#### RESEARCH ARTICLE

# Lactiplantibacillus plantarum h32: A bifunctional starter culture that effectively degrades nitrite and enhances flavor in kimchi fermentation

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Kimchi, a traditional fermented vegetable food, exhibits nitrite accumulation during spontaneous fermentation, posing health risks due to carcinogenic nitrosamine formation and methemoglobinemia. While lactic acid bacteria (LAB) can degrade nitrite, developing multifunctional starter cultures that simultaneously enhance safety and flavor remains critical. In this research, *Lactiplantibacillus plantarum* h32 was isolated from kimchi brine and evaluated its nitrite degradation capacity through tolerance assays, degradation kinetics, and acid production. Its application in kimchi fermentation was compared with spontaneous fermentation (SF) by monitoring pH, nitrite levels, and volatile compounds *via* the combination of headspace extraction, solid-phase microextraction, and gas chromatography—mass spectrometry (HS-SPME/GC-MS). The results showed that *L. plantarum* h32 exhibited high nitrite tolerance with OD<sub>600</sub> > 1.58 at 300 mg/L and degraded 99% of nitrite (150 mg/L) within 48 h. Inoculation eliminated the nitrite peak of 282 mg/kg in SF at 36 h and reduced pH to 3.56 by 48 h, accelerating fermentation. HS-SPME/GC-MS analysis revealed enhanced flavor complexity in inoculated kimchi with increased aldehydes of hexanal and nonanal and esters, novel compounds of 2-methyl-n-butyraldehyde and (E)-2-hexenal, and reduced alkane dominance. These results demonstrated *L. plantarum* h32 as a bifunctional starter culture for safer, high-quality kimchi production.

**Keywords:** Lactiplantibacillus plantarum; nitrite degradation; Kimchi; flavor; starter culture.

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#### Introduction

Kimchi, a traditional fermented vegetable product, is widely favored for its distinctive soursalty taste, crisp texture, and vibrant coloration [1, 2]. However, nitrite formation inevitably occurs during the fermenting process. Excessive nitrite intake poses significant health risks due to its reaction with secondary amines in gastric contents to form nitrosamines, compounds demonstrating potent carcinogenic effects [3]. Furthermore, acute nitrite toxicity manifests through hemoglobin oxidation, inducing

methemoglobinemia that impairs oxygen transport, potentially leading to tissue hypoxia and life-threatening systemic effects [4]. These health concerns have motivated intensive research on nitrite degradation strategies, positioning this field as critical for food safety innovation.

Current nitrite reduction technologies encompass physical, chemical, and biological approaches with lactic acid bacteria (LAB)-based degradation emerging as particularly promising for fermented food applications [5, 6]. LAB

species naturally dominate kimchi fermentation ecosystems, providing multifunctional benefits beyond nitrite metabolism. They enhance product characteristics through acid production and flavor compound synthesis [7], while conferring health benefits including gastrointestinal tolerance, immune modulation, cholesterol reduction, and intestinal microbiota regulation [8, 9]. In addition, LAB accelerates pH reduction and secretes bacteriocins to suppress competitor microorganisms, thereby inhibiting nitrate conversion and promoting nitrite degradation [10, 11]. Zeng et al. found that Limosilactobacillus fermentum RC4 demonstrated 82% nitrite reduction at 150 mg/L within 14 h [12], while Liu et al. confirmed that Lactobacillus casei subsp. Rhamnosus exhibited effective degradation in both Lactobacillus de Man, Rogosa, and Sharpe (MRS) broth and fermented vegetables [13]. Recent advances further highlighted LAB's capacity to enhance product quality with L. plantarum strains shown to increase volatile compound diversity including acids, alcohols, esters, phenols in kimchi [14], while L. plantarum FM 17 significantly reduced nitrite levels with improving sensory attributes [15]. Notably, key species including Lactobacillus brevis, Leuconostoc mesenteroides, and multiple L. plantarum variants collectively orchestrate the complex biochemical dynamics of kimchi fermentation [16-18].

While LAB inoculation enhances kimchi quality, identifying strains that concurrently achieve efficient nitrite degradation and enhancement remains challenging. This study aimed to isolate and characterize LAB strains with superior nitrite degradation capacity from kimchi brine and screen for nitrite degradation. The top candidate was identified molecularly with its growth, acidification, nitrite tolerance, and degradation kinetics all being assessed in vitro. Its impact on nitrite levels, pH dynamics, and volatile flavor compound profiles during kimchi fermentation was also evaluated in comparison to spontaneous fermentation (SF) using the combination of headspace extraction, solidphase microextraction, and gas

chromatography—mass spectrometry (HS-SPME/GC-MS) methods. This study provided theoretical foundations for developing starter cultures with enhanced nitrite-reducing potential and industrial applicability for safer, higher-quality kimchi production.

#### Materials and methods

#### Microbial sources

Traditional homemade Chinese kimchi prepared using Chinese cabbage purchased from local market in Zhumadian, Henan, China and 6% (w/v) NaCl, 3% (w/v) sugar, 0.5% (w/v) ginger, 0.5% (w/v) garlic, and 0.1% (w/v) chili powder fermented spontaneously at ambient temperature of approximal 20 - 25°C for 7 days was used as the microbial source.

#### LAB isolation and purification

LAB isolation was performed by inoculating 25 mL of kimchi brine into 50 mL MRS broth (Solarbio Science & Technology Co., Ltd., Beijing, China) followed by 24 h enrichment at 37°C. Serial dilutions of 10<sup>-1</sup> to 10<sup>-7</sup> of the enriched culture were prepared using sterile saline solution (0.85% NaCl) and spread-plated onto MRS agar supplemented with 0.1% (w/v) CaCO<sub>3</sub>. After 48 h incubation at 37°C, colonies exhibiting calcium dissolution halos were streak-purified on fresh MRS agar. Pure cultures were examined microscopically for cellular morphology and Gram-staining characteristics. Gram-positive isolates were preserved in glycerol stocks at 40% (v/v) at -80°C for further analysis.

# **Screening of nitrite-degrading LAB**

Nitrite quantification was conducted *via* the colorimetric N-(1-naphthyl) ethylenediamine dihydrochloride method [19]. Overnight cultures of LAB strains were inoculated at 2% (v/v) into MRS broth containing 150 mg/L NaNO<sub>2</sub> and incubated at 37°C, 180 rpm, for 48 h. For nitrite extraction, 5 mL of fermented broth was mixed with 12.5 mL saturated borax solution (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and 40 mL distilled water

followed by 15 min boiling and cooling. Protein precipitation was achieved by sequential addition of 5 mL potassium ferrocyanide and 5 mL zinc acetate with subsequent 30 min settling. The supernatant was filtered, and 2 mL filtrate was mixed with 40 mL distilled water, 2 mL sulfanilic acid, and 1 mL N-(1-naphthyl) ethylenediamine dihydrochloride. After 15 min dark incubation, absorbance was measured at 538 nm using a UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Nitrite degradation efficiency (%) was calculated as:

$$R = (C_0 - C_t) / C_0 \times 100$$

where C<sub>0</sub> and C<sub>t</sub> were initial and residual nitrite concentrations (mg/L), respectively.

#### Molecular identification of LAB isolates

Bacterial genomic DNA was extracted using a genomic DNA extraction kit (Solarbio Science & Technology Co., Ltd., Beijing, China). 16S rDNA amplification was performed with universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Polymerase chain reaction (PCR) was performed at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1.5 min, 72°C for 1 min, and then 72°C for 10 min. Sequencing was conducted by Sangon Biotech Co., Ltd. (Shanghai, China), and sequences were aligned against the National Center for Biotechnology Information (NCBI) database via **BLAST** (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was reconstructed with MEGA 7.0 software (<a href="https://www.megasoftware.net/">https://www.megasoftware.net/</a>).

# **Determination of growth curve**

Strains were cultured overnight in MRS broth medium and then transferred to fresh MRS broth at a 2% (v/v) inoculation volume. The cultures were incubated at 37°C, 180 rpm, for 24 h, and the optical density at 600 nm was measured every 2 h.

# **Determination of acid production capacity**

The pH and total acidity were determined according to the method described by Yang et al.

with some modifications [20]. After overnight incubation in MRS broth, *L. plantarum* h32 was inoculated into fresh MRS broth at 2% (v/v) and cultured at  $37^{\circ}$ C, 180 rpm, for 72 h. The pH and total acidity of the fermentation broth were measured at 0, 4, 8, 12, 24, 36, 48, 60, and 72 h.

#### Nitrite degradation kinetics

L. plantarum h32 was cultured overnight in MRS broth and then inoculated at 2% (v/v) into MRS broth containing 150 mg/L NaNO<sub>2</sub>. The cultures were incubated at 37°C, 180 rpm, for 72 h. The nitrite degradation rate was determined at 0, 4, 8, 12, 24, 36, 48, 60, and 72 h.

#### Nitrite tolerance assessment

Nitrite tolerance was assessed using the method of Zeng *et al.* with some modifications [12]. *L. plantarum* h32 was inoculated overnight in MRS broth and then transferred at 2% (v/v) into MRS broth supplemented with 50, 100, 150, 200, 250, and 300 mg/L NaNO<sub>2</sub>. A control group without NaNO<sub>2</sub> was included. After 24 h of incubation at 37°C, the OD<sub>600</sub> values were determined.

# Application of *L. plantarum* h32 in kimchi fermentation

#### (1) Nitrite content and pH in kimchi

Fresh cabbage was washed, drained, and packed into 200 mL fermentation bottles. A 6% sterile saline solution was added, and 2% (v/v) of logphase lactic acid bacteria was inoculated. Fermentation was conducted at 28°C for 108 h with spontaneous fermentation as the control. The nitrite content and pH of the kimchi were measured every 12 h.

# (2) Analysis of volatile flavor compounds

Volatile compounds were analyzed according to the methods of Bao et~al. with some modifications [21]. The 3.0 g samples of the control group (spontaneous fermentation (SF)) and the experimental group (with added L.~plantarum~h32~(LP)) were homogenized and placed in a 20 mL headspace vial, respectively. A solid phase microextraction (SPME) fiber assembly (50/30  $\mu$ m DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA) was inserted into the vial

and exposed to the headspace at 50°C for 30 min. The fiber was desorbed in the Agilent 7890B Gas Chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) inlet at 250°C for 3 min. Analysis was performed using a DB-Wax capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) installed in the GC coupled with an Agilent 5977B Mass Spectrometer (MS) (Agilent Technologies, Santa Clara, CA, USA) with helium carrier gas at a constant flow rate of 0.8 mL/min. Electron ionization was applied at 70 eV with ion source and quadrupole temperatures set to 200°C and 150°C, respectively. Mass spectra were acquired in the range of 30 – 500 m/z.

### Data analysis

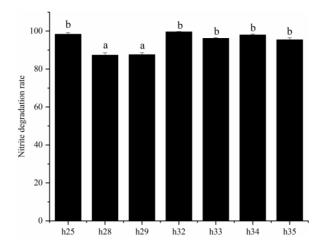
All experiments were performed in triplicate. Data was analyzed by one-way ANOVA with Duncan's test using SPSS version 26.0 (IBM, Armonk, NY, USA). Statistical significance was defined as P value less than 0.05.

#### Results

# Screening and identification of nitrite-degrading lactic acid bacteria

Thirty-six bacterial strains were isolated from kimchi brine via the streak plate method using MRS agar supplemented with 0.1% (w/v) CaCO<sub>3</sub>. All strains were Gram-positive and preliminarily identified as lactic acid bacteria. Seven strains including h25, h28, h29, h32, h33, h34, h35 exhibited strong nitrite degradation capabilities with strain h32 achieving the highest degradation rate of 99.57% (Figure 1). Consequently, strain h32 was selected for subsequent experiments to further characterize its nitrite-reducing capacity and evaluate its potential application in mitigating nitrite accumulation during fermentation Genomic DNA processes. extraction quality was validated by agarose gel electrophoresis. The DNA sample of strain h32 exhibited a distinct band within the 1,500 – 2,000 bp range, indicating high-integrity genomic DNA suitable for 16S rDNA amplification and sequencing (Figure 2A). Phylogenetic analysis demonstrated that strain h32 shared 100%

sequence homology with *L. plantarum* JCM1149, confirming its taxonomic classification as *L. plantarum* (Figure 2B).



**Figure 1.** Nitrite degradation ability of 7 suspected lactic acid bacteria strains. Lowercase letters indicated statistically significant differences (P < 0.05).

# **Growth curve analysis**

L. plantarum h32 demonstrated good growth potential. The logarithmic growth phase was from 6 to 12 h during the cultivation process, and it entered the stationary phase after 12 h of cultivation (Figure 3).

#### Acid production capacity

When L. plantarum h32 fermented for 12 h, the pH value of the fermentation broth had decreased to 4.41. When it fermented for 24 h, the pH dropped to 3.78. The total titratable acid in the strain fermentation broth accumulated rapidly within 4 to 36 h, and reached 1.07 g/L at 36 h, causing the pH value of the fermentation broth to drop rapidly. After 36 h, the total acid and pH of the strain fermentation broth tended to be stable. When the fermentation reached 72 h, the pH of the fermentation broth reached 3.65, and the total titratable acid was 1.11 g/L (Figure 4). The acid production of the strain fermentation could reduce the pH of the medium to about 3.65, which was beneficial for the fermentation acidification of kimchi and the inhibition of miscellaneous bacteria.

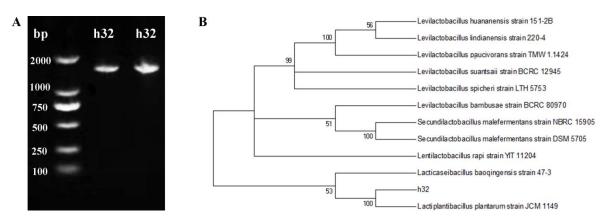


Figure 2. Identification of L. plantarum h32. A. Electrophoretogram. B. Phylogenetic tree.

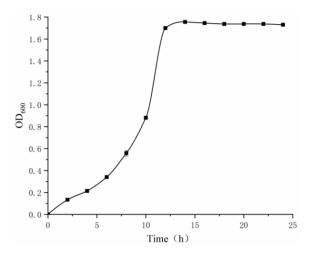


Figure 3. Growth curves of L. plantarum h32.

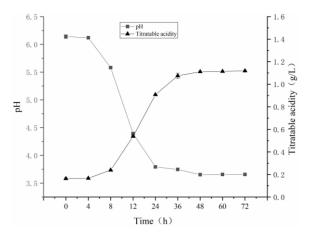
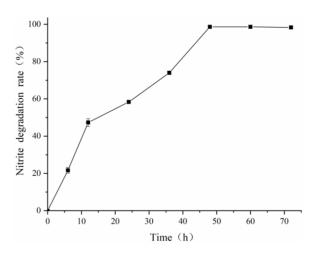


Figure 4. Changes of pH value and acidity of *L. plantarum* h32 in growth.

# Nitrite degradation kinetics

The degradation rate of *L. plantarum* h32 could reach 49% after 12 h of fermentation (Figure 5). When the fermentation reached 48 h, the degradation rate reached 99%. With continuous fermentation, the sodium nitrite in the fermentation broth was almost completely degraded. The results showed that this strain had an obvious effect on degrading nitrite and could be used to explore its application in degrading nitrite in kimchi products.



**Figure 5.** Nitrite degradation rate during the culture of *L. plantarum* h32

#### Nitrite tolerance

After growing for 24 h, *L. plantarum* h32 cultured with 50 and 100 mg/L NaNO<sub>2</sub> in the media demonstrated no significant difference comparing to the control group (without NaNO<sub>2</sub>)

with their OD<sub>600</sub> values reaching 1.69 and above. When the mass concentration of NaNO<sub>2</sub> was 150 and 200 mg/L, the OD<sub>600</sub> of L. plantarum h32 was between 1.64 and 1.63, while, when the mass concentration of NaNO2 reached 250 and 300 mg/L, the OD600 of L. plantarum h32 was between 1.59 and 1.58, which was significantly lower than the OD<sub>600</sub> of *L. plantarum* h32 when in the mass concentration of NaNO₂ at 200 mg/L. Since lactic acid bacteria face the problem of increased nitrite content during the kimchi fermentation process, the inoculation and fermentation of lactic acid bacteria require a certain degree of nitrite tolerance. L. plantarum h32 demonstrated a strong tolerance to NaNO<sub>2</sub> less than 200 mg/L and a relatively high tolerance to NaNO₂ between 250 and 300 mg/L (Figure 6). Therefore, L. plantarum h32 had the potential to be used as an inoculum for fermenting kimchi.

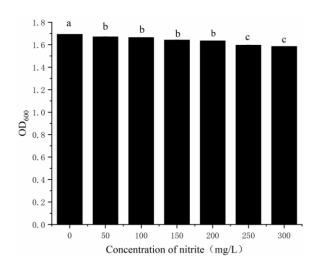


Figure 6. The growth of L. plantarum h32 under different concentrations of NaNO<sub>2</sub>. Lowercase letters indicated statistically significant differences (P < 0.05).

# Application of lactic acid bacteria (1) Changes in nitrite content in kimchi

The starter plays an important role in nitrite degradation during kimchi fermentation. The nitrite content in the kimchi fermented with *L. plantarum* h32 was lower than that in the SF kimchi (Figure 7). In the early stage of fermentation, nitrate was transformed into

nitrite due to the growth of nitrate reducing bacteria [15]. When the kimchi was fermented for 72 h, the nitrite was no longer detected in the kimchi, and there was no nitrite peak during the whole fermentation process. However, when the SF reached 36 h, a nitrite peak appeared with a value of 282 mg/kg. The nitrite content during the whole fermentation process was higher than that in the kimchi fermented with *L. plantarum* h32. After the fermentation reached 108 h, the nitrite content decreased to 10 mg/kg, which was lower than the maximum limit of 20 mg/kg [20], and the degradation rate was generally slow.

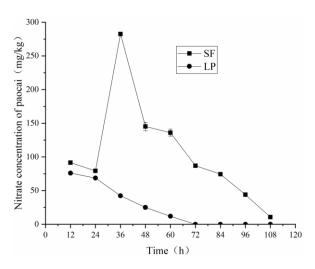


Figure 7. Changes in nitrite content of kimchi during fermentation.

#### (2) Changes in pH value in kimchi

The results showed that, with the increase of time, the pH values of both types of kimchi gradually decreased. The pH values in L. plantarum h32-inoculated kimchi were consistently significantly lower than those in SF throughout fermentation (P < 0.05). When the fermentation reached 48 h, the pH value of kimchi fermented with L. plantarum h32 decreased to 3.56. After 48 h, the change in its pH value was relatively small. At 108 h, the pH value was 3.15. The pH value of the SF decreased rapidly after 72 h and reached 3.22 at 96 h. At 108 h, the pH value was 3.17, which was approximately the same as the pH value of kimchi fermented with L. plantarum h32 (Figure 8).

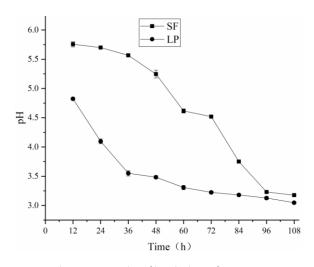


Figure 8. Changes in pH value of kimchi during fermentation.

# (3) Changes in flavor compounds in kimchi

In this study, HS-SPME/GC-MS was used to conduct a flavor analysis on the two groups of samples. According to the similarity and peak area, the flavor compounds were sorted, and the flavor compounds were studied. A total of 71 volatile components were found including 44 alkanes, 4 aromatic hydrocarbons, 2 esters, 3 alcohols, 7 aldehydes, 1 acid, and 10 other compounds. Among them, for the SF, 47 volatile substances were analyzed as flavor compounds including 35 alkanes, 1 ester, 2 alcohols, 7 aldehydes, 1 acid, and 1 other compound. For sample LP, 55 volatile substances were analyzed as flavor compounds including 35 alkanes, 2 esters, 2 alcohols, 9 aldehydes, 2 acids, and 5 other compounds. Generally, a high content of alkanes in the flavor components of food does not have obvious benefits. Alkanes are usually not the main flavor contributors to food, and excessive alkanes may have an adverse effect on the taste and quality of food [22]. Aldehydes may impart some fruity, grassy, and other odors to food, and at the same time, they may also affect the taste and the complexity of the overall flavor of food. Different aldehydes as well as their contents and interactions in food will lead to different flavor performances. For sample LP, the proportion of the content of alkane types decreased, which was beneficial for better enhancing the flavor contribution.

proportions of aldehydes, esters, and other substances, which have a greater impact on the flavor of food, increased, playing an important role in enhancing the flavor. The addition of L. plantarum h32 resulted in the formation of two novel aldehyde compounds, 2-methyl-nbutyraldehyde and (E)-2-hexenal, simultaneously inducing significant increases in the concentrations of hexanal, heptanal, benzaldehyde, nonanal, octanal, and decanal. Concurrently, two previously undetected sulfate esters, butyl trialkyl sulfate and butyl tetradecyl sulfate, were synthesized through bioprocess. These findings demonstrated that microbial inoculation not only expanded the of flavor-impacting chemical diversity constituents through de novo biosynthesis but also enhanced the quantitative presence of existing aromatic aldehydes. This dual mechanism of qualitative compound generation and quantitative content elevation effectively optimized the organoleptic properties, thereby significantly improving the overall flavor profile of the fermented vegetable product.

### Discussion

Nitrites are recognized carcinogens, and their presence has been detected in most kimchi products. LAB not only serves as essential starter cultures for kimchi fermentation but also demonstrates significant capability in nitrite degradation, thereby mitigating associated health risks [23, 24]. Nowadays, lactic acid bacteria that are capable of degrading nitrite have been isolated and screened for application in kimchi samples [15]. Kim et al. confirmed that Lactobacillus sakei, Lactobacillus curvatus, and Lactobacillus brevis could effectively degrade nitrite in fermented foods [25]. In this study, L. plantarum h32 isolated from kimchi brine demonstrated exceptional nitrite degradation efficiency and rapid acidification kinetics, positioning it as a promising candidate for enhancing food safety and quality in fermented vegetable production. These findings aligned with previous reports, highlighting L. plantarum

strains as key contributors to nitrite reduction [26]. However, the strain-specific variations in degradation efficiency remain underexplored. The superior nitrite degradation capacity of L. plantarum h32 surpassed earlier findings for L. fermentum RC4 [27], likely attributable to its rapid acid production and metabolic adaptability. pH and titrable acidity were the important indicators that might affect fermentation [28]. The strain's ability to reduce broth pH to 3.65 within 24 h was aligned with studies demonstrating that LAB mediated acidification accelerates nitrite degradation by inhibiting nitrate-reducing bacteria and promoting denitrification pathways [26]. Notably, the absence of a nitrite peak in h32-inoculated contrasted sharply with kimchi natural nitrite fermentation, where accumulation peaked at 36 h. This suppression of the "nitrite hump" suggested that early-stage inoculation with h32 disrupted the ecological succession of nitrate-reducing microbes, a critical advantage for industrial applications requiring predictable safety profiles. In addition to its degradation efficiency, L. plantarum h32 also showed high tolerance to nitrite concentrations with its tolerance to nitrite concentrations up to 300 mg/L, which highlighted its application potential. While growth inhibition occurred at ≥ 200 mg/L  $NaNO_2$ , the retained viability (OD<sub>600</sub> > 1.58) suggested metabolic adaptation mechanisms, possibly involving nitrite reductase activity or stress-responsive efflux systems [29]. This tolerance was pivotal for starter cultures as earlystage fermentation often coincided with transient nitrite spikes [30]. Furthermore, the rapid pH decline induced by h32 at 48 h shortened the kimchi ripening period compared to natural fermentation at 96 h, indicating potential for reduced production cycles.

Flavor constitutes a critical determinant in assessing the quality attributes of fermented food products. In the case of kimchi, the development of desirable flavor profiles directly influences consumer acceptability and purchasing decisions [31, 32]. It is noteworthy that traditional spontaneous fermentation

processes exhibit substantial instability in product quality, primarily due to uncontrolled proliferation of spoilage microorganisms during fermentation. This microbial imbalance frequently leads to undesirable metabolite formation, ultimately compromising organoleptic characteristics and food safety parameters [33]. The inoculation of L. plantarum h32 significantly altered the volatile compound profile, enriching aldehydes like hexanal and nonanal and esters while reducing alkane dominance. These shifts correlated with enhanced sensory quality as aldehydes imparted fruity and grassy notes, and esters contributed to aromatic complexity [34]. The de novo synthesis of 2-methyl-n-butyraldehyde and (E)-2-hexenal in h32-fermented kimchi suggested strainspecific enzymatic activities such as lipoxygenase or alcohol dehydrogenase pathways, which warranted further mechanistic investigation [35]. L. plantarum h32 fermentation enriched the flavor of kimchi and enhanced its aroma, thereby strengthening the dual role of lactic acid bacteria in terms of safety and quality improvement.

This study demonstrated that L. plantarum h32 isolated from traditional kimchi exhibited exceptional nitrite degradation capacity as 99% degradation of 150 mg/L nitrite within 48 h in vitro and robust fermentation performance. Application in kimchi fermentation effectively eliminated the nitrite accumulation peak observed in spontaneous fermentation, ensuring compliance with safety thresholds (< 20 mg/kg) by 72 h. Its rapid acidification with pH reaching 3.56 at 48 h accelerated fermentation kinetics, while high nitrite tolerance (OD<sub>600</sub> > 1.58 at 300 NaNO₂) underscored mg/L industrial applicability. Critically, inoculation significantly enriched flavor complexity by increasing aldehyde and ester contents, introducing novel compounds such as 2-methyl-n-butyraldehyde and (E)-2-hexenal, and reducing alkane dominance, thereby enhancing fruity, grassy, and aromatic notes. These findings highlighted the dual role of L. plantarum h32 in mitigating nitriterelated health risks and improving sensory quality, positioning it as a promising starter culture. Future studies should investigate its long-term storage stability, gastrointestinal survival, and metabolic interactions in complex fermentation systems to optimize its application in commercial kimchi production. This work advanced the development of tailored starter cultures for safer, higher-quality fermented vegetables.

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