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Optimization of Chemical Defined Medium for *Lactobacillus brevis* Based on Artificial Intelligence

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Keywords: industrial fermentation | *Lactobacillus brevis* | medium optimization | nutrient requirements | synthetic medium

ABSTRACT: *Lactobacillus brevis* (*L. brevis*) is a bacterium known for its lactic acid production and probiotic properties. However, the limited research on industrial fermentation of *L. brevis* and its low productivity pose challenges. This work aimed to develop a cost-effective synthetic medium as an alternative to a complex medium for its large-scale production. We first investigated the nutrient requirements of *L. brevis* in terms of amino acids, vitamins, and nucleotides using the single nutrient omission method. Artificial intelligence algorithms combined with the Growth Profiler 960 (GP960) were then employed to optimize the concentration in the synthetic medium. The results indicate that *L. brevis* has diverse nutritional demands, including 18 essential amino acids (excluding aspartic acid and glutamine) and specific vitamins (pantothenic acid, nicotinic acid, vitamin B6, riboflavin) and nucleotides. Furthermore, the developed synthetic medium supported equivalent growth of *L. brevis* to that of the complex medium. The specific growth rate of *L. brevis* in synthetic medium could reach 0.297 h^{-1}. The cost of synthetic medium was 57.52% lower than that of fermentation medium. These results provide a solid foundation for further research on industrial fermentation of *L. brevis*.

1 | Introduction

Lactobacillus brevis (L. brevis) is a probiotic microorganism known for its diverse beneficial effects on human health. It significantly contributes to the balance of gut microbiota [1], and possesses notable anti-inflammatory and antibacterial properties [2]. Additionally, *L. brevis* plays a crucial role in maintaining oral health [3] and boosting the body's anti-viral and immune defenses [4]. Due to these benefits, *L. brevis* is widely used in various products, including fermented foods [5], medications [6], and health supplements [7]. However, in the process of industrial production, the key bottleneck of this strain is the high production cost caused by low fermentation efficiency, which seriously restricts its large-scale application prospects [8]. The root cause is that although the complex medium (such as the formula containing yeast extract and peptone) used in the existing fermentation process can maintain a high number of viable bacteria [9], its composition complexity and batch differences directly lead to poor control of the fermentation process and unstable product quality [10]. Therefore, it is necessary to develop a synthetic medium with low cost, clear composition, and suitable for industrial scale-up.

Abbreviations: GP960, Growth Profiler 960; L. brevis, *Lactobacillus brevis*; LAB, lactic acid bacteria; RAMOS, Respiration Activity MOnitoring System.

Summary

- Determine the requirements of *Lactobacillus brevis* growth for various types of amino acids, vitamins, and nucleotides.
- A combination of artificial intelligence algorithms and Growth Profiler 960 (GP960) equipment is used to optimize the medium at high throughput.
- No restrictions are placed on the factors and levels of medium optimization.
- Compared to complex medium, the components of the synthetic medium for *Lactobacillus brevis* are clearer and less expensive.

Synthetic medium is composed of pure chemicals in precisely known proportions [11]. This synthetic medium can be easily expanded for industrial use as a reliable alternative to composite medium. However, *L. brevis* has a high demand for some specific amino acids, vitamins, and other nutrients. Existing synthetic media are based on formulations like the lactic acid bacteria (LAB) synthetic medium [12, 13]. It cannot fully meet the specific nutritional requirements of *L. brevis*. The yield of viable bacteria was significantly lower than that of complex medium. Therefore, there is an urgent need to develop an optimized synthetic medium tailored for *L. brevis*.

Currently, the response surface methodology (RSM) is the primary method for optimizing fermentation medium. Qin [14] studied the effect of the concentration of maltose, yeast extract powder, and dipotassium hydrogen phosphate on fermentation by Box-Behnken test with three factors and three levels, and determined the optimal concentration combination by RSM. Zhang [15] used the same method to optimize the addition amount of glucose, distiller's lees, and yeast extract powder in LAB fermentation medium. When a study involves a limited number of variable factors, the RSM is widely adopted due to its simplicity and high efficiency. Standard RSM models usually fit quadratic polynomials with data obtained in experiments. However, there are limitations in the face of the complexity of microbial non-linear metabolic processes. In particular, it is difficult to establish an accurate model under multi-variable conditions [16]. Therefore, in order to overcome the limitations of RSM, it is necessary to adopt machine learning methods, such as AI-driven experimental design systems. Many new technologies have been successfully developed today that take advantage of big data and artificial intelligence algorithms to provide more accurate models for trial design and optimization. In recent years, medium optimization methods based on artificial neural networks (ANN) have gained traction. Wang et al. [17] demonstrated that using ANN to optimize the *Bacillus subtilis* fermentation medium for *L*-asparaginase production resulted in a 90.9% improvement. Sun et al. [18] successfully predicted the interaction between different strains in LAB starter culture through a high-precision semi-supervised learning method, providing a new strategy for the efficient development of starter culture. Bayer et al. [19] developed a hybrid model that can be used to describe the fermentation process and integrated the neural network model with the first-principle-based dynamic model to characterize the dynamic characteristics of the fermentation process. Artificial intelligence can identify complex non-linear relationships and patterns, facilitating the development of more precise medium formulations to enhance microbial growth rates and yields. However, it remains unknown whether AI-based optimization methods can effectively optimize *L. brevis* synthetic medium, which can achieve the goal of replacing complex medium.

This study aims to develop a synthetic medium capable of replacing complex medium for *L. brevis* fermentation. Firstly, single nutrient omission experiments will be conducted to determine the specific amino acid, vitamin, and nucleotide requirements of *L. brevis*. High-throughput equipment and artificial intelligence methods were combined to optimize concentrations of medium components. This approach aims to achieve high-throughput screening from multifactorial and multilevel perspectives, ultimately facilitating the industrial fermentation of *L. brevis*.

2 | Materials and Methods

2.1 | Strains

L. brevis (Provided by the Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, China).

2.2 | Medium

2.2.1 | *L. brevis* Preculture Medium

20 g/L glucose (Sterilized individually and add before inoculation), 10 g/L peptone, 9 g/L yeast extract FM503(Angel Yeast, China), 2 g/L K_2HPO_4, 2 g/L ammonium dihydrogen citrate, 5 g/L sodium acetate trihydrate, 0.58 g/L $MgSO_4{\cdot}7H_2O$, 0.25 g/L $MnSO_4{\cdot}4H_2O$, 1 g/L Tween-80. The medium is sterilized at a temperature of 115°C for 30 min. The agar medium is prepared by incorporating 15 g/L of agar into the above medium.

2.2.2 | *L. brevis* Complex Medium

8 g/L glucose (GLC), 10 g/L fructose (Fru), 7 g/L soy peptone, 13 g/L yeast extract FM503, 17 g/L K_2HPO_4, 2.2 g/L ammonium dihydrogen citrate (ACD), 1.5 g/L sodium acetate trihydrate (SAT), 0.05 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $MnSO_4{\cdot}4H_2O$, 1 g/L Tween-80.

2.2.3 | *L. brevis* Synthetic Medium

The synthetic medium used in this study is derived based on *Lactobacillus plantarum* synthetic medium (LPSM) [20], LAB basal synthetic medium (BM) [13]. The medium composition before and after optimization are detailed in Table 1. Among them, $FeSO_4{\cdot}7H_2O$, amino acids, vitamins, and nucleotides were dissolved in a solution and then filter sterilized with a 0.45 μm filter. Other components were steam sterilized at 115°C for 30 min.

TABLE 1 | The composition of synthetic medium before and after optimization. All amino acid (Ami) and vitamin (Vit) concentrations in the start synthetic medium were from LPSM, and all nucleotide (Nuc) concentrations were from BM.

Constituent	Concentration (g/L)	
	Start synthetic medium	Optimized synthetic medium
Glucose	8	14.5
Fructose	10	7
Sodium acetate trihydrate	5	8
K_2HPO_4	10	13
Ammonium dihydrogen citrate	0.2	1.2
$MgSO_4 \cdot 7H_2O$	0.15	0.45
$MnSO_4 \cdot 4H_2O$	0.02	0.06
$FeSO_4 \cdot 7H_2O$	0.01	0.01
Tween-80	1	1
L-Glycine	0.2	0.32
L-Glutamic acid	0.15	0.24
L-Alanine	0.2	0.32
L-Arginine	0.3	0.48
L-Asparagine	0.2	0.32
L-Aspartic acid	0.2	
L-Cysteine	0.2	0.32
L-Phenylalanine	0.2	0.32
L-Histidine	0.2	0.32
L-Isoleucine	0.2	0.32
L-Leucine	0.3	0.48
L-Lysine monohydrate	0.3	0.48
L-Methionine	0.2	0.32
L-Proline	0.2	0.32
L-Serine	0.2	0.32
L-Tyrosine	0.2	0.32
L-Threonine	0.2	0.32
L-Tryptophan	0.2	0.32
L-Valine	0.3	0.48
p-Aminobenzoic acid	0.01	
Vitamin B12	0.001	
D-biotin	0.01	
Calcium pantothenate	0.001	0.0023
Folic acid	0.001	
Nicotinic acid	0.001	0.0023
Vitamin B6 HCl	0.001	0.0023
Thiamine	0.001	
Riboflavin	0.001	0.0023
Adenine	0.05	0.035
Cytosine	0.05	0.035
Deoxyguanosine	0.05	0.035
Guanine	0.05	0.035
Thymine	0.05	0.035
Uracil	0.05	0.035

2.3 | Growth Conditions

2.3.1 | Preculture

L. brevis—preserved in glycerol tubes at −80°C was defrosted. At 2% inoculation amount, the seed solution with OD_{600} of 0.9 ± 0.11 was inserted into the preculture medium, and then incubated at 37°C for 6 h.

2.3.2 | 96-Well Microplate Culture

The Growth Profiler 960 (GP960, EnzyScreen, Netherlands) is a high-throughput device designed to monitor the real-time growth of bacteria that are cultured in the individual wells of ten 96-well plates. This allows for efficient high-throughput screening tasks. The device utilizes a camera positioned beneath the well plates, capturing images every 20 min. Each image contains RGB (Red, Green, Blue) pixel values, which correspond to the bacterial concentration in the wells. By analyzing the RGB values against a standard curve of bacterial concentration, the system provides real-time growth data for each well. Using the GP960, Sanne [21] successfully performed a detailed analysis of the growth characteristics of yeast. This method eliminates the need for manual sampling and the associated risk of bacterial contamination, ensuring accurate and timely data acquisition. In order to eliminate the interference of background noise, we corrected the RGB data at each point in time. The specific method is to subtract the RGB values of the initial time point (the first time point) from the RGB values of each time point. Using the GP960, researchers can optimize the concentration of various components in the culture medium by monitoring bacterial growth in wells with different medium compositions. Before inoculation, *L. brevis* cells from preculture medium are washed twice with sterile ultrapure water to prevent contamination and ensure accurate results. An inoculum volume of 2% is then used to inoculate the wells. The cultures are incubated at 37°C, shaking at 140 rpm, until the RGB values reach their maximum and cease to increase, indicating that bacterial growth has reached its plateau.

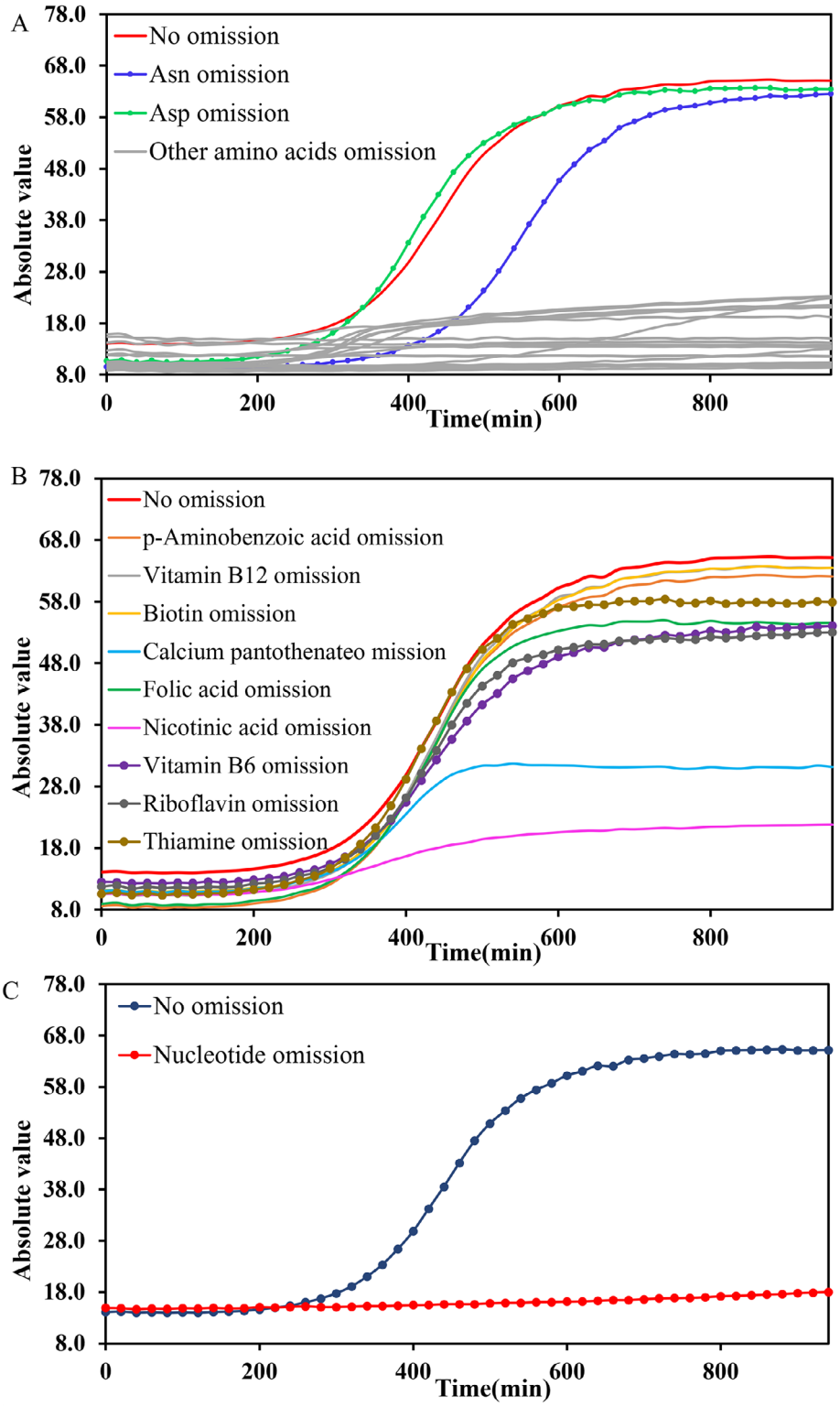

FIGURE 1 | The growth of *L. brevis* in different nutrients omission medium. (A) The growth of *L. brevis* in amino acid omission medium. Asn, Asparagine; Asp, Aspartic acid. (B) The growth of *L. brevis* in vitamin omission medium. (C) The growth of *L. brevis* in nucleotide group omission medium.

2.3.3 | Monitoring Respiration Activity in Flask Culture

RAMOS (Respiration Activity MOnitoring System, HITEC ZANG, Germany) is a device designed for real-time monitoring of respiratory metabolic activity during microbial cultivation. It finds extensive application in fermentation optimization, metabolic engineering, and physiological studies. Janina et al. [10] successfully used RAMOS to optimize the synthetic medium. The core principle of this system involves indirectly quantifying the respiratory intensity of microorganisms by continuously

measuring the transfer rates of oxygen (O_2) and carbon dioxide (CO_2) online. RAMOS has the ability to monitor the respiration activity of microbial cells cultured in the flask, so that the right moment when the highest cell amount can be determined easily by viewing the respiration activity of cells. Seed, washed by sterile ultrapure water, was inoculated into the RAMOS flask with an inoculum volume of 2%. And the culture was maintained at 37°C and a shaking speed of 140 rpm. When the maximum oxygen uptake rate is reached, samples were processed for cell counting.

In summary, the RAMOS and the GP960 are two sets of equipment that operate independently. The GP960 facilitates the selection and optimization of medium components at high throughput via automated optical monitoring. The RAMOS evaluates the metabolic activity of microorganisms in the GP960-optimized medium compared to the conventional medium by monitoring the oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) in real time, which serve as indicators of bacterial growth and decline. The validity of this optimization strategy is confirmed through plate counting analysis.

2.4 | Determination of Essential Amino Acid, Vitamin, and Nucleotide

To determine the essential nutrients that *L. brevis* requires for growth, we used the single nutrient omission method [22]. The growth of *L. brevis* was monitored in individual wells with different medium composition in the GP960. A start synthetic medium (Table 1) without any nutrient omission was used as control, and three groups were identified according to the relative final biomass amount in wells with one single nutrient omission medium compared to the control. For wells that the relative final biomass reaches 0% to 30%, the corresponding missing nutrient is considered essential for the growth of *L. brevis*, while when it reaches 30% to 70%, the corresponding missing nutrient is considered to have a stimulating effect on the growth of *L. brevis*, if it is more than 70% the nutrient is considered unnecessary for growth.

Based on the results of single nutrient omission experiment, nutrient-combined omission experiments were also conducted on vitamin components, among this study it includes vitamin B1, riboflavin, vitamin B6, biotin, folic acid, and vitamin B12. The effects of missing two, three, four and five vitamins from these six vitamins on growth of *L. brevis* were explored.

2.5 | High-Throughput Medium Optimization Based on Artificial Intelligence System

The artificial intelligence system used in this study was an experimental design system (not yet published) that integrated artificial intelligence optimization techniques, including the Random Hyperparameter Search algorithm, Extreme Gradient Boosting (XGBoost) algorithm, and Upper Confidence Bound (UCB) algorithm. The workflow began with constructing an XGBoost model based on individual medium components and their concentration ranges in the initial synthesis medium. The Random Hyperparameter Search algorithm was then employed to train the XGBoost model. This algorithm involved generating n candidate models by randomly sampling from the XGBoost hyperparameter space, which included core parameters such as learning_rate, max_depth, colsample_bytree, and n_estimators. The experimental results from the initial round were partitioned into training and test sets at a 4:1 ratio, followed by systematic training and testing of all XGBoost models. Through performance evaluation, m top-performing model architectures were selected and used to predict the next-round 96 culture medium combinations. Finally, the UCB algorithm was applied to sort the prediction results, and the top 96 culture medium combinations were selected for the subsequent experimental round.

To simplify experimental operations, we categorized all nutrients, except carbon sources and other metal salts and Tween-80, into three groups: amino acids, vitamins, and nucleotides. In the synthetic medium, the initial concentrations of these three groups were set to 1× concentration (The 1× concentration is the middle value). In order to carry out multiple optimizations in the subsequent component concentration optimization process, to reduce the complexity of the experiment.

In the artificial intelligence experimental design system, the start synthetic medium concentrations of 12 components–glucose, fructose, K_2HPO_4, ammonium dihydrogen citrate, sodium acetate trihydrate, $MgSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}4H_2O$, $FeSO_4{\cdot}7H_2O$, Tween-80, amino acid group, vitamin group, and nucleotide group–were set as central levels for medium optimization. Concentrations were designed to increase and decrease sequentially, resulting in multiple concentration levels. By inputting these 12 factors and multiple levels into the system. Ninety-six synthetic medium combinations will be randomly generated in the first round. Combined with the GP960, 96 synthetic medium combinations were performed in a 96-well microplate. The liquid volume in a 96-well microplate cannot exceed 250 μL. In order to avoid component concentration errors caused by too small addition amounts during the sample addition process. The artificial intelligence technology system will use 1 mL as the total volume of the medium to generate 96 groups of addition amounts of each component and 12 groups of mother solution concentrations. The GP960 medium was prepared and inoculated in 96 separate 2 mL centrifuge tubes, and then distributed the inoculated medium into two 96-well microplates to conduct parallel group experiments. Added 200 μL of medium to each well of each microplate. The experimental results were input into the system. Based on the results, the artificial intelligence technology system output new concentrations for each component and the second round of 96 synthetic medium combinations, and then the next experiment is conducted, and so on.

3 | Results and Discussion

3.1 | Determination of Essential Amino Acids for *L. brevis*

During fermentation, microorganisms require amino acids to synthesize proteins necessary for their growth and metabolic activities. To investigate the specific amino acid requirements of *L. brevis*, we conducted single amino acid omission experiments, using a complete medium as the control group. Figure 1A shows

TABLE 2 | Growth of *L. brevis* in amino acid omission medium and its requirement for different types of amino acids.

Omitted amino acid	Absolute value	Amino acid effect	Growth condition
No omission	51.042 ± 0.979		
L-Glycine	NG	E	−2.15%
L-Glutamic acid	NG	E	−4.26%
L-Alanine	NG	E	−0.65%
L-Arginine	5.036 ± 0.236	E	9.87%
L-Histidine	11.097 ± 0.567	E	21.74%
L-Lysine	1.582 ± 0.203	E	3.10%
L-Tyrosine	9.144 ± 0.33	E	17.91%
L-Aspartic acid	52.696 ± 0.400	NE	103.24%
L-Asparagine	53.004 ± 1.248	S	103.85%
L-Cysteine	3.906 ± 0.121	E	7.65%
L-Phenylalanine	12.503 ± 0.204	E	24.50%
L-Isoleucine	13.588 ± 0.06	E	26.62%
L-Leucine	1.503 ± 0.079	E	2.94%
L-Methionine	11.639 ± 0.237	E	22.80%
L-Proline	NG	E	−0.25%
L-Serine	4.22 ± 0.034	E	8.27%
L-Threonine	NG	E	0.92%
L-Tryptophan	13.346 ± 1.239	E	26.15%
L-Valine	NG	E	0.12%

Abbreviations: E, essential; NE, nonessential; NG, no growth; S, stimulatory.

TABLE 3 | Growth of *L. brevis* in vitamin omission medium and its requirement for different type of vitamins.

Omitted vitamin	Absolute value	Vitamin effect	Growth condition
No omission	51.042 ± 0.979		
p-Aminobenzoic acid	53.518 ± 0.454	NE	104.85%
Vitamin B12	52.926 ± 0.177	NE	103.69%
Biotin	52.32 ± 0.413	NE	102.50%
Calcium pantothenate	20.171 ± 0.178	S	39.52%
Folic acid	45.633 ± 0.182	NE	89.40%
Nicotinic acid	11.185 ± 0.109	E	21.91%
Vitamin B6	41.595 ± 0.631	NE	81.49%
Riboflavin	41.243 ± 0.471	NE	80.80%
Thiamine	47.363 ± 0.326	NE	92.79%

growth in medium lacking individual amino acids. Table 2 summarizes the requirements.

L. brevis demonstrated a broad requirement for various amino acids. Only when asparagine or aspartic acid was omitted, the final biomass level of *L. brevis* was comparable to the control group. The absence of aspartic acid didn't affect the bacterial growth rate. However, the lack of asparagine in the synthetic medium led to a decrease in growth rate and an increase of the lag phase, indicating that asparagine has a stimulatory effect on the growth of *L. brevis*. The omission of any other 17 amino acids caused 75% growth reduction or no growth. This indicates that these amino acids are essential for the growth of *L. brevis*.

The lag phase when asparagine is omitted may be due to the weak transport ability of *L. brevis* for aspartic acid. LAB utilize asparagine through a high-affinity transport system [23]. The asparagine ingested by the bacteria is preferentially converted to aspartic acid, which is then transaminated to generate oxaloacetate. And oxaloacetate is ultimately degraded to pyruvate [24]. Although the lack of aspartic acid or asparagine will not affect the final biomass amount of *L. brevis*, both amino acids are precursors for the synthesis of some essential amino acids. Therefore, asparagine needs to be retained in the medium. In summary, we exclude only aspartic acid from the synthetic medium while retaining the other 18 amino acids.

The growth of *L. brevis* is highly dependent on specific amino acids. Similarly, Lin et al. [25] found that the strains of *Li. panis* and *Le. buchneri* had nutritional deficiencies of 11 and 4 amino acids, respectively. Pot et al. [26] studies show that the growth of LAB relies on nutrient-rich environments. This dependency has caused them to lose the ability to synthesize certain nutrients during evolution [27]. The genetic decay results in a substantial loss of carbohydrate metabolism, amino acid, and cofactor biosynthesis, resulting in fussy and varied nutritional requirements [28].

3.2 | Essential Vitamins for *L. brevis*

To determine the essential vitamins for *L. brevis*, a single vitamin omission experiment was conducted. The growth of *L. brevis* was examined in media lacking each of nine individual vitamins (Figure 1B), and the essential vitamins were identified (Table 3). The absence of calcium pantothenate or niacin significantly impacted *L. brevis* growth. Specifically, when nicotinic acid was omitted, the final biomass dropped to 21.91% of the control, indicating its essentiality. Similarly, the absence of calcium pantothenate reduced the final biomass to 39.52% of the control, highlighting its stimulatory effect on *L. brevis* growth. The other seven vitamins seem to be non-essential.

According to the analysis of vitamin requirements of *L. brevis* (Table 3), it could be seen that the bacteria can grow normally in the absence of *p*-aminobenzoic acid, which indicates that *p*-aminobenzoic acid is a non-essential vitamin. However, when calcium pantothenate or nicotinic acid was missing, bacterial growth was significantly inhibited. Based on these observations, we designed a series of combined omissions of the other six vitamins: vitamin B1, riboflavin, vitamin B6, biotin, folic acid, and vitamin B12, in the absence of *p*-aminobenzoic acid and the presence of calcium pantothenate and nicotinic acid (Figure 2).

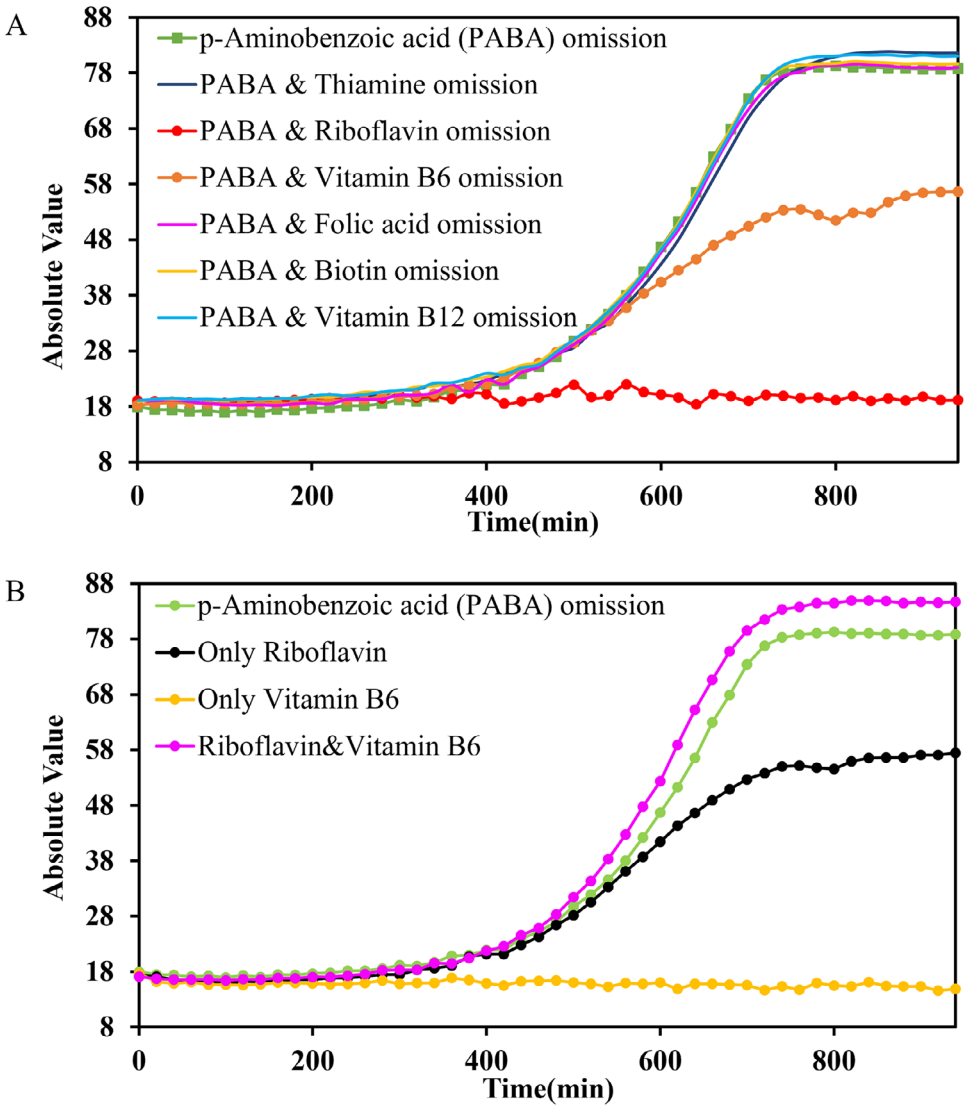

FIGURE 2 | The growth of *L. brevis* in vitamins combined with the omissions of the other six vitamins medium. (A) The growth of *L. brevis* in p-aminobenzoic acid and one of the other six vitamins omission medium. (B) The growth of *L. brevis* in supplemented with vitamin B6 or Riboflavin in the presence of calcium pantothenate and nicotinic acid medium.

Figure 2A shows the results with only single vitamin omitted among the six vitamins. It is observed that *L. brevis* is unable to grow normally when riboflavin is omitted, and it only grows to 63.11% compared to the control when vitamin B6 is omitted. While omission of any of the other four vitamins shows no effect to the final biomass of *L. brevis*. Further investigation shows synergy between riboflavin and B6 on *L. brevis* growth (Figure 2B). The bacteria grow poor with only riboflavin or B6, but grow well with both these two ingredients in the medium. Similar results were observed for *Lactobacillus delbrueckii* [29], as nicotinic acid, calcium pantothenate, and vitamin B6 are necessary for the growth of *L. delbrueckii*, and vitamin B1 and riboflavin have a stimulating effect on growth. During the fermentation process of LAB, vitamin B family acts as a component of coenzymes. They participate in the energy metabolism of LAB, promote substrate metabolism and product production, thereby generating adenosine triphosphate (ATP) energy supply. Nancib et al. [30] studied the joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production. Xue et al. [31] studied the effects of vitamins B_1, riboflavin, pantothenic acid, vitamins B_6 and biotin on the fermentation of lactic acid by *Lactobacillus paracasei*.

L. brevis, a facultative anaerobe, relies on several metabolic pathways for energy generation: oxidative phosphorylation, substrate-level phosphorylation, fermentation, and amino acid decarboxylation. Oxidative phosphorylation, the most efficient ATP production method, involves electron carriers like NAD (nicotinamide adenine dinucleotide), FMN (riboflavin-5-phosphate sodium), and FAD (flavin adenine dinucleotide), transferring electrons through the respiratory chain to oxygen molecules, synthesizing ATP. Nicotinic acid and riboflavin, are precursors to these carriers, playing a pivotal role in electron transfer and the energy production process [32]. Some LAB require external riboflavin supplementation due to limited synthesis capabilities [33]. Thus, adding nicotinic acid and riboflavin to synthetic media can enhance electron transfer, metabolic flux, and growth energy. However, LAB may also rely on substrate-phosphorylation, espe-

TABLE 4 | Growth of *L. brevis* in nucleotide group omission medium.

Omitted nucleotide	Absolute value	Nucleotides effect	Growth condition
No omission	51.042 ± 0.979		
Nucleotides	3.208 ± 0.271	E	6.28%

cially when their electron transport systems are inadequate [34, 35]. Coenzyme A, derived from pantothenic acid, is crucial in substrate-level phosphorylation, pyruvate decarboxylation, and citric acid cycle, further underlining pantothenic acid's importance in LAB energy metabolism and cell membrane formation [36, 37]. Amino acid decarboxylation, facilitated by pyridoxal phosphate (PLP), the active form of vitamin B6, also supports LAB growth by converting amino acids into energy substrates. PLP's role in amino acid metabolism emphasizes the necessity of vitamin B6 for *L. brevis* [38]. In addition, riboflavin and vitamin B6 exhibit a synergistic effect on the growth of *L. brevis*. This synergy may be attributed to the complementarity in coenzyme synthesis and the cross-regulation of metabolic pathways. Specifically, FAD derived from riboflavin is involved in the metabolism of vitamin B6 to PLP. Meanwhile, riboflavin-driven redox reactions are closely associated with vitamin B6-mediated amino acid metabolism.

3.3 | Nucleotides Requirement for *L. brevis*

To determine the effect of nucleotides on *L. brevis* growth, we performed a complete set of nucleotide group omission experiment. Figure 1C shows growth in medium lacking nucleotide group. Table 4 summarizes the requirement. *L. brevis* was unable to grow when nucleotides were missing, indicating that nucleotides are essential nutrients for *L. brevis*. This may be due to the mutational inactivation of pyrR, a gene related to pyrimidine synthesis, resulting in the inability of LAB to synthesize nucleotides by themselves [39]. In summary, during the fermentation process of *L. brevis*, nucleotides need to be added to support its normal growth. In addition, some LAB have additional requirements for deoxyribonucleotides because they lack the ability to reduce ribonucleotides to deoxyribonucleotides. The latter is a key component of DNA synthesis. This characteristic requires that the addition of corresponding deoxynucleotides must be considered when designing synthetic medium to support the normal growth and synthesis of genetic material of these lactic acid strains [40].

Different microorganisms have different nutritional requirements for nucleotides. *Gluconobacter oxydans* was reported to be able to independently synthesize nucleotides during its fermentation process, thus classifying nucleotides as non-essential nutrients [41]. Similarly, the growth and metabolic activities of *Bacillus pumilus* do not require the supplementation of exogenous nucleotides [10]. However, many LAB are unable to synthesize sufficient purines and pyrimidines on their own during growth, so they exhibit auxotrophic deficiencies in nucleotide nutrients. The variation in nucleotide requirements among LAB primarily stems from a combination of genomic characteristics, metabolic capabilities, evolutionary adaptations,

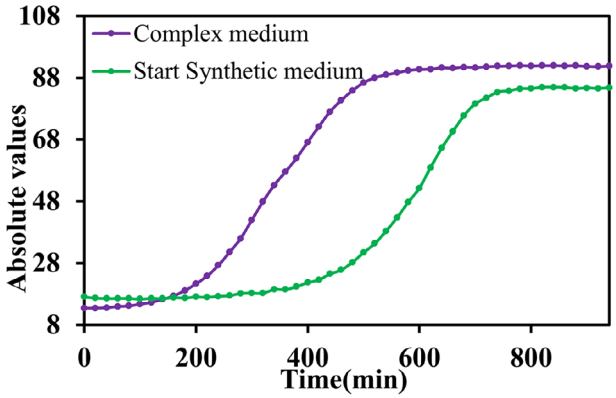

FIGURE 3 | The growth of *L. brevis* in start synthetic and complex medium after nutrient composition has been determined.

and environmental conditions. The evolutionary history and ecological niche differentiation of LAB have significantly shaped their nutritional demands. Many LAB strains, predominantly isolated from fermented foods, have adapted to environments rich in exogenous nucleotides. Consequently, the genomes of these strains have undergone a degree of degeneration, resulting in a reduced capacity for endogenous nucleotide synthesis. However, certain LAB strains thrive in nutrient-poor environments where nucleotides are scarce. In such settings, the ability to synthesize nucleotides autonomously confers a substantial survival advantage, enabling these strains to exhibit enhanced viability and maintain robust growth.

3.4 | Optimization of Synthetic Medium Composition

Based on the above experiments, we determined all required amino acids, vitamins and nucleotides for *L. brevis* growth. However, the concentrations of individual ingredients should be optimized to reach the same results with complex medium. It is shown that with the start synthetic medium of the essential nutrients *L. brevis* can only reach 85.89% of that with complex medium (Figure 3). Therefore, a method combining the artificial intelligence experimental design system with GP960 was used to further optimize the concentration of each component of the synthetic medium.

Using the high-throughput medium optimization system based on artificial intelligence, we did the component concentration optimization using GP960. Twelve components of the synthetic medium and multiple concentration gradients for each component were tested. The 12 components include K_2HPO_4, glucose, fructose, ammonium dihydrogen citrate, sodium acetate trihydrate, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, Tween-80, amino acid group, vitamin group, and nucleotide group (the start concentration is shown in Table 1). Following the iterative steps shown in Section 2.5, the optimized concentration combinations of the synthetic medium were obtained after three rounds of experiments (Figure 4A).

Figure 4A shows the trend in optimization results for the component concentrations of the synthetic medium for *L. brevis*. It is noted that the first round has a wide spread of the final

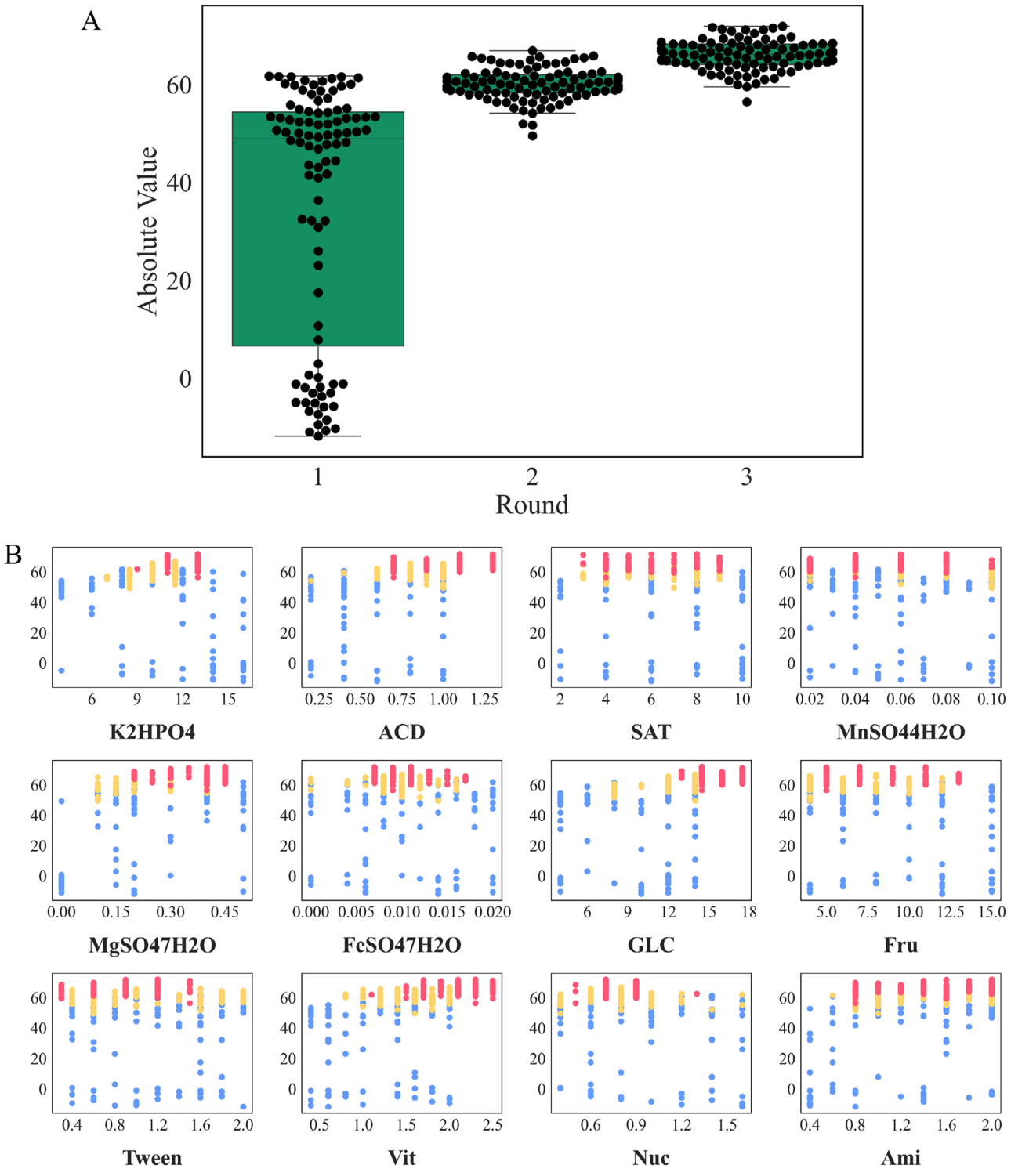

FIGURE 4 | The results are obtained after three rounds of concentration optimization using an artificial intelligence system. (A) Box plot of the iterative optimization process using artificial intelligence models. There are three rounds of iteration, in which each black point represents one experiment with a specific medium composition. The results of each round are depicted as a set of box plots. (B) Concentration distribution diagram of the 12 tested components in synthetic medium for *L. brevis*. Each small graph represented a component, the abscissa of the small graph was the concentration range of the component, and each point represents the result of a specific medium combination. The blue points represent the results of the first round of experiments. The yellow points represent the results of the second round of experiments. The red points represent the results of the third experiments.

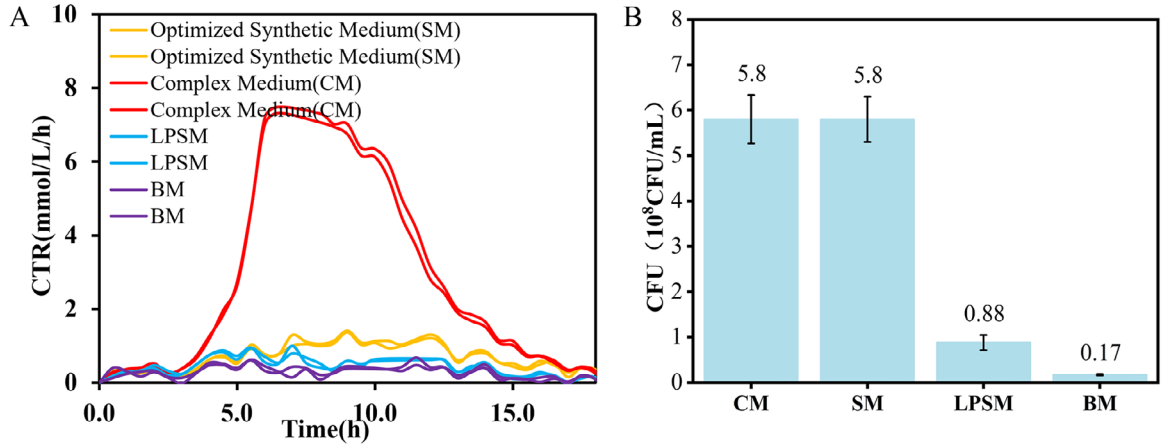

FIGURE 5 | Comparison of *L. brevis* in four medium. SM represents the optimized synthetic medium in this article. CM stands for the complex medium of *L. brevis*. LPSM and BM are the two synthetic medium for *L. brevis* mentioned in Section 2.2.3. (A) The curve of carbon dioxide production during the growth of *L. brevis* in four different medium. (B) The results of plate counting conducted when the decline of *L. brevis* was indicated by the tail gas data.

growth results, as all concentration combinations selected are based on the Random Hyperparameter Search algorithm. As the XGBoost model learn the relationship between the final biomass with individual medium component, the second and third rounds of experiments show narrow final biomass results. The figure indicates that *L. brevis* is unable to grow in certain medium combinations. In-depth analysis found that the concentration of $MgSO_4 \cdot 7H_2O$ was zero in these media, suggesting that Mg^{2+} is essential for the growth of *L. brevis*. In contrast, the absence of $FeSO_4 \cdot 7H_2O$ did not affect the growth of *L. brevis*. This is because *L. brevis* has a low demand for Fe^{2+} in nucleotide-rich medium [42]. After the second round of concentration optimization experiment, it was observed that although the demand for Fe^{2+} by *L. brevis* is low, the presence of Fe^{2+} can lead to enhanced growth. Ultimately, the optimal synthetic medium for *L. brevis* was obtained. Figure 4B displays the concentration distribution diagram of the 12 components. The final optimized synthetic medium is presented in Table 1. The specific growth rate can reach 0.297 h^{-1}, calculated based on the growth curve distributed by the profiles of relative biomass values of *L. brevis* in the optimal concentration medium combination. The algorithm is able to capture the non-linear relationship between the biomass concentration and the composition of the medium; at the same time, it also found that the synergistic effect of different nutrients, which can be seen in Figure 4B. It showed that the concentration effects of K_2HPO_4, GLC, and Nuc are relatively independent on other nutrients, as their effects are best in a narrow concentration range, while the best responses of other nutrients are scattered across a wide concentration range, which means there is a strong synergistic effect among these nutrients.

3.5 | Validation of Effect of the Optimized Synthetic Medium

Validation experiments of optimized synthetic medium were conducted in the RAMOS. Proper sampling time for viable cell count is determined by the oxygen uptake profile during the fermentation process in the RAMOS. It is found that the viable cell count reaches its highest value when the oxygen uptake rate reaches the highest. Figure 5A shows the carbon dioxide escape rate profiles of *L. brevis* under different medium. For complex medium, samples were taken at 7 h (when the highest CTR occur), while samples for synthetic medium were taken at 10 h (when the CTR begin to drop). As shown in Figure 5B, the viable cell count of *L. brevis* in the complex medium is 5.80×10^8 CFU/mL, and similarly, the viable cell count in the optimized synthetic medium also reaches 5.80×10^8 CFU/mL. This indicates that the number of viable bacteria in both the optimized synthetic medium and the complex medium was the same, demonstrating that the fermentation of *L. brevis* in synthetic medium had similar efficacy to that in complex medium.

TABLE 5 | Comparison of the cost of synthetic medium and complex medium.

	Medium cost (¥/L)	Viable cell (10^{11}CFU/L)	Average cost (¥/10^{11}CFU)
Complex medium	1.60	5.80	0.276
Optimized Synthetic medium	0.68	5.80	0.117

According to Table 5, the cost per unit of *L. brevis* cells fermented in a complex medium is 0.276 RMB, whereas the cost per unit in the optimized synthetic medium is only 0.117 RMB. This represents a 57.52% reduction in fermentation cost when using the optimized synthetic medium compared to complex medium. The cost of complex medium is higher than that of synthetic medium, primarily attributed to the elevated costs of yeast extract and peptone included in the formulation. Therefore, this study successfully developed a synthetic medium formulation that can replace the complex medium for *L. brevis* fermentation.

LAB, as significant industrial microbial cell factories, have been successfully employed in the production of high-value compounds, antimicrobial agents, sweeteners, and essential industrial enzymes [43]. Many LAB strains have been designated

as GRAS (Generally Recognized as Safe) by the U.S. Food and Drug Administration (FDA), making them ideal candidates for applications in food and pharmaceuticals. LAB are extensively used as starters in fermented foods and incorporated as functional ingredients into raw materials to enhance the nutritional value and safety of the final products [44]. Moreover, numerous LAB strains have been proposed as potential vaccine delivery vehicles to elicit appropriate immune responses in humans and farm animals [45]. This study, through the optimization of the culture medium components for *L. brevis*, has significantly increased the viable cell count of LAB and reduced fermentation costs, thereby further unlocking the substantial potential of LAB in industrial and medical fields.

4 | Conclusions

L. brevis is a probiotic that produce a variety of beneficial metabolites during fermentation. Therefore, it is widely used in food, medical, and health fields. In order to promote its wide application, the fermentation medium is optimized to improve production efficiency and reduce production costs.

This study first identified the essential nutrients for its growth through single and combined omission experiments of amino acids, vitamins, and nucleotides. The results show that, except for aspartic acid, the other 18 amino acids are essential for *L. brevis*. Pantothenic acid, nicotinic acid, vitamin B6, and riboflavin are identified as essential vitamins. Additionally, *L. brevis* requires nucleotides for growth. Based on these findings, we optimized the component concentrations of the synthetic medium using an artificial intelligence experimental design system and the GP960. The viable cell count of *L. brevis* in the optimized synthetic medium matched that in the complex medium, and the cost of fermentation per unit cell was reduced by 57.52%. This study successfully developed a low-cost synthetic medium that can replace the complex medium for *L. brevis*.

While this study achieved significant results in optimizing the synthetic medium for *L. brevis*, further research is needed to address some remaining issues. The concentrations of each amino acid, vitamin, and nucleotide in the synthetic medium can be further refined, and alternatives to high-cost components can be explored to enhance the industrial viability of *L. brevis* fermentation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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