophila*, *acsCD* encoding an iron–sulfur protein from *M. thermoacetica*, and *acsE* encoding a methyltransferase from *M. thermoacetica* were synthesized and codon optimized for *E. coli* by GENEWIZ (Genewiz Biotech Co. Ltd., China) (Table S4). All strains, plasmids, and primers used in this study are listed in the supplementary materials. A one-step clone kit (TransGen Biotech, Beijing, China) was used for plasmid construction. Folic acid, THF, 5-MTHF, and 5,10-methylenetetrahydrofolate were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Vitamin B12, sodium sulfide nonahydrate, nickel chloride hexahydrate, and ammonium iron(II) sulfate hexahydrate were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Recombinant human gamma-glutamyl hydrolase was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Cultivation conditions

LB medium served as the seed culture, whereas M9 medium was used for flask-scale fermentation. For seed cultivation, a single colony was transferred to 5 mL of LB broth and cultivated at 37 °C and 200 rpm. After approximately 12 h of cultivation, 1 mL of the seed culture was added to 100 mL of M9 medium containing 20 g/L glucose and 2 mM $MgSO_4$ in a 500 mL flask. These flasks were incubated at 37 °C for approximately 6 h at 200 rpm. For protein expression, 0.5 mM IPTG was added to induce protein expression when the cell density reached 0.6–0.8 at 600 nm (OD_{600}), and 2 mg/L vitamin B12, 200 μM sodium sulfide, 10 μM nickel chloride, and 100 μM ammonium iron(II) sulfate hexahydrate were added to the culture at a final concentration. After 48 h of cultivation at 25 °C and 200 rpm, the cells were harvested for further testing. All the experiments were conducted in triplicate. Chloramphenicol (34 mg/L), kanamycin

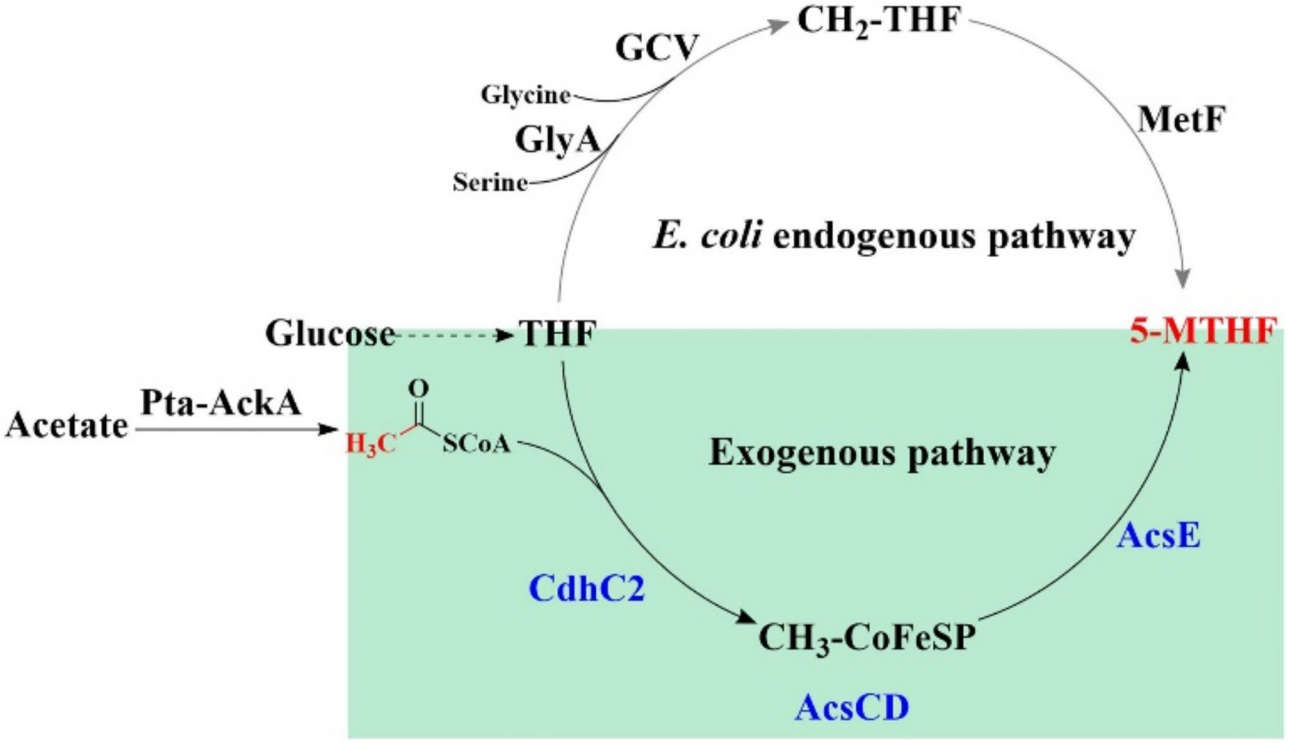

Fig. 2 Design and construction of the novel methyl supply pathway. Pta, phosphate acetyltransferase. AckA, acetate kinase. CdhC2, acetyl-CoA decarbonylase/synthase β subunit from *M. thermophila*. AcsCD, iron–sulfur protein from *M. thermoacetica*. AcsE, methyltransferase from *M. thermoacetica*

(50 mg/L) and ampicillin (100 mg/L) were added to the culture medium when needed.

In vitro enzymatic catalysis by cell lysates

The methodology employed for cell cultivation adheres closely to the procedures outlined in the preceding section, with minor deviations as detailed subsequently. For arabinose induction, 30 mM arabinose was added to the culture medium approximately 2 h after inoculation. After 16 h of cultivation at 16 °C and 200 rpm, the cells were collected via centrifugation. The cell pellet was washed twice with sterile dH_2O, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0) and lysed via a high-pressure cell crusher. Following centrifugation (13000 × g, 4 °C, 30 min), the supernatant was collected for enzymatic catalysis.

The exogenous pathway for synthesizing 5-MTHF in vitro involves multiple cofactors and precursors, such as acetate, ATP, CoA, cobalamin, and THF. The in vitro assays for the C1 pathway were performed at 30 °C in a 1000 µL total reaction volume containing 100 mM Tris-HCl buffer (pH 8.0), 5 mM CoA, 10 mM ATP, 5 mM acetate, 5 mM THF, and 500 µL of cell lysate. The identity of the formed acetyl-CoA and 5-MTHF was further confirmed through UHPLC–MS (see supplementary materials).

Genome modification

The λ Red recombination system was used for genome modification. To delete *metH/metE* in *E. coli* BL21(DE3), the knockout fragment was subsequently amplified from the isogenic *E. coli* K-12 KEIO collection of the Genome Analysis Project in Japan [25], 5 µL of which was then added to 100 µL of electrocompetent cells harboring the plasmid pKD46, and electroporation was performed via a Gene Pulser X-cell electroporation system (Bio-Rad Laboratories) and ice-cold 2 mm electroporation gap cuvettes (Bio-Rad Laboratories). Recombinants were confirmed by colony PCR using two primers outside of the homologous arms of the target gene.

Extraction and metabolite analysis

5-MTHF is usually accumulated in the *E. coli* intracellular space during fermentation and then extracted by cell lysis. For 5-MTHF extraction, the cell pellets were resuspended to half of the original culture volume in extraction buffer composed of 20 mM ammonium acetate and 0.2% ascorbic acid, and the cells were then lysed via a high-pressure cell crusher. 1% (v/v) recombinant human gamma-glutamyl hydrolase was added to the cell lysates, and the mixture was incubated at 37 °C for 3 h to deconjugate the glutamate tail of intracellular 5-MTHF [14, 26]. The hydrolysis products were then mixed with the same volume of methanol and immediately centrifuged at 10,000 × g for 5 min [27]. The supernatants were then filtered through a 0.22 µm nylon membrane for HPLC measurement.

For 5-MTHF analysis, samples were analyzed using high-performance liquid chromatography (HPLC; Agilent Technologies 1260 Infinity II, Santa Clara, CA, USA) equipped with a fluorescence detector (295 nm excitation, 356 nm emission). The chromatographic column used was an SB-C18 (Agilent). HPLC analysis was conducted with a mobile phase composed of 7% acetonitrile and 93% water containing 30 mM KH_2PO_4 (pH 2.3) at a temperature of 23 °C and a flow rate of 0.5 mL/min. The injection volume was set to 10 µL. Standard solutions of 5-MTHF were prepared in extraction buffer and demonstrated excellent linear response within a concentration range of 0.1–10 mg/L.

Microbial production of 5-MTHF by fed-batch fermentation

For fed-batch fermentation, a single colony was picked from the plate, inoculated into 5 mL of LB broth as the initial seed culture, and then cultivated at 37 °C and 200 rpm for 12 h. Then, 1 mL of the seed culture was transferred into 100 mL of M9 medium with 20 g/L glucose and 2 mM $MgSO_4$ in a 500 mL shake flask and cultivated at 37 °C and 200 rpm for approximately 8 h. The M9 culture was poured into a 5 L bioreactor with 2 L fermentation medium containing 20 g/L glucose, 9.8 g/L $K_2HPO_4{\cdot}3H_2O$, 2.1 g/L citric acid$\cdot H_2O$, 3 g/L $(NH_4)_2SO_4$, 0.3 g/L ammonium ferric citrate, 2 mM $MgSO_4$, and 2 mL trace element solution. The trace element mixture contained 3.7 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 2.9 g/L $ZnSO_4{\cdot}7H_2O$, 24.7 g/L H_3BO_3, 15.8 g/L $MnCl_2{\cdot}4H_2O$, 2.4 g/L $NiCl_2{\cdot}6H_2O$, and 2.5 g/L $CuSO_4{\cdot}5H_2O$. When the residual glucose level fell below 1 g/L, a feeding solution with 500 g/L glucose was used to maintain the glucose concentration between 0 and 1 g/L. The pH was maintained at 7.0 by automatically adding NH_4OH (25%, v/v). Kanamycin (50 mg/L) and ampicillin (100 mg/L) were added to the culture medium when needed. When the OD_{600} reached approximately 30, 0.5 mM IPTG, 2 mg/L vitamin B12, 200 µM sodium sulfide, 10 µM nickel chloride, and 100 µM ammonium iron (II) sulfate hexahydrate were together added to the culture at a final concentration. The fermentation temperature was maintained at 25 °C after induction.

Results and discussion

Design and construction of the novel methyl supply pathway

The native 5-MTHF synthesis pathway in *E. coli* can be divided into THF synthesis and C1 supply; however, insufficient methyl donors limit the efficient synthesis of 5-MTHF. In this study, a novel C1 transfer pathway was designed by introducing enzymes from the

Wood–Ljungdahl pathway for 5-MTHF synthesis (Fig. 2). This pathway combines the acetyl-CoA decarbonylase/synthase β subunit CdhC2, iron-sulfur protein AcsCD, and methyltransferase AcsE to transfer the methyl group from acetyl-CoA breakdown to 5-MTHF through the intermediate CH_3-CoFeSP. Specifically, CdhC2 features an unusual hexameric cluster comprised of an iron–sulfur cluster (Fe_4S_4) and two nickel ions, which catalyzes the cleavage of the acetyl group C–C bond and facilitates the synthesis of acetyl–CoA during the growth of *M. thermophila* [28–30]. The CdhC subunit from *M. thermophila* (methanogenic archaeon) TM-1 was expressed in *E. coli*, and its catalytic activity was analyzed. The results showed that CdhC could be expressed in a soluble form in *E. coli*, and the C-terminal-truncated CdhC* exhibited better solubility expression and catalytic activity [29]. AcsE belongs to the vitamin B12-dependent methyltransferase family and catalyzes the conversion of 5-MTHF and CoFeSP into THF and CH_3-CoFeSP [31]. AcsE catalyzes reversible methyl transfer between 5-MTHF and CH_3-CoFeSP. In the forward direction, it methylates CoFeSP to yield CH_3-CoFeSP, using 5-MTHF as a methyl donor. Conversely, in the reverse direction, it methylates THF to produce 5-MTHF, with CH_3-CoFeSP serving as the methyl donor. The enzymatic characteristics of AcsE from *M. thermoacetica* have been extensively investigated, and the results revealed that the enzyme was insensitive to oxygen and could be heterologously expressed in *E. coli* [32, 33]. Despite potential challenges from oxygen sensitivity and misfolding risks, the anaerobic bacterial proteins CdhC2 and AcsCDE were successfully expressed in *E. coli* with minimal inclusion body formation, demonstrating their catalytic applicability (Fig. S1).

In vitro enzyme catalysis experiments were conducted to verify the activity of the exogenous pathway by supplementing cofactors and precursors. In these experiments, acetyl-CoA was synthesized from acetate through Pta-AckA enzymatic steps and further used for the synthesis of 5-MTHF through the exogenous C1 supply pathway. Compared with the control strain M1011A, the strain M1012A, which expresses the exogenous C1 supply pathway, did not significantly improve the 5-MTHF synthesis capacity (Fig. S2). This may be attributed to the requirement of cobalamin as a cofactor for the catalytic function of the proteins employed in this study. Although *E. coli* BL21(DE3) contains an endogenous gene cluster encoding cobalamin transport proteins, this gene cluster may be silent or expressed at a low level, which can affect the activity of the expressed exogenous proteins.

Increasing cobalamin uptake to obtain methyl supply pathway activity

Based on possible cobalamin requirements, the capacity for cobalamin uptake was aimed to be increased by overexpressing the cobalamin transporters in *E. coli*. The gene cluster was constructed on a plasmid following methods reported before, and the expression of related proteins was controlled by an arabinose promoter [34]. A cobalamin uptake enhancement experiment was conducted using strains M1016 and M1017. Compared with the control strain M1016, strain M1017, which overexpresses the cobalamin transport protein complex BtuCEDFB, exhibited noticeable pink coloration, demonstrating successful enhancement of cobalamin uptake in *E. coli* BL21(DE3) (Fig. S3).

After enhancing cobalamin uptake, further comparisons were made to evaluate the impact of introducing a novel methyl supply pathway on 5-MTHF synthesis. A comparison of the ion intensity of the 5-MTHF precursor via UHPLC-MS/MS revealed that in the M1017A strain, which has increased cobalamin uptake and an exogenous methyl supply pathway, the ion intensity of 5-MTHF was approximately 2.4 times greater than that of the control strain M1015A after a 1.5 h reaction (Fig. 3A&B). This preliminary result suggested that the novel methyl supply pathway, which serves as an alternative route to the serine and glycine degradation pathway, successfully enhanced 5-MTHF synthesis by supplementing the C1 source.

Isotope tracing of the methyl transfer process

To verify the transfer of the designed methyl group pathway, isotope labeling experiments were implemented to confirm the methyl transfer process. Isotope labeling experiments were conducted using strains M1015A and M1017A, and the methyl donor in the reaction mixture in vitro was replaced with deuterated (D-labeled) acetate, with all other conditions remaining the same. Based on the mass spectrometry results, the parent ion and fragment ions of D-labeled acetyl-CoA, which has a molecular weight 3 units higher than that of unlabeled acetyl-CoA (due to the replacement of hydrogen with deuterium in the acetyl group), were detected in both groups, confirming the successful synthesis of D-labeled acetyl-CoA (Fig. 3C). D-labeled 5-MTHF was detected only in strain M1017A, with an increase in the parent ion and fragment ions corresponding to strain M1015A (Fig. 3D & Fig. S4). Both groups also presented the presence of unlabeled 5-MTHF, with no significant differences observed (Fig. S4).

Both groups successfully synthesized D-labeled acetyl-CoA; however, D-labeled 5-MTHF was detected only in strain M1017A. The M1015A strain contained genes related to acetate utilization (*pta* and *ackA*) capable of

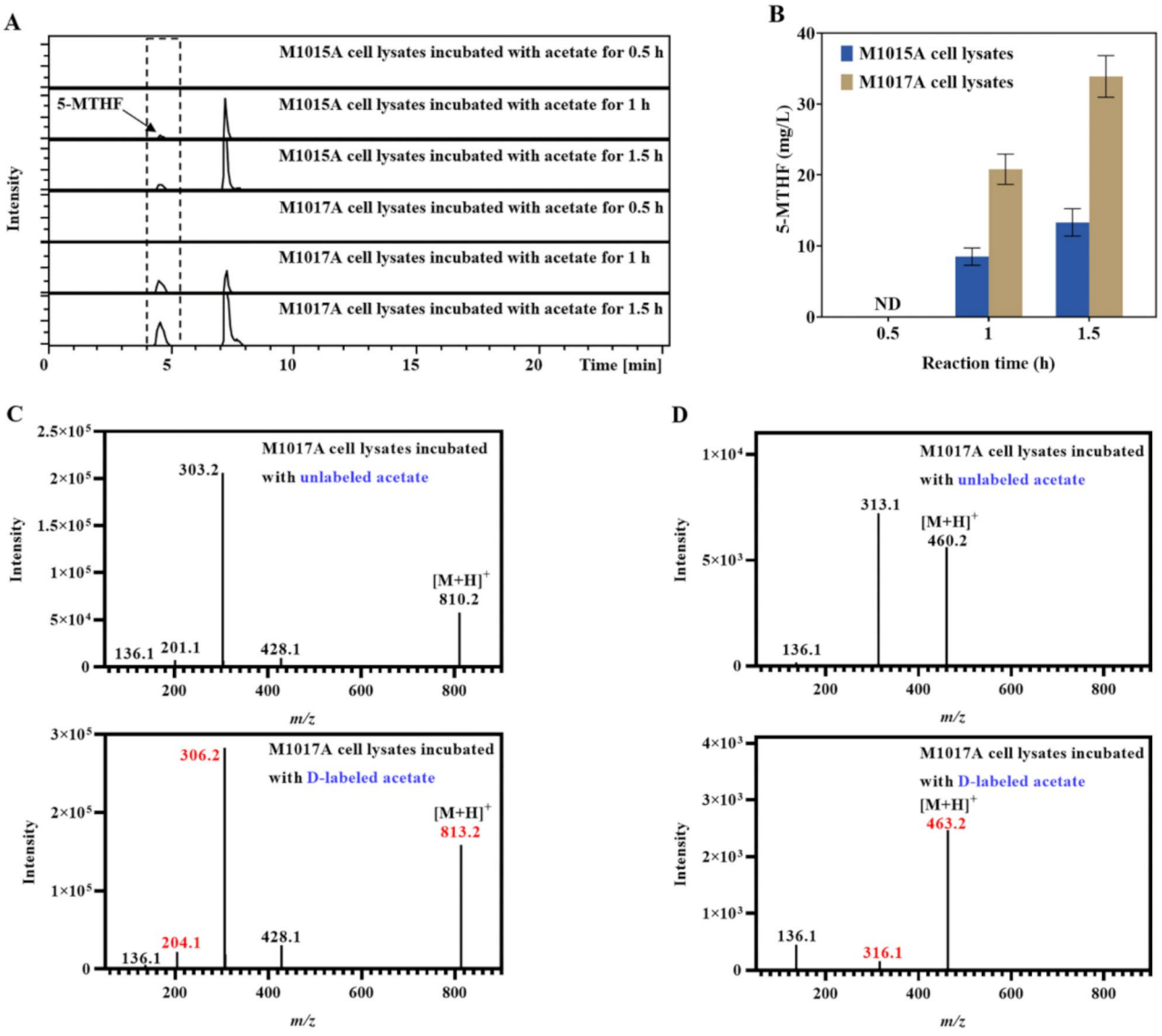

Fig. 3 LC-MS/MS analysis of 5-MTHF and isotope tracing of methyl transfer process. **(A)** Comparison and **(B)** quantitative comparison of 5-MTHF synthesis by cell lysates in vitro before and after the introduction of the exogenous methyl supply pathway. Tandem mass spectrometry fragmentation profile of **(C)** acetyl-CoA and **(D)** 5-MTHF in M1017A cell lysates incubated with unlabeled acetate (above) and D-labeled acetate (below) in positive ion mode. ND, not detected. The red numbers represent the precursor and fragment ions that differ in mass spectrum

synthesizing acetyl-CoA from acetate, leading to the detection of D-labeled acetyl-CoA, which is consistent with the obtained results. Compared with the control group, the M1017A strain differed only in the expression of the exogenous C1 pathway. Through isotope labeling experiments, the ability of the CdhC2 and AcsCDE proteins to catalyze the transfer of methyl groups from acetyl-CoA to 5-MTHF was preliminarily validated.

Construction of chassis cells for 5-MTHF production in vivo

E. coli naturally contains pathways for the synthesis of THF and 5-MTHF. This study further introduced a novel methyl supply pathway for 5-MTHF synthesis in *E. coli* by incorporating genes from the Wood–Ljungdahl pathway to augment the C1 supply.

Initial analysis and comparison of 5-MTHF synthesis via shake-flask fermentation across several strains were conducted to identify high-producing strains suitable as chassis cells for subsequent metabolic engineering modifications. Strain M1011, lacking the novel methyl supply pathway, did not exhibit detectable 5-MTHF synthesis, potentially because of the unmodified baseline level of endogenous 5-MTHF synthesis in *E. coli* (Fig. 4B). As shown in Fig. 4B, the strains that incorporated the novel methyl supply pathway produced 5-MTHF at concentrations ranging from 0.1 mg/L to 0.4 mg/L, with M1012

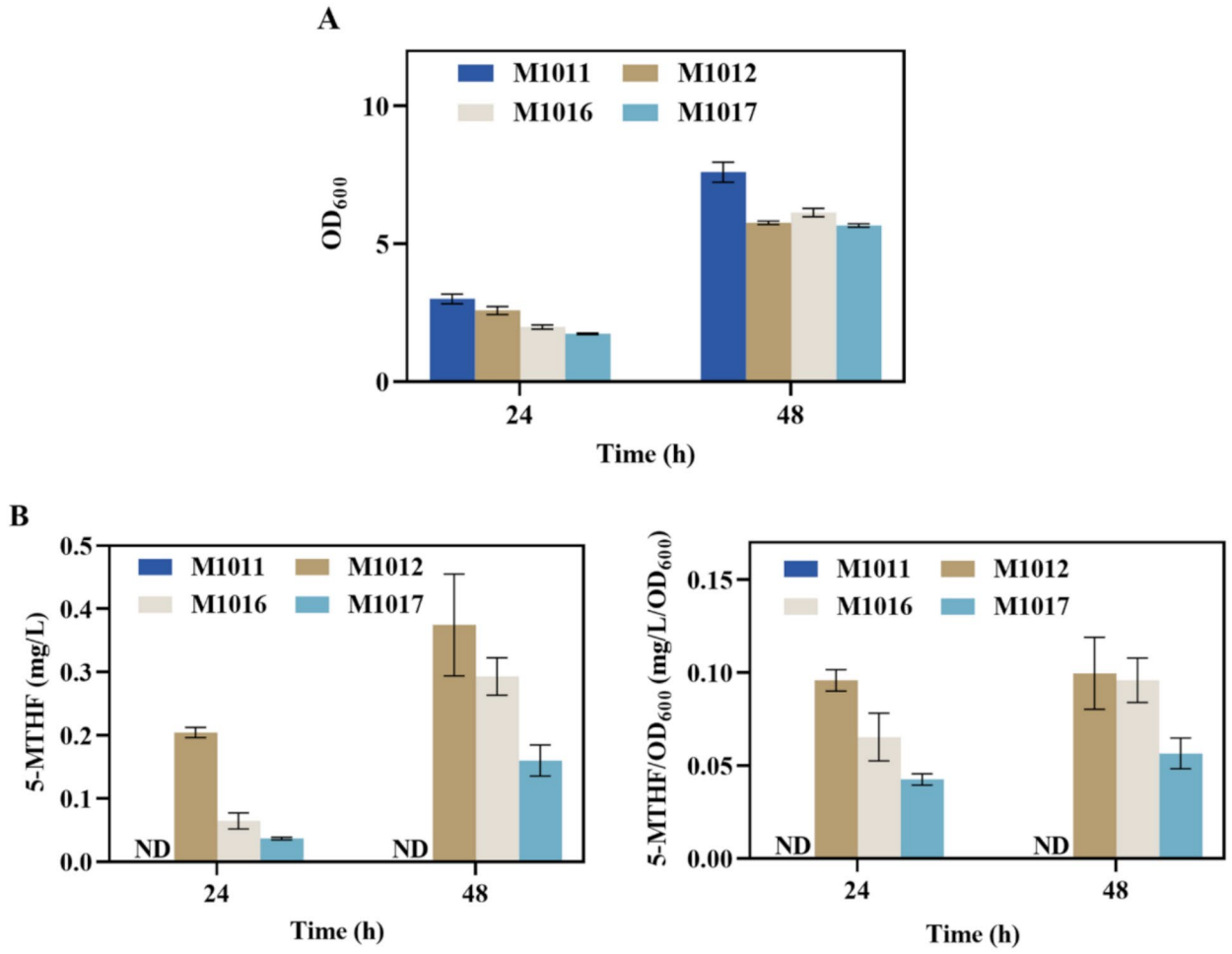

Fig. 4 Construction of the 5-MTHF synthesis chassis cells. Presentation of **(A)** cell growth and **(B)** 5-MTHF production in different strains. ND, not detected

achieving the highest 5-MTHF titer of 0.37 mg/L by 48 h. Conversely, the M1017 strain, which overexpresses cobalamin transport proteins, presented a decrease in 5-MTHF synthesis, possibly attributed to metabolic stress or an imbalance in the strain due to the overexpression of cobalamin transport proteins.

Since 5-MTHF cannot be secreted into the extracellular space, the level of 5-MTHF accumulation is positively correlated with biomass to a certain extent. The concentration of 5-MTHF per unit cell was assessed to compare the 5-MTHF accumulation capacity per cell among different engineered strains, which served as a crucial reference indicator (Fig. 4B). Notably, strain M1012, which exhibited the highest 5-MTHF accumulation per unit cell during the chassis cell selection process, outperformed the other strains in terms of both the accumulation speed and the amount of 5-MTHF concentration per unit cell, leading to its selection as the chassis cell for further metabolic engineering modifications.

Increasing the precursor supply to improve 5-MTHF production

Initially, efforts were made to enhance the capability of *E. coli* to assimilate folate from the culture medium for the synthesis of 5-MTHF. *E. coli* is incapable of absorbing folate independently, and engineered *E. coli* can potentially take up folate through the introduction of the exogenous folate transport protein ECF-FolT2 from *Lactobacillus delbrueckii subsp. bulgaricus* (Fig. 5A) [35, 36]. Initially, the expression of transport proteins was regulated by the T7 promoter, resulting in significant growth inhibition of the cells postinduction. After switching to the arabinose promoter, the strain was able to grow normally following arabinose induction (Fig. S5). The introduction of folate transport proteins resulted in the M3006 strain, which presented increased 5-MTHF accumulation compared with that of the control strain M1012 after 24 h of shake-flask fermentation (Fig. 5C). Nevertheless, after 48 h of fermentation, the production of 5-MTHF in the two strains had reversed, suggesting that the folate transport proteins accelerated the

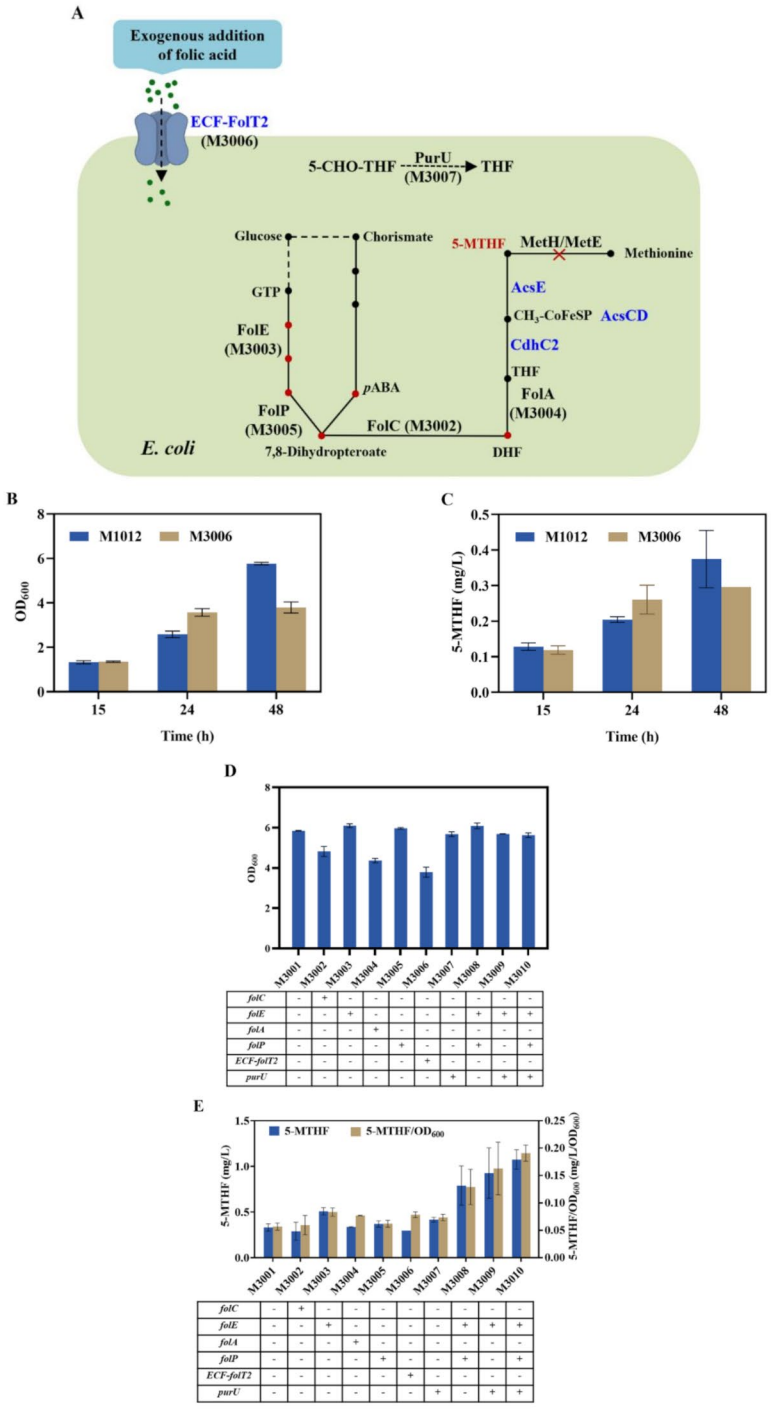

Fig. 5 Enhancement of 5-MTHF biosynthesis through metabolic engineering modifications. **(A)** Schematic of metabolic engineering modifications. Comparison of the differences in **(B)** OD_{600} and **(C)** 5-MTHF production before and after the introduction of folate transport proteins. Comparison of different engineered strains on the basis of **(D)** OD_{600} and **(E)** 5-MTHF concentration. The red dots represent the overexpressed genes in related pathways

accumulation of 5-MTHF in strain M3006. The cell density of strain M3006 was significantly lower than that of strain M1012. This may be attributed to the expression of channel proteins imposing a certain burden on cell growth, leading to reduced biomass and subsequently affecting the accumulation of 5-MTHF.

As the introduction of folate transport proteins did not significantly increase the production of 5-MTHF in the engineered strain, our focus shifted to metabolic

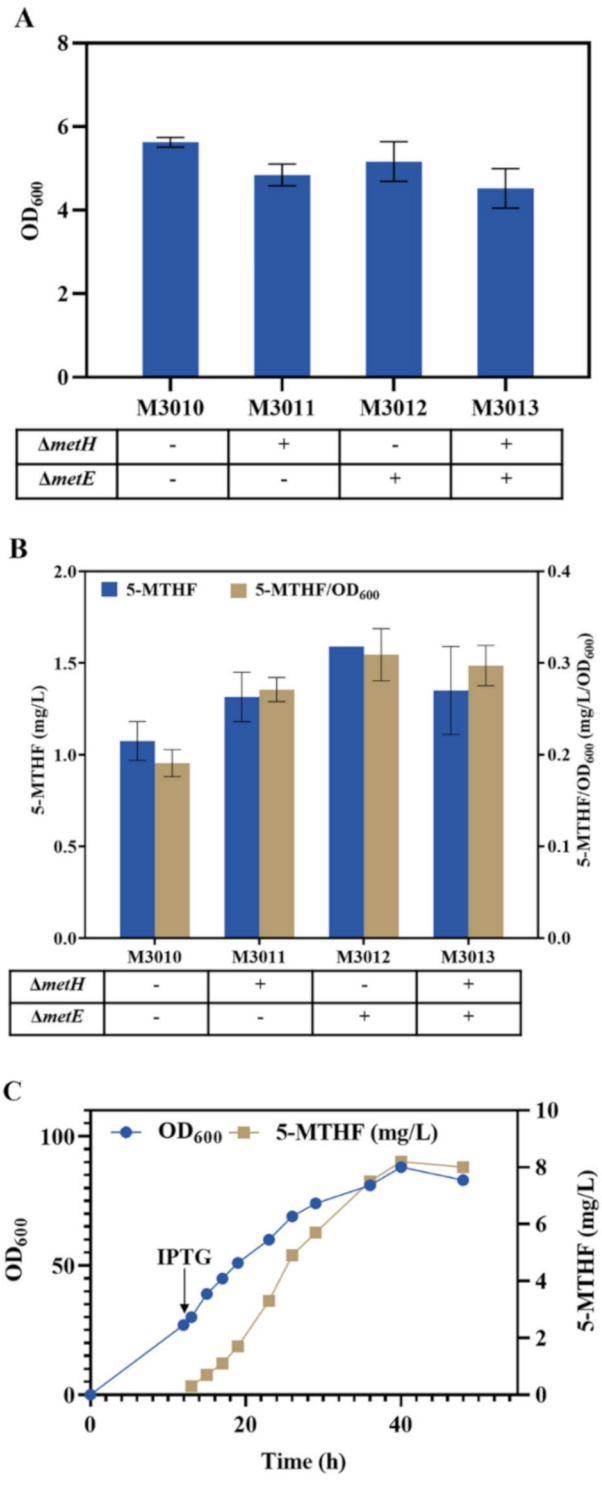

Fig. 6 Effects of knocking out the 5-MTHF degradation pathway on the **(A)** OD_{600} and **(B)** 5-MTHF concentration. **(C)** Fed-batch fermentation process curve of strain M3012 in a 5 L bioreactor

engineering modifications of the THF synthesis pathway to improve the endogenous precursor supply of 5-MTHF synthesis in *E. coli* (Fig. 5A). Drawing from previous studies, we proceeded to overexpress several key genes within the 5-MTHF synthesis pathway [1, 18, 19]. The findings revealed that the overexpression of GTP cyclohydrolase encoded by *folE* (strain M3003), dihydropteroate synthase encoded by *folP* (strain M3005), and formyltetrahydrofolate deformylase encoded by *purU* (strain M3007) led to various increases in 5-MTHF production, and the concentrations of 5-MTHF increased by 105%, 53%, and 73%, respectively, compared with that of the control strain M3001 (Fig. 5E). The effects of various combinations of gene overexpression involving *folE*, *folP*, and *purU* on the synthesis of 5-MTHF were subsequently evaluated, and the results indicated that co-overexpression of FolE and FolP, or FolE and PurU, could further increase the accumulation of 5-MTHF. Strain M3012, which overexpresses all three genes, achieved the highest 5-MTHF concentration of 1.075 mg/L. Considering the influence of biomass on 5-MTHF accumulation, the ratio of the 5-MTHF concentration per unit cell density was compared among the different engineered strains. The results indicated that the ratio trends among the different strains were almost identical to those of the concentration of 5-MTHF (Fig. 5E).

Reducing the 5-MTHF degradation pathway to improve production

On the basis of the metabolic engineering mentioned above, the accumulation of 5-MTHF can be further increased by knocking out the catabolic pathway of 5-MTHF. In *E. coli*, 5-MTHF serves as a methyl donor for methionine synthesis, producing THF in the process, and this step is catalyzed by both MetH and MetE, which facilitate the transfer of methyl group from 5-MTHF to methionine. A comparison of the levels of 5-MTHF synthesis among different gene knockout strains revealed that all of them increased the accumulation of 5-MTHF, with the highest concentration of 1.59 mg/L observed in strain M3012 (Fig. 6B). Knockout of the *metH* gene slightly inhibited the growth of strains M3011 and M3013, which unavoidably decreased the final concentration of 5-MTHF compared with that of strain M3012 (Fig. 6A).

Maximum 5-MTHF production via fed-batch fermentation

The shake flask experiments described above yielded one of the most promising strains, M3012. To further evaluate the fermentation potential of the best strain under fed-batch conditions, fed-batch fermentation was implemented in a 5 L bioreactor. In the fed-batch fermentation experiments, the strategy of maintaining a low concentration of residual glucose was adopted after the initial

glucose consumption, in which 40 g/L *L*-methionine and 500 g/L glucose were fed together. The changes in cell growth and the 5-MTHF titer with time throughout the fermentation process are shown in Fig. 6C. The cell growth was relatively stable without significant fluctuations during the fed-batch fermentation process. Following induction, the titer of 5-MTHF increased gradually with cell growth, ultimately reaching a maximum concentration of 8.2 mg/L after 40 h of fed-batch fermentation. The level of 5-MTHF production was also the highest reported production to date, and this 5-MTHF biosynthetic strain has potential for the industrial production of 5-MTHF. In the future, to further increase the 5-MTHF yield, promoting the export of 5-MTHF from the cytoplasm to the extracellular space and preventing the oxidation of 5-MTHF during fermentation might be important [1, 37].

Conclusion

This study successfully developed a high-yield strain for the synthesis of 5-MTHF through several strategies. These strategies include (1) the introduction of exogenous enzymes to create a novel C1 transfer pathway from acetyl-CoA cleavage to provide a methyl group for 5-MTHF synthesis, (2) the overexpression of key enzymes to increase the carbon flux of 5-MTHF synthesis, and (3) the knockout of the 5-MTHF degradation pathway to further increase 5-MTHF production. In a 5 L bioreactor, the M3012 strain accumulated 8.2 mg/L 5-MTHF, the highest reported titer for microbial production of 5-MTHF to date. In conclusion, these strategies synergistically enhanced the production of 5-MTHF in engineered *E. coli*. This research highlights the need for further development in the efficient microbial synthesis of 5-MTHF, with potential implications for industrial-scale production.

Supplementary Information

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Supplementary Material 1

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Author contributions
Wen Liu: Writing– original draft, investigation, formal analysis, conceptualization. Jing Guo: Writing– review & editing, investigation, formal analysis. Wei Lu: Validation, formal analysis. Tao Cheng: Validation, investigation. Yiting Li: Writing– review & editing, investigation, resources. Mo Xian: Supervision, resources. Rubing Zhang: Writing– review & editing, supervision, resources, funding acquisition, conceptualization.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animal performed by any of the authors.

Consent for publication
All authors have read and approved this manuscript to publish.

Competing interests
The authors declare no competing interests.

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