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Elucidating the Capacity and Mechanism of *Lactiplantibacillus plantarum* in Synthesizing Essential Amino Acids from Non-Essential Amino Acids in a Novel Severely Deficient Medium

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Abstract: The gut microbiota plays a vital role in human physiology and nutrient metabolism. However, its capacity to synthesize essential amino acids (EAAs) as a nutrient source remains insufficiently characterized, with genomic evidence suggesting this potential but lacking direct in vitro validation. To address this, we developed an artificial medium comprising 78 components, enabling *Lactiplantibacillus plantarum* (ATCC 8014) to achieve growth comparable to that in conventional MRS broth. Through systematic depletion of individual and multiple EAAs, leucine, isoleucine, phenylalanine, tryptophan, and valine were identified as critical for the survival and proliferation of this strain. Subsequent analysis revealed that lysine and threonine were synthesized and secreted into the medium after 48 h of culturing in medium lacking these EAAs, using aspartic acid as a major precursor. Notably, in response to methionine deficiency, cysteine seemed to be converted to methionine via the transsulfuration pathway, with vitamin B6 serving as an essential cofactor. Collectively, our findings demonstrated the ability of *L. plantarum* to synthesize and provide lysine and threonine in these EAA-restricted conditions. This ability to serve EAAs to the environment provides a basis for future studies to further investigate the role of intestinal microbiota as a potential source of EAAs in host animals.

Keywords: gut microbiota; amino acids; *Lactiplantibacillus plantarum*; essential amino acids; methionine; lysine; threonine

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1. Introduction

Despite significant advancements in food technology, protein malnutrition continues to pose a global public health challenge, with 147,672,757 cases and 212,242 associated deaths reported in 2019 [1]. Ensuring the intake of high-quality protein in adequate quantities across all meals is vital for optimal growth, development, and health at every stage of life.

Protein nutritional quality is defined by its ability to supply essential amino acids (EAAs), which cannot be synthesized by the human body and must be obtained through diet. Nonetheless, many staple crops exhibit insufficient levels of EAAs to meet dietary requirements. For instance, rice, a primary food source for nearly 50% of the global population [2] and especially in developing nations, is deficient primarily in lysine [3], whereas animal proteins provide a complete profile of EAAs [4]. As the global population

is projected to reach 10 billion by 2050, an estimated 1250 million tons of animal-derived protein will be required annually if current consumption levels are maintained, posing significant challenges to both food security and environmental sustainability [5,6]. In response, food production from alternative proteins has advanced markedly, with plant-, fungi-, and insect-based products now available in grocery stores and meat substitutes increasingly featured in restaurant menus. Despite recent advancements, there remain several challenges that need to be addressed before these products can achieve widespread accessibility [7]. Consequently, exploring potential protein sources, particularly those that can provide EAAs, is crucial for expanding the spectrum of future dietary options and addressing human nutritional needs.

The literature emphasizes the intricate and multifaceted relationship between intestinal microbiota and protein/amino acid (AA) metabolism, which significantly influences the host's AA landscape [8]. Studies in murine models, particularly those comparing metabolite profiles between germ-free and specific-pathogen-free mice or via fecal microbiota transplantation, have demonstrated that gut bacterial colonization impacts the profiles of free AAs within both the gastrointestinal tract and circulation [9,10]. This alteration is attributed to the modified availability of AA and efficiency of AA absorption in the intestines [11]. In an animal model, stable isotope analysis using carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) has revealed that approximately 44% of EAAs in the red blood cells of predominantly granivorous mammals are synthesized by their gut microbiota [12]. In pigs, the contributions of gut microbiota to EAA absorption have been quantified, with daily uptake rates of valine at 1.8 g, isoleucine at 0.8 g, leucine at 2.0 g, phenylalanine at 0.3 g, and lysine at 0.9 g [13]. In humans, a recent study has demonstrated a positive correlation between gut microbial biosynthesis of branched-chain amino acids (BCAAs)—specifically leucine and valine—and circulating BCAA levels in a healthy population [14]. This observation implies that AAs synthesized by the gut microbiota translocate into the bloodstream from the intestine, thereby modulating systemic AA concentrations in the host.

In vitro studies have further demonstrated the activity of the BCAA biosynthesis pathway in *Streptococcus thermophilus* [15] and have revealed that a variety of lactic acid bacteria (LAB) exhibit the enzymatic capability to synthesize a wide spectrum of EAAs, including methionine, lysine, threonine, and tryptophan [16]. Despite this, the specific metabolic precursors and pathways responsible for the biosynthesis of these EAAs in LAB remain incompletely understood and warrant further investigation. In parallel, gene expression studies have indicated that gut bacteria can enzymatically convert non-essential amino acids (NEAAs) into EAAs, thus serving as an essential nutrient source. For example, DNA and RNA sequencing analyses from *Lactobacillus paracasei* FAM18149 have demonstrated that cysteine can be converted into methionine via the intermediate cystathionine. Although relevant genes have been identified in microbial genomes, their functionality cannot be assumed without further verification [17]. Taken together, while the intestinal microbiota has been shown to possess the capability to synthesize EAAs, the precise origins and extent of this production are yet to be fully quantified [17], with no definitive evidence proving that the microbiota can convert NEAAs into EAAs in both in vitro and in vivo studies.

Herein, we utilized six LAB strains, each known for their potential to produce EAAs [15,18–22], to investigate LAB de novo synthesis capabilities and adaptive mechanisms for converting NEAAs into EAAs under nutrient-limited conditions. To this end, we developed a multi-deficiency EAA medium and cultured each strain in that medium. Our findings demonstrate that *Lactiplantibacillus plantarum* (ATCC 8014, hereafter referred to as *L. plantarum*) is capable of synthesizing lysine and threonine, and aspartic acid was identified as a key precursor utilized by this strain in the biosynthesis of these EAAs. Notably, there appears to be preliminary evidence suggesting the presence of a transsulfu-

ration pathway in *L. plantarum* that may facilitate the conversion of cysteine to methionine, regulated by vitamin B6; however, its capacity for methionine production remains inconclusive. Collectively, our findings highlight a physiologically significant function of intestinal microbiota in synthesizing EAAs from NEAAs under nutrient-restricted conditions, laying a foundation for future studies to validate this function and investigate its physiological implications in multi-strain polyculture in vitro systems and animal models.

2. Materials and Methods

2.1. Microbial Strains

All parental strains listed in Table 1 were obtained from the Biological Resource Center, NITE (NBRC), and subsequently cultivated in MRS (de Man–Rogosa–Sharpe) broth (Difco 288130, Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA). Individual colonies were meticulously isolated from MRS agar plates (Difco 288210, BD) and reinoculated into fresh MRS broth. The bacterial cells were cryopreserved in 80% MRS and 20% glycerol (075-00616, FUJIFILM Wako Pure Chemical Industries, Tokyo, Japan) solution and subsequently stored at −80 °C until use.

Table 1. Bacterial strains used in this study.

Bacterial Strains	ATCC [1]	NBRC [2]	Isolation Sources
Lactiplantibacillus plantarum	8014	3070	Unclear
Lactobacillus delbrueckii	9649	3202	Sour grain mash
Lactocaseibacillus rhamnosus	7469a	3435	Existing strain (ATCC 7469) [3]
Lactobacillus delbrueckii	11842	13953	Dairy products (Bulgarian yogurt)
Lactocaseibacillus casei	393	15883	Dairy products
Lactocaseibacillus paracasei subsp. *Paracasei*	25302	15889	Dairy products

[1] ATCC: American Type Culture Collection. [2] NBRC: Biological Resource Center, NITE. [3] Isolation source of ATCC 7468 is unclear.

2.2. Preparation of Artificial Medium Combinations

Building upon the formulation of MRS broth, an enriched and selective medium specifically optimized for the cultivation of LAB species, we developed a new artificial medium, with its detailed composition provided in Table S1. The procedure involved 5 primary steps: weighing the ingredients to a predetermined amount, dissolving and thoroughly mixing them, adjusting the pH, and filtering the medium for sterilization before inoculating it with LAB (Figure 1). Most components were prepared as concentrated stock solutions and stored at −20 °C, except for $FeSO_4 \cdot 7H_2O$ and the 4 ribonucleotides (adenylic acid, thymidylic acid, guanylic acid, and uridylic acid), which were freshly prepared prior to each use. Additionally, D-(+)-glucose, polyoxyethylene sorbitan monooleate, ammonium citrate dibasic, sodium acetate, magnesium sulfate, manganese (II) sulfate, and dipotassium hydrogen phosphate were mixed, autoclaved, and stored at 4 °C. Water-insoluble AAs, vitamins, minerals, and nucleotides were dissolved in alkaline (5N NaOH) solutions. All stock solutions were prepared with distilled water unless specified otherwise. The artificial medium was assembled by sequentially adding the following solutions: AAs, vitamins and minerals, the autoclaved mixture, ribonucleotides, and distilled water. The pH was adjusted to 6.0 ± 0.05, consistent with freshly prepared MRS broth, using 6N HCl and 5N NaOH, followed by the addition of an unstable component. Finally, the medium was sterilized using a 0.2 μm syringe-driven filter (No. 95463-DEU-1, Minisart Syringe Filter, Sartorius Stedim Biotech, Goettingen, Germany). The complete artificial media were prepared immediately prior to use. A series of nutrient deprivation and supplementation experiments were conducted for individual nutrients, and nutrient groups were systematically excluded or supplemented in the artificial medium.

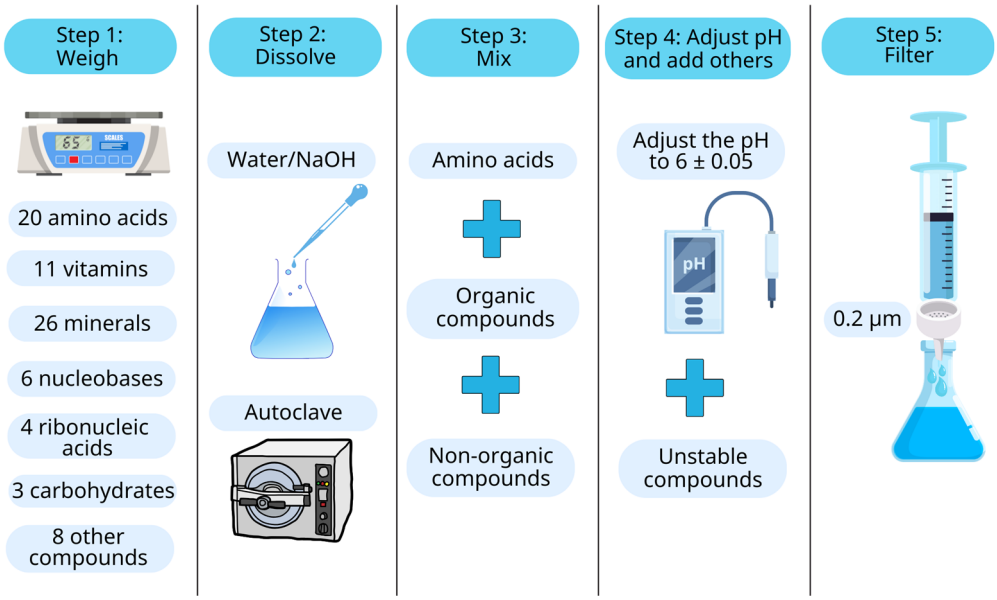

Figure 1. Flow chart of the artificial medium production process. Figure created using Canva; www.canva.com (accessed on 2 December 2024).

2.3. Measurement of LAB Strain Growth Curves

All strains were streaked from freezer stocks and incubated in MRS broth for 24 h at 37 °C. After growth, bacterial cells were harvested by centrifugation (12,000× *g*, 3 min) and washed twice with phosphate-buffered saline (PBS). The cell suspension was adjusted to an OD600 of 0.1 ± 0.05 in PBS using an Eppendorf BioSpectrometer (Model No. 6135 000, Eppendorf AG, Hamburg, Germany), and 100 µL of the bacterial solution was inoculated into 8 mL of artificial medium contained in a 15 mL conical tube. The artificial medium was incubated at 37 °C without agitation and in triplicate, with MRS broth serving as the control. Growth curves were assessed using a 96-well microtiter plate by transferring 200 µL of the artificial medium from the 15 mL conical tube, with turbidity measurements at 570 nm taken using the iMark Microplate Reader (BIO-RAD Laboratories, Tokyo, Japan).

2.4. Measurement of Amino Acid Concentrations

Samples were collected before and after 48 h of culturing by removing 1 mL aliquots from the artificial cultures. The aliquots were centrifuged to a supernatant of medium and pellets of cells; after removal of the supernatant, the cell pellets were washed with 5% mannitol and centrifuged again to re-pellet the cells. Both the cell pellets and the medium supernatant were stored at −20 °C until further analysis. AAs were extracted as previously reported, and AA concentrations were determined via capillary electrophoresis–mass spectrometry (CE-MS), according to established methodologies described in prior studies [23,24]. Intracellular AA concentrations were adjusted by colony number in culture medium samples.

2.5. Data Analysis

All experiments were conducted in triplicate, and the data are presented as the mean ± standard deviation (SD) from three independent replicates. For comparisons between 2 groups, statistical significance was assessed using Student's *t*-test, with a significance threshold of $p < 0.05$. For analyses involving more than 2 groups, a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post hoc test was applied. Statistical analyses were performed using GraphPad Prism 10.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Development of an Artificial Medium for LAB Strain Growth

To investigate the de novo synthesis capabilities of EAAs from NEAAs in LAB, we developed an artificial medium utilizing 78 components, including the 20 proteinogenic AAs, based on previous research [25–28] and the composition of MRS broth (Table S1). All investigated strains except for *Lactobacillus delbrueckii* (ATCC 11842) grew in the artificial medium, and they exhibited differential growth dynamics (Figure 2). Among these, *L. plantarum* consistently exhibited the highest growth rate (Figure 2A). Additionally, the growth rate of *L. plantarum* in the artificial medium appeared nearly equivalent to that observed in MRS broth at both the 24 h and 48 h time points, suggesting that the medium adequately supports the nutritional needs of this strain. Collectively, our artificial medium supported the growth of five LAB strains, and we used it for making a multi-deficiency EAA medium thereafter in this study.

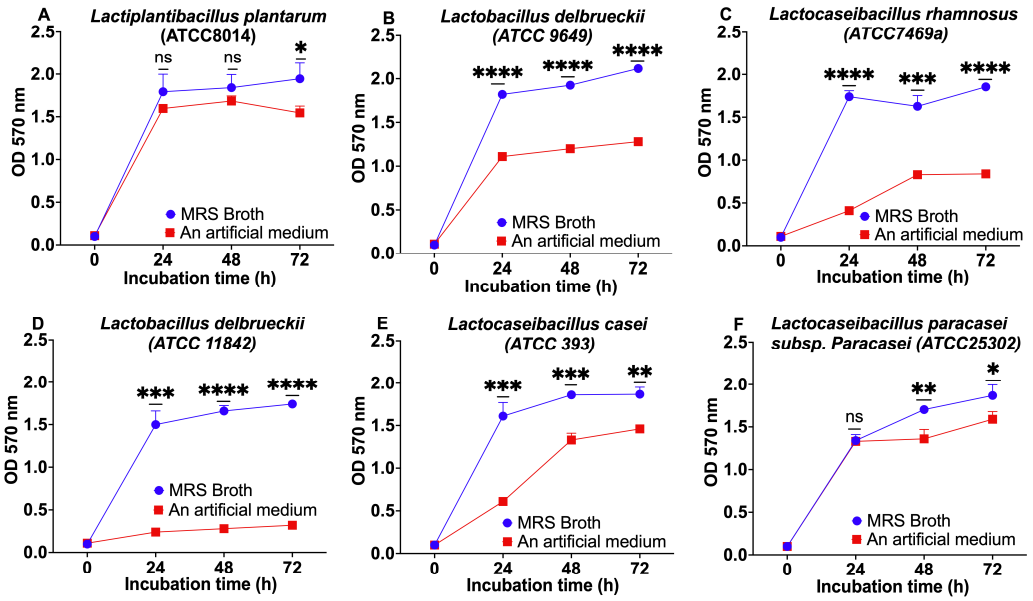

Figure 2. Growth curve analyses of 6 strains at 37 °C in MRS broth and artificial medium. Figure 2 compares the growth curves of 6 LAB strains cultured in MRS broth (blue circles) and a complete artificial medium (red squares) over 72 h of incubation. The horizontal axis represents time (hours, h), while the vertical axis depicts bacterial growth, measured as optical density (OD) at 570 nm (OD 570 nm). Panels (**A**–**F**) illustrate growth patterns for the following LAB strains: (**A**) *Lactiplantibacillus plantarum* (ATCC 8014), (**B**) *Lactobacillus delbrueckii* (ATCC 9649), (**C**) *Lactocaseibacillus rhamnosus* (ATCC 7469a), (**D**) *Lactobacillus delbrueckii* (ATCC 11842), (**E**) Lactocaseibacillus casei (ATCC 393), and (**F**) *Lactocaseibacillus paracasei* subsp. *Paracasei* (ATCC 25302). All of the experiments were repeated in triplicate. Significance was calculated by unpaired Student's *t*-test; error bars indicate standard deviation; ^{ns} (not significant) for $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2. Determination of EAAs Required for the Growth of LAB Strains

To explore the potential for EAA production, we conducted subsequent assays in media devoid of single and grouped EAAs to determine which EAAs are indispensable for the growth of these LAB strains. The exclusion of individual AAs identified leucine, isoleucine, phenylalanine, tryptophan, and valine as indispensable for the growth of all strains tested (Figure S1). For the remaining four EAAs, growth responses varied depending on the AA omitted (Figure 3A–D). Notably, while the absence of histidine, lysine, and threonine affected growth to varying degrees across the strains, the omission of methionine resulted in a significant reduction in the specific growth rate across all strains. *L. plantarum* was the only strain to exhibit growth (i.e., 66.8% of the OD 570 value of the complete

artificial medium containing 20 proteinogenic AAs) after 72 h in methionine-deficient medium (Figure 3B). Moreover, methionine deprivation significantly prolonged the lag phase, indicating that the cells required additional time to adapt to methionine depletion. In contrast, threonine and lysine omission had a relatively minor impact on growth in five strains, suggesting that environmental threonine and lysine are not strictly essential for their growth (Figure 3C,D).

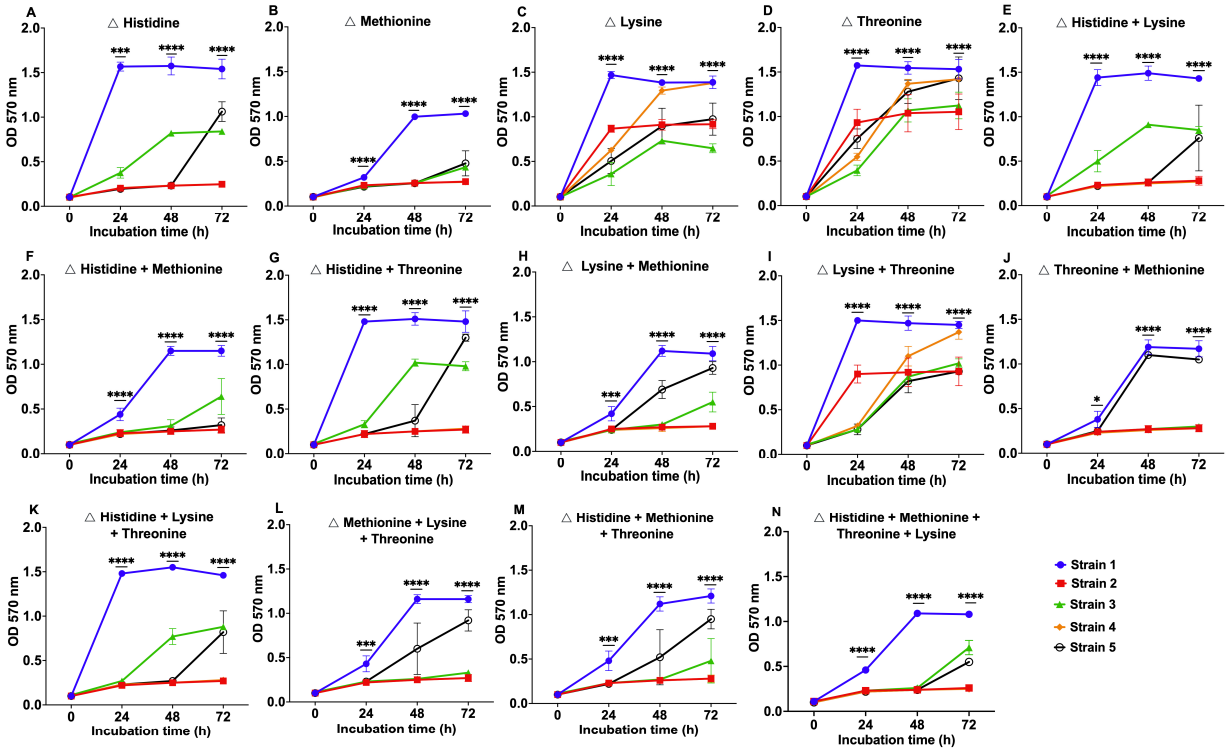

Figure 3. The effect of single, double, triple, and quadruple EAA omission on the growth of LAB strains in an artificial medium. Changes in OD 570 nm of each bacteria strain in media with single (**A**–**D**), double (**E**–**J**), triple (**K**–**M**), and quadruple (**N**) EAA omissions for culturing over the indicated time. Strain 1: *Lactiplantibacillus plantarum* (ATCC 8014); Strain 2: *Lactobacillus delbrueckii* (ATCC 9649); Strain 3: *Lactocaseibacillus rhamnosus* (ATCC 7469a); Strain 4: *Lactocaseibacillus casei* (ATCC 393); Strain 5: *Lactocaseibacillus paracasei* subsp. *Paracasei* (ATCC 25302). The symbol Δ indicates that a specific EAA was omitted from the artificial medium. Error bars show the SD. Data represent the mean ± SD from 3 independent experiments performed in triplicate. Statistical significance was assessed using a one-way ANOVA followed by Tukey's multiple comparisons test, with the following significance thresholds: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Distinct from previous studies that focused solely on single AA omissions [29,30], we expanded our analysis by systematically eliminating combinations of methionine, lysine, threonine, and histidine—AAs that were not deemed absolutely essential for growth. In general, the removal of two or more of those three EAAs did not affect the growth rate across all strains. The combination of EAA exclusions revealed two distinct growth patterns: a methionine-deficient trend and a methionine-present trend (Figure 3E–N). In methionine-deprived media, strains consistently showed lower growth rates and an extended lag phase compared with conditions where methionine was available, suggesting that methionine plays a pivotal role in promoting efficient and timely growth, particularly under nutrient-limited circumstances. Notably, among the five strains tested, *L. plantarum* exhibited the highest growth rate across all EAA-deficient medium conditions. These findings further demonstrate that methionine, lysine, threonine, and histidine are not essential for the

survival and growth of *L. plantarum*, and we therefore used this strain in subsequent experimental assays.

3.3. Assessment of the Ability of L. plantarum to Synthesize Excluded EAAs in an Artificial Medium

While AAs are essential for bacterial growth, elevated concentrations of certain AAs, such as glutamine and serine, can affect the metabolism of other AAs and may exert inhibitory effects [31,32]. Next, we investigated the effects of removing serine and glutamine from an artificial medium on the growth and production of EAAs by *L. plantarum* under conditions of limited EAA availability. *L. plantarum* exhibited a slightly greater growth rate in the medium lacking serine and glutamine compared with the control medium at 48 h (Figure 4A). To explore the ability of *L. plantarum* to biosynthesize EAAs under nutrient-limited conditions, we utilized a medium lacking six AAs for AA profiling. Our analysis revealed a significant increase in the extracellular concentrations of lysine, threonine, and histidine after 48 h, with methionine being the sole exception (Figure 4B–H). Lysine exhibited the highest concentration, followed by threonine, while histidine was present in minimal amounts. To rigorously validate these findings and eliminate potential interference from intracellular residual AAs originating from MRS broth, we repeated the experiment with a refined experimental design. In this iteration, cell pellets of *L. plantarum* were harvested after 48 h of culture in the artificial medium devoid of the six AAs, rather than from MRS broth, and were then reintroduced into the same AA-excluded medium for continued culturing. Viewed from this angle, this approach ensured that both the intracellular and extracellular AA pools accurately reflected the nutrient-limited environment, enabling a more precise evaluation of the strain's intrinsic capabilities for AA synthesis and secretion. In the results, the growth curve and the capacity of *L. plantarum* to produce the excluded AAs mirrored the previously observed outcomes (Figure S2). Similarly, normalizing the extracellular concentrations of excluded AAs to the colony count (Figure S3) yielded results comparable to those normalized to the culture medium volume (Figure 4C–H). Collectively, our findings confirm that *L. plantarum* is capable of synthesizing and secreting EAAs from another component into the extracellular environment.

3.4. Elucidation of Aspartic Acid-Derived Lysine and Threonine Biosynthesis in L. plantarum

To elucidate the precursors involved in the biosynthesis of lysine and threonine, we hypothesized that *L. plantarum* utilizes aspartic acid as a key substrate. This hypothesis is supported by a genome study that demonstrated that bacteria have a catalog of genes related to the synthesis of these EAAs from aspartic acid [17]. Moreover, the observed metabolic activity in *L. plantarum* aligns with this pathway, as our data indicate that the aspartic acid consumption rate was one of the highest among the proteinogenic NEAAs (Figure 4B). In fact, the omission of aspartic acid reduced the growth of *L. plantarum* relative to the control group (Figure 5A). The absence of aspartic acid resulted in a significant reduction in lysine and threonine secretion after 48 h of incubation (Figure 5B). A comparable trend was observed intracellularly; however, threonine levels in the medium containing aspartic acid were not significantly higher than those in the deficient group (Figure 5C). Furthermore, no statistically significant differences were observed between the two groups regarding the levels of other AAs, either extracellularly or intracellularly, at the 48 h mark, with the exception of asparagine and aspartic acid (Figure 5B–E). Notably, there was a slight increase in the aspartic acid concentration at 48 h in the aspartic acid-depleted medium (Figure 5D), which may explain the observed production of lysine (Figure 5B). In summary, our study provides robust evidence that aspartic acid is a crucial substrate for the production and secretion of lysine and threonine in growing *L. plantarum*.

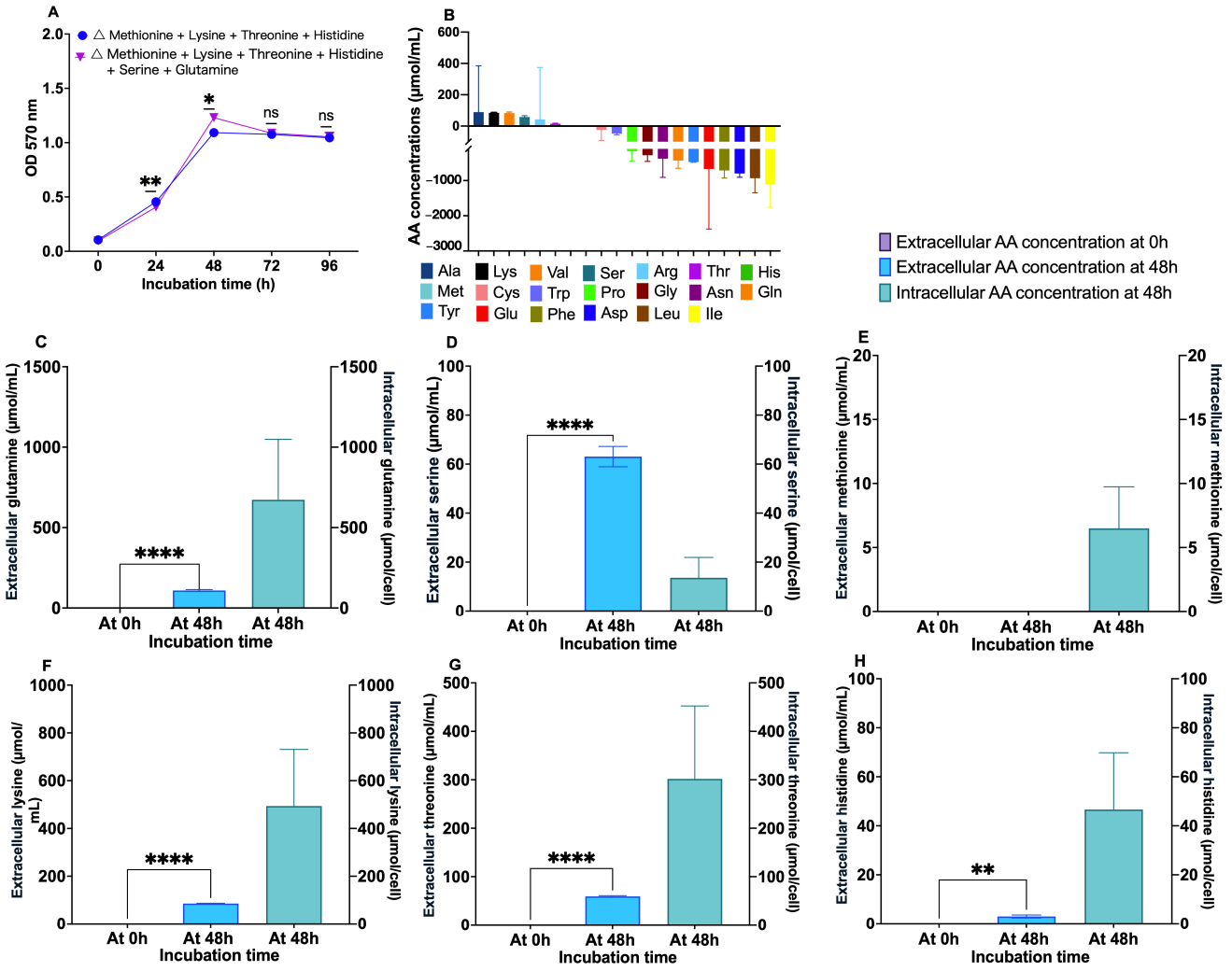

Figure 4. The effect of AA deprivation on growth and intracellular and extracellular AA concentrations in *L. plantarum*. (**A**) Growth curves of *L. plantarum* in 4 EAA-deficient artificial media (Δ methionine, Δ lysine, Δ threonine, and Δ histidine), with and without serine and glutamine. (**B**) Changes in the AA concentrations before and after 48 h cultivation of *L. plantarum* in the medium simultaneously lacking methionine, lysine, threonine, histidine, serine, and glutamine. (**C**–**H**) A quantitative comparison of the extracellular concentrations of AAs at 0 h (prior to inoculation with *L. plantarum*) and 48 h post-inoculation, alongside the analysis of intracellular concentrations of AAs at 48 h. Extracellular AA concentrations were normalized to the volume of the culture medium, while intracellular AA concentrations were normalized to the colony count in culture medium samples. Error bars show the SD. All of the experiments were repeated in triplicate. Significance was calculated by unpaired Student's *t*-test; ^{ns} (not significant) for $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Ala (alanine), Arg (arginine), Asn (asparagine), Asp (aspartic acid), Cys (cysteine), Gln (glutamine), Glu (glutamic acid), Gly (glycine), His (histidine), Ile (isoleucine), Leu (leucine), Lys (lysine), Met (methionine), Phe (phenylalanine), Pro (proline), Ser (serine), Thr (threonine), Trp (tryptophan), Tyr (tyrosine), Val (valine).

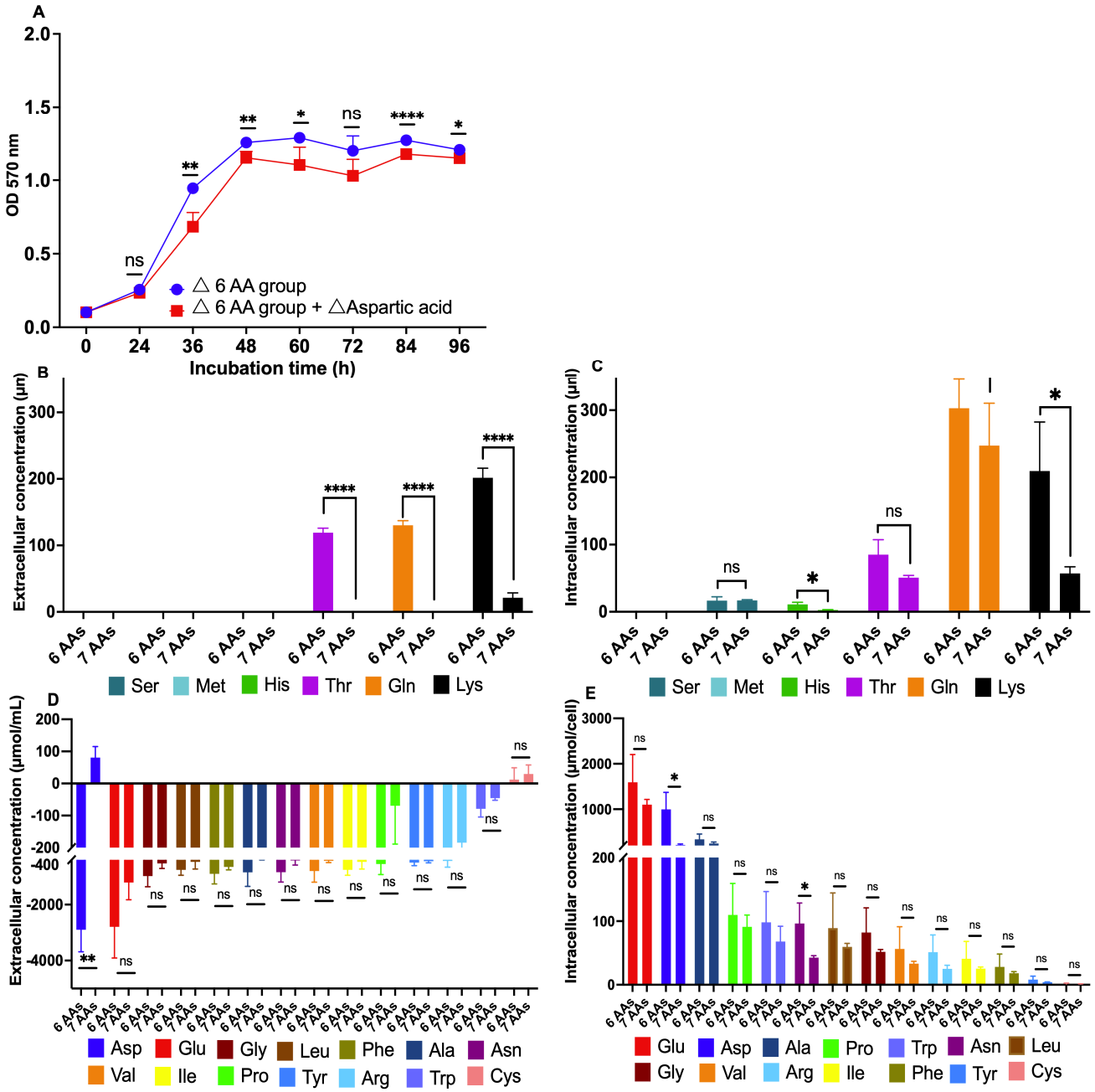

Figure 5. Effect of aspartic acid deprivation on growth and AA synthesis in L. plantarum. (**A**) Growth curve of L. plantarum with or without aspartic acid in medium simultaneously lacking methionine, lysine, threonine, histidine, serine, and glutamine (AA composition in the medium is shown in Figure 4B). The symbol Δ indicates that a specific compound was omitted from the artificial medium. (**B**,**C**) Comparative analysis of extracellular AA (**B**) and intracellular AA concentrations (**C**) in 2 preparations of deficient artificial media, with (lacking 6 AAs) and without aspartic acid (lacking 7 AAs), at 48 h post-culture. Specifically, this measured the concentrations of the 6 AAs missing from the media. (**D**,**E**) Changes before and after 48 h cultivation of L. plantarum in the extracellular AA (**D**) and intracellular AA concentrations (**E**) between 2 deficient artificial media with (lacking 6 AAs) and without aspartic acid (lacking 7 AAs) at 48 h post-culture. Significance was calculated by unpaired Student's *t*-test; error bar indicates the SD; ^{ns} (not significant) for $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

3.5. Vitamin B6 Modulates the Conversion of Cysteine to Methionine in L. plantarum to Facilitate Adaptation to a Methionine-Deficient Synthetic Medium

Methionine is an EAA universally required for the initiation of protein synthesis in all known organisms. Although methionine was undetectable extracellularly (Figures 4E and 5B) and appeared at very low intracellular levels as determined by CE-MS (Figure 4E), we hypothesized that *L. plantarum* synthesizes methionine from cysteine via a series of enzymatic reactions, producing levels minimally sufficient to support cellular survival and proliferation. To elucidate the regulatory mechanisms governing methionine biosynthesis in *L. plantarum*, we initially excluded both methionine and cysteine from the artificial media, which led to a complete inhibition of *L. plantarum* growth (Figure 6A). Previous studies have indicated that vitamin B6 is a critical coenzyme for transsulfuration pathways [33,34]. To further investigate its role, we excluded the vitamin B6 components from the medium. While vitamin B6 deficiency did not affect the growth of *L. plantarum* in a medium containing all 20 proteinogenic AAs, it significantly impaired proliferation under methionine-deprived conditions, even in the presence of cysteine (Figure 6B). In media lacking methionine but supplemented with homocysteine (24 mg/100 mL), which serves as a precursor to methionine, *L. plantarum* demonstrated significantly enhanced growth compared to media without homocysteine supplementation (Figure 6C). Moreover, in the medium deficient in both methionine and cysteine, the addition of homocysteine (24 mg/100 mL) partially alleviated the growth impairment of *L. plantarum* under vitamin B6-deficient conditions (Figure 6D). Notably, increasing the concentration of homocysteine to 96 mg/100 mL fully restored the growth capacity of *L. plantarum* to levels observed in a methionine-deficient medium (Figure 6D). In concordance with these findings, cysteine accumulation was observed, while methionine was undetectable in the medium deprived of both cysteine and methionine, following supplementation with homocysteine (96 mg/100 mL) after 48 h of culture (Figure 6E,F). These results suggest that both cysteine and methionine are essential nutrients for *L. plantarum* optimal growth. This is likely attributable to the role of cysteine in the formation of disulfide bonds, which are crucial for proper protein folding, structural stability, and functional activity [35].

Collectively, our findings indicate that *L. plantarum* may possess both remethylation and transsulfuration pathways, enabling the conversion of cysteine to methionine and homocysteine to cysteine (Figure 6G). Furthermore, these results suggest that *L. plantarum* demonstrates a limited ability to synthesize methionine at minimal levels sufficient for survival in methionine-deprived environments.

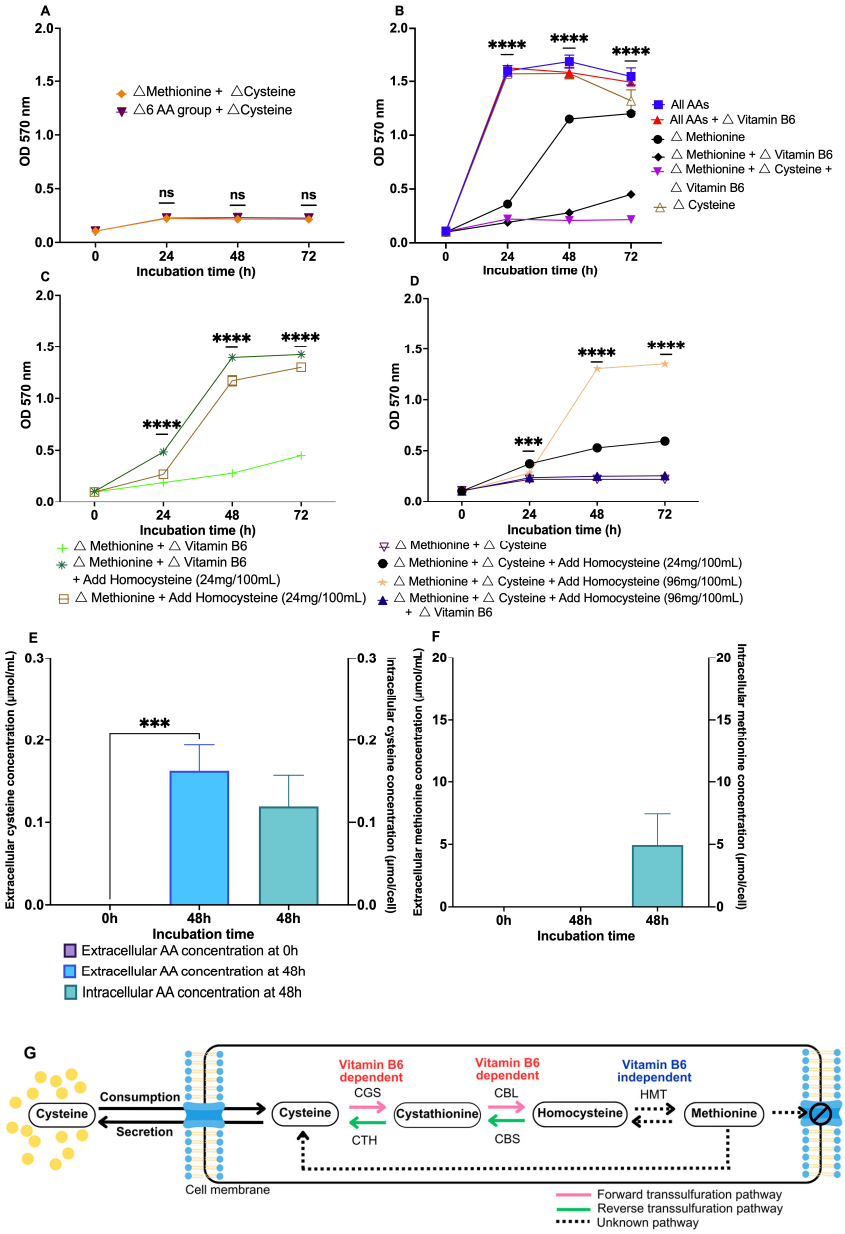

Figure 6. Characterization of vitamin B6-dependent methionine synthesis in *L. plantarum* under EAA-deficient conditions. (**A**) Effect of cysteine and methionine exclusion on growth of *L. plantarum*. 6AA group: methionine, lysine, threonine, histidine, serine, and glutamine deprivation. (**B**) Effect of vitamin B6 exclusion on growth of *L. plantarum* with or without methionine and cysteine. (**C**) Effect of homocysteine supplementation on growth of *L. plantarum* in each methionine-deprived conditioned medium. (**D**) Effect of homocysteine supplementation on growth of *L. plantarum* in each methionine- and cysteine-deprived conditioned medium. (**E**,**F**) Quantification of extracellular and intracellular cysteine (**E**) and methionine (**F**) levels in a medium depleted of methionine and cysteine, supplemented with 96 mg/100 mL homocysteine at both 0 h and 48 h post-culture. (**G**) Proposed pathway for the conversion of cysteine to methionine by *L. plantarum* to adapt to EAA-deficient conditions. Data represent 3 independent experiments. This figure was created using Canva; www.canva.com (accessed on 4 December 2024). The symbol Δ indicates that a compound was omitted from the artificial medium. Error bars show the SD. Significance was calculated by unpaired Student's *t*-test between 2 groups and one-way ANOVA followed by Tukey's multiple comparisons test for more than 3 groups, with the following significance thresholds: ^{ns} (not significant) for $p > 0.05$, *** $p < 0.001$, **** $p < 0.0001$. CGS: cystathionine gamma-synthase (metB, metI); CBL: cystathionine beta-lyase (patB); CBS: cystathionine beta-synthase; CTH: cystathionine gamma-lyase; HMT: 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (metE).

4. Discussion

The gut microbiota is recognized for its ability to synthesize EAAs [17], although additional evidence is needed to better understand this process. Our study focused on examining the EAA biosynthetic capacity of *L. plantarum* and the role of substrates and cofactors in regulating multi-EAA deficiency responses. Our findings indicate that *L. plantarum* can survive in an artificial medium devoid of all of the following EAAs: lysine, threonine, histidine, and methionine. In response to this deprivation, the strain appears to convert cysteine into methionine via the transsulfuration pathway, with vitamin B6 serving as a crucial cofactor in facilitating this metabolic conversion. Additionally, we demonstrated that the strain synthesizes lysine and threonine from aspartic acid when cultured in a medium deficient in these EAAs. These results suggest that *L. plantarum* may serve as a supplier of lysine and threonine within the gut microbiota.

Our initial aim was to elucidate the capacity of *L. plantarum* to synthesize EAAs de novo in a multi-EAA-deficient medium. By analyzing lysine and threonine concentrations at 48 h post-inoculation, we verified that *L. plantarum* can convert aspartic acid into EAAs, achieving production levels estimated at 12.5 mg/mL for lysine and 7.06 mg/mL for threonine in 3.27×10^8 CFU/mL (Figure 3F,G). This biosynthetic capability is consistent with previous findings, although EAA production levels by LAB vary due to influences from strain-specific genetic pathways, culture conditions, and the methods used for quantitative AA analysis. For example, studies employing whole cow's milk as a culture medium indicated that *Lactobacillus bulgaricus* 2-11 released 5.44 mg·(100 g medium)$^{-1}$/24 h of lysine and 1.26 mg (100 g medium)$^{-1}$/24 h of threonine [36]. In skim milk medium, *Lactococcus lactis* C15 did not produce lysine but synthesized 0.46 mg·(100 g medium)$^{-1}$/21 h of threonine, and *Lactobacillus helveticus* MP12 released 0.64 mg·(100 g medium)$^{-1}$/21 h of lysine without threonine production [37]. Furthermore, stable-isotope combined $^{15}N_2$ urea and $^{15}NH_4Cl$ tracing studies highlight the substantial role of gut microbiota in augmenting host EAA pools, with microbial lysine contributions estimated at between 11 and 130 mg/kg/day in humans [38] and 21.3 mg/kg/day in conventional rats [39]. Similarly, microbial threonine contributions in humans range from 21 to 44.7 mg/kg/day [40], with microbial synthesis accounting for an estimated 17% of muscle threonine levels in mice [41]. Viewed from the perspective that microbially derived EAAs contribute to the host AA homeostasis as a nutritional resource, this may partly elucidate how *Lactiplantibacillus plantarum* WJL supports muscle mass accrual in undernourished infant mice [42] and how *Lactobacillus plantarum* TWK10 facilitates significant muscle mass enhancement in healthy humans [43]. In general, existing evidence supports the role of microbial communities in the production and absorption of EAAs in both humans and animals; however, the specific biological impact of intestinal microbial AA synthesis on meeting host metabolic EAA demands remains an open question. Metabolic activities and interspecies interactions within complex natural ecosystems such as auxotrophy and cross-feeding have been observed in the human gut microbiome and in vitro studies [44–46]. Moreover, each gut commensal strain displays competition among bacteria with overlapping nutrient requirements [47]. Consequently, further research is warranted to investigate the capacity of *L. plantarum* to supply EAAs to the host from within the gut microbiome, providing microbiome-targeted therapeutic interventions for conditions related to inadequate protein intake.

In contrast to DNA-based estimation techniques of bacterial phenotyping, our approach directly quantifies the production of specific EAAs. In our study, *L. plantarum* showed a marked prolongation of the lag phase in the methionine-deficient artificial medium even though it may have three genes in the transsulfuration pathway (Figures 3 and 6). The results from the vitamin B6-deprived medium also support that vitamin B6-dependent enzymes, such as cystathionine γ-synthase (metB or metI) and cys-

tathionine β-lyase (patB), could work in this pathway [17]. Moreover, the homocysteine supplementation experiment suggests that the growth retardation of *L. plantarum* in methionine depletion results from a lower capacity of homocysteine methyltransferase (metE) activity than that required for normal growth. Our CE-MS analysis further showed that the markedly lower intracellular methionine concentration in *L. plantarum* compared to its extracellular level in the methionine-supplemented medium (Figure S4) indicates potential inefficiencies in methionine transport, retention, or metabolic processing, which may limit the ability of *L. plantarum* to effectively convert methionine into cysteine. Collectively, studies using our EAA-deprived artificial media can add information on the activity of metabolic enzymes found in genomic sequence-based studies.

Our study also has several limitations. First, a key limitation of our approach is its dependence on the bacteria's ability to survive and grow in an artificial medium. For those species that require more tailored conditions, additional adjustments to the medium components and culture parameters may be necessary. Second, the capability for EAA production appears to be specific to *L. plantarum*, although further investigation across additional strains is necessary to substantiate our observation. Finally, the construction of predicted de novo pathways for the conversion of cysteine to methionine remains constrained. Although predictions for several single-step reactions have been established, the design of complete synthetic pathways, such as those necessary for cystathionine biosynthesis, has not yet been fully elucidated. Additionally, the specific genes involved in each step of substrate or EAA biosynthesis in *L. plantarum* have yet to be identified. Therefore, further studies are needed to identify the key genes necessary to complete the NEAA-to-EAA biosynthesis pathways in *L. plantarum*.

5. Conclusions

In summary, our findings demonstrate that *L. plantarum* possesses the metabolic capability to synthesize lysine and threonine from aspartic acid under conditions of deficiencies in those AAs. In response to methionine-deprived conditions, where methionine levels are insufficient to support the growth of bacteria, vitamin B6 appears to function as a coenzyme, catalyzing the biosynthesis of methionine from cysteine via the transsulfuration pathway. To further substantiate these findings, additional preclinical research is required to validate the EAA production capacity of gut microbiota in multi-strain polyculture in vitro studies and animal models, and to elucidate the mechanistic pathways by which this function contributes to host metabolic homeostasis. We propose that advancing our understanding of microbial functions related to the biosynthesis, conversion, and metabolism of EAAs within the gut could pave the way for novel therapeutic strategies targeting disorders associated with deficiencies in AA and protein metabolism.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol5010016/s1, Table S1: Ingredients of artificial medium; Figure S1: The effect of single EAA omission on the growth of LAB strains in an artificial medium; Figure S2: The effect of AA deprivation on growth and intracellular and extracellular AA concentration in *L. plantarum*; Figure S3: The effect of AA deprivation on extracellular AA concentrations in *L. plantarum*; Figure S4: The effect of cysteine and vitamin B6 deprivation on AA concentration in *L. plantarum*.

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Data Availability Statement: The data generated or analyzed during this study are provided in this published article and Supplemental Information. The datasets generated during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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