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Lactic Acid-Enriched Fermentate from Marine-Derived *Lacticaseibacillus casei* R-1 as a Biological Preservative for Bread

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Abstract

Marine-derived *Lacticaseibacillus casei* R-1 was employed to produce a fermentation product with dual preservative and quality-enhancing functions for bread applications. Through single-factor optimization, the optimal fermentation conditions were determined as 8% whey protein, 2% sucrose, 2% inoculum, and 72 h of fermentation. Under these conditions, a multi-component antimicrobial system was obtained, with significantly enriched lactic acid (4399.65 mg/L), total phenolics (22.86 mg/mL), peptides (0.29 mg/mL), and free amino acids (3.25 mg/L). The lyophilized fermentate was incorporated into bread at 0.3–1.0% (*w*/*w*), and its effects on microbial stability, texture, and flavor were systematically evaluated during ambient storage. A clear dose-dependent response was established across three functional dimensions. At the preservation level, 0.8–1.0% supplementation eliminated visible mold within 7 days, reduced microbial loads by 1–2 log cycles, and extended shelf life by 100%. For quality enhancement, 0.6–0.8% delivered optimal textural improvement (23–31% hardness reduction, 15–18% springiness increase) and superior crust coloration. For flavor, 0.5% achieved the most balanced profile through selective enrichment of esters and alcohols. Collectively, the 0.6–0.8% range emerges as the optimal compromise between preservation efficacy and overall quality enhancement. This study demonstrates that the marine-origin *L. casei* R-1 fermentate offers a directly applicable, safe, and effective solution for bread preservation, achieving shelf-life extension without compromising product quality, while addressing the underexplored potential of marine-derived LAB fermentates in baked goods.

Keywords: *Lacticaseibacillus casei* R-1; marine-derived lactic acid bacteria; biological preservative; bread shelf-life; clean-label bakery products

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Academic Editor: Massimo Iorizzo

Received: 29 September 2026
Revised: 28 October 2026
Accepted: 29 October 2026
Published: 7 November 2026

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1. Introduction

Bakery products are staple foods worldwide, and the baking industry continues to expand, driven by consumer demand for convenient, functional, and diverse offerings [1,2]. The global bakery market reached USD 209.55 billion in 2025, within which industrially produced pre-packaged bread—requiring preservatives to maintain shelf stability during long-distance distribution—represents a major category. However, this requirement for

preservatives is increasingly challenged by a growing consumer preference for "clean-label" foods—those with minimal additives, no synthetic preservatives, and transparent ingredient lists [3]. This shift challenges conventional formulation systems that rely on chemical preservatives such as potassium sorbate and calcium propionate. Although these chemicals are effective in controlling spoilage, their use is increasingly met with consumer resistance, prompting an urgent need for safer, greener preservation technologies. Among the emerging alternatives, lactic acid bacteria (LAB) fermentation products stand out as a promising solution, aligning with the clean-label paradigm while offering multifunctional benefits [4].

Microbial spoilage remains one of the most critical threats to baked goods, underscoring the urgent need for effective natural preservation strategies. Mold contamination, predominantly by *Aspergillus*, *Penicillium*, and *Rhizopus* species, not only causes visible deterioration but also raises safety concerns due to potential mycotoxin production [5]. Bacterial spoilage, particularly ropy bread caused by *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and members of the *Bacillus cereus* group, further compromises quality during ambient storage [6,7]. In this context, LAB fermentation products—complex mixtures containing organic acids, short peptides, exopolysaccharides, and other bioactive metabolites—offer a particularly attractive solution [8]. They exert broad-spectrum antimicrobial effects through multiple mechanisms, including acidification, membrane disruption, and interference with energy metabolism [9,10]. Moreover, unlike live cultures, these products remain stable under baking and storage conditions and can be declared on labels as "fermentation products" or "fermented whey protein," thus meeting clean-label requirements [11].

Despite the demonstrated potential of LAB fermentation products, current research on LAB-based biopreservation for baked goods has largely focused on terrestrial strains, with several studies demonstrating the efficacy of fermented whey using *Lactobacillus plantarum* and other conventional LAB in extending bread shelf life [12,13]. However, systematic investigations of marine-derived LAB strains remain scarce. Compared with their terrestrial counterparts, microorganisms originating from marine environments—such as mangrove sediments and coastal areas—have adapted to high salinity, osmotic stress, and complex microbial interactions, and thus often possess distinctive metabolic pathways and tolerance traits [14,15]. Their fermentation products may exhibit unique antimicrobial spectra, organic acid profiles, and flavor precursor compositions, potentially offering dual benefits of preservation and quality enhancement for bakery products [16,17]. Nevertheless, the specific antifungal activities and baking-relevant functionalities of marine-derived LAB fermentates remain poorly understood, and their application in bread systems has not been systematically evaluated [18,19].

To address this knowledge gap, the marine-derived strain *L. casei* R-1 was employed. This strain was independently isolated and purified from mangrove sediments in our laboratory. The strain was used to produce a LAB-based fermentation product with combined preservative and quality-enhancing properties. The fermentation conditions were systematically optimized, and the resulting lyophilized fermentate was applied to bread to evaluate its effects on microbial inhibition, shelf-life extension, textural parameters, and volatile flavor profiles. This approach offers a practical technical pathway that reduces dependence on synthetic preservatives while simultaneously improving bread quality. The findings are expected to fill the current gap in knowledge regarding the application of marine-derived LAB fermentates in baked goods and to support the development of natural, multifunctional preservation systems for the baking industry.

2. Materials and Methods

2.1. Materials

L. casei R-1, a marine-derived strain, was used in this study. The strain has been deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number CGMCC No. 33871. The indicator strains—*Staphylococcus aureus*, *Escherichia coli*, *B. subtilis*, and *Aspergillus niger* were obtained from the laboratory stock culture collection.

MRS broth (CAS No. 027312) was purchased from Guangdong Huankai Microbial Technology Co., Ltd. (Guangzhou, China). Whey protein (CAS No. 9013-90-5) was obtained from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). LB broth was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Plate count agar (PCA), Baird-Parker (BP) agar, rose Bengal agar and potato dextrose agar (PDA) were sourced from Qingdao Hopebio Marine Biotechnology Co., Ltd. (Qingdao, China). Sucrose (analytical grade), the BCA protein assay kit, and o-phthalaldehyde (OPA) were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Folin–Ciocalteu reagent was obtained from Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Standard solutions of lactic acid, acetic acid, propionic acid, and butyric acid (chromatographic grade) were purchased from Xilong Scientific Co., Ltd. (Guangzhou, China).

2.2. Strain Activation, Single-Factor Optimization, and Antimicrobial Activity Assessment

L. casei R-1, stored at −80 °C, was streaked onto MRS agar plates and incubated at 37 °C for 24 h. A single colony was transferred into MRS broth and cultured at 37 °C with shaking at 180 rpm for 18 h. This pre-culture was inoculated (1%, v/v) into fresh MRS broth and incubated under the same conditions for another 18 h to obtain the seed culture (approximately 10^8 CFU/mL).

Fermentation was carried out in a whey protein-based medium. The basal formulation consisted of 10% (w/v) whey protein, 6% (w/v) sucrose, and 5% (v/v) inoculum. To optimize the fermentation conditions, three key parameters—whey protein concentration, sucrose concentration, and inoculum size—were optimized sequentially using a single-factor-at-a-time approach. Specifically, whey protein concentration (2%, 4%, 6%, 8%, and 10%, w/v) was evaluated first; the optimal level was then fixed while sucrose concentration (2%, 4%, 6%, and 8%, w/v) was evaluated; finally, both concentrations were held constant while inoculum size (2%, 3%, 4%, and 5%, v/v) was evaluated. For each condition, an uninoculated control was prepared by replacing the seed culture with an equal volume of sterile purified water and was processed identically thereafter, to account for the background contribution of the medium matrix. The uninoculated medium filtrate (UMF) used as the matrix control in the supernatant-based assays was prepared from uninoculated whey protein medium subjected to the same sterilization, incubation, centrifugation, and filtration steps as the fermented samples, except that the seed culture was replaced with an equal volume of sterile purified water. For each condition, whey protein powder and sucrose were dissolved in 300 mL purified water, and the resulting medium was dispensed into 500 mL Erlenmeyer flasks sterilized at 115 °C for 20 min. Each fermentation was initiated by inoculating the seed culture into the whey protein-based medium and conducted in 500 mL Erlenmeyer flasks at 37 °C with shaking at 180 rpm for 72 h. Three independent replicates were prepared for each condition, and pH was measured every 24 h. At the end of fermentation, the broth was centrifuged at 6000 rpm for 15 min at 4 °C. The resulting supernatant was filter-sterilized through a 0.22 μm membrane and stored at 4 °C for subsequent analyses.

The antimicrobial activity of the fermentation supernatant obtained under each condition was evaluated against three bacterial indicators (*S. aureus*, *E. coli*, and *B. subtilis*) and

one fungal indicator (*A. niger*) using the agar well diffusion method. For bacterial strains, each was activated for 16–24 h and inoculated (1%, *v*/*v*) into LB semi-solid agar (8 g/L agar). The mixture was poured into Petri dishes, and wells of 6 mm diameter were bored after solidification. An aliquot of 100 µL of the fermentation supernatant was added to each well. The plates were kept at 4 °C for 2 h to allow diffusion, followed by incubation at 37 °C for 10–24 h, after which the diameters of inhibition zones were measured. For the bacterial agar well diffusion assay, the matrix control consisted of an equal volume (100 µL) of UMF applied instead of the fermentation supernatant; all other procedures were identical to those of the sample group.

The antimicrobial activity assays were performed in three independent replicates ($n = 3$), and the results are expressed as mean ± standard deviation. For the bacterial agar well diffusion assay, inhibition zone diameters were measured with a digital vernier caliper (0.01 mm resolution) using the cross method: each zone was measured along two perpendicular axes, and the arithmetic mean of the two readings was recorded as the zone diameter for that plate. The reported inhibition zone diameters are total diameters including the 6 mm well; the well diameter was not subtracted.

For *A. niger*, a donor PDA plate was inoculated with 1 mL of spore suspension (10^6 CFU/mL), spread evenly, and incubated at 28 °C for 72 h to allow full mycelial coverage. A fresh PDA plate was then prepared. One milliliter of the fermentation supernatant was evenly spread over the surface of the fresh PDA plate, and a 6 mm mycelial plug cut from the edge of the donor colony was placed at the center of the plate. For the matrix control, an equal volume (1 mL) of UMF was spread over a fresh PDA plate in place of the fermentation supernatant, and the same procedure was followed. The plates were further incubated at 28 °C for 3 days. Colony diameters were measured using the same caliper and cross method as described above for the bacterial inhibition zones, with the 6 mm inoculum plug excluded. Radial growth inhibition (%) was calculated as (D_control − D_treated)/D_control × 100, where D_control and D_treated are the colony diameters of the matrix control (UMF) and the fermented-supernatant group, respectively. Mycelial growth was also monitored visually.

Following the identification of optimal fermentation conditions (8% whey protein, 2% sucrose, and 2% inoculum) from the single-factor experiments, fermentation was performed in 500 mL Erlenmeyer flasks using the optimized conditions at 37 °C with shaking at 180 rpm for 72 h. The fermentation broth was centrifuged at 6000 rpm for 15 min at 4 °C, and the supernatant was freeze-dried at −50 °C under ≤10 Pa for 96 h. The resulting lyophilized powder was sealed and stored at −20 °C until further use.

2.3. Determination of Bioactive Components in Fermentation Supernatant

All measurements in this section were performed in three independent replicates ($n = 3$), and all values are reported as the mean ± standard deviation of three independent determinations.

Total phenolic content was determined using the Folin–Ciocalteu method [20]. Briefly, 1 mL of fermentation supernatant was mixed sequentially with 1 mL of Folin–Ciocalteu reagent and 2 mL of 12% (*w*/*v*) Na_2CO_3 solution. The mixture was diluted to 10 mL with purified water and incubated at room temperature in the dark for 1 h. The absorbance was measured at 765 nm. UMF served as the matrix control and was assayed identically. Gallic acid was used as the standard, and the results were expressed as mg of gallic acid equivalents (GAE) per mL.

Peptide content was determined using a BCA protein assay kit following the manufacturer's instructions. Absorbance was measured at 562 nm, and bovine serum albumin (BSA)

was used as the standard. UMF served as the matrix control and was assayed identically. Results were expressed as mg/mL.

Free amino acid content was determined using the OPA method [21]. The OPA reagent was prepared as follows: solution A (80 mg OPA dissolved in 4 mL ethanol); solution B (3.81 g sodium tetraborate, 100 mg sodium dodecyl sulfate (SDS), and 88 mg dithiothreitol (DTT) dissolved in 150 mL purified water). Solution A was transferred into solution B, mixed well, and diluted to 100 mL with purified water. For the assay, 20 µL of sample was mixed with 200 µL of OPA reagent and incubated at room temperature in the dark for 20 min. Absorbance was measured at 340 nm. UMF served as the matrix control and was assayed identically. Serine was used as the standard, and the results were expressed as mmol/L.

Organic acid content (lactic, acetic, propionic, and butyric acids) was determined by high-performance liquid chromatography (HPLC) using a Waters 2695 system equipped with a refractive index detector. Separation was performed on an Aminex HPX-87H column (300 mm × 7.8 mm). The mobile phase was 5 mmol/L H_2SO_4 at a flow rate of 0.6 mL/min. The column temperature was maintained at 40 °C, and the injection volume was 10 µL. UMF served as the matrix control and was assayed identically. Quantification was carried out using an external standard method with a mixed standard solution of lactic, acetic, propionic, and butyric acids.

2.4. Application of Fermented Freeze-Dried Powder in Bread

2.4.1. Bread Preparation Method

The bread base used in this study was a conventional soft bread ("rich dough") formula. Relative to 250 g of flour, the levels of sucrose (5 g), whole egg (50 g), and butter (30 g) are those commonly used in soft bread to improve flavor, color, and eating quality; the product was still manufactured by the conventional bread process and is therefore a rich bread dough rather than a pastry formulation. Dough was prepared by mixing high-gluten (strong) wheat flour (bread flour; protein content ≥ 12%, dry basis; wet gluten content ≥ 30%) (250 g), white granulated sugar (5 g), edible salt (2 g), active dry yeast (4 g), egg (50 g), and water (170 mL) at low speed for 5 min. For the experimental groups, the R-1 fermented lyophilized powder (obtained as described in Section 2.2) was added at concentrations of 0.3–1.0% (*w*/*w*), followed by mixing at medium speed for 5 min. Butter (30 g) was then added, and mixing continued for another 5 min until a smooth dough formed. The control group consisted of bread prepared without the addition of the R-1 fermented lyophilized powder, while all other ingredients and processing steps were identical to those of the experimental groups.

For each supplementation level, one independent batch was prepared from 250 g of flour; three independent batches were prepared per level ($n = 3$). This amount of dough was sufficient for all measurements of that level, and no additional dough preparation was required. The dough was fermented at 30 °C with 80% relative humidity for 50 min. After degassing and shaping, a second fermentation was carried out under the same conditions for 60 min. Each batch of dough was baked as a single loaf in one mold; the dough was not divided into separate portions for baking. The dough was then baked in an oven at 200 °C for 30 min. Immediately after baking, the bread was removed from the mold and cooled at room temperature for 30 min, then sliced, packaged, and stored for further analysis.

2.4.2. Physicochemical Property Determination

All determinations in this section were performed in three independent replicates ($n = 3$), and the results are expressed as mean ± standard deviation.

pH and titratable acidity: During storage, samples were taken every 2 days. The control group consisted of bread prepared without the R-1 fermented lyophilized powder, while all other ingredients and processing steps were identical to those of the experimental groups. Bread crumb (1 g) was homogenized with 9 mL of purified water, and the pH was measured directly using a pH meter. Titratable acidity was determined using phenolphthalein as an indicator and titration with 0.1 mol/L NaOH according to our previous study [19]. Acidity was expressed in Thorner degrees (°T), defined as the volume (mL) of 0.1 mol/L NaOH standard solution required to titrate the extract equivalent to 100 g of bread crumb to the phenolphthalein endpoint (a faint pink color persisting for 30 s). A blank determination was run in parallel. The titer was converted to °T using the equation: °T = (V_sample − V_blank) × (c/0.1) × (100/m), where V_sample is the volume of NaOH solution consumed by the sample (mL), V_blank is the volume consumed in the blank (mL), c is the exact concentration of the NaOH standard solution (mol/L), and m is the mass of the bread crumb sample (g; m = 1 g in this study).

Microbiological analysis: Determinations were performed following our previous study [19]. The control group consisted of bread prepared without the R-1 fermented lyophilized powder, and all other conditions were identical to those of the experimental groups. Briefly, bread samples (1 g) were homogenized with 9 mL of sterile saline, and ten-fold serial dilutions were prepared. Appropriate dilutions were spread onto: plate count agar (PCA) for bacterial enumeration (incubation at 37 °C for 48 h), rose bengal agar for mold enumeration (incubation at 28 °C for 72 h), and Baird-Parker agar for *S. aureus* enumeration (incubation at 37 °C for 48 h). Colonies were regularly observed and counted, and results were expressed as lg (CFU/g).

2.4.3. Sensory and Quality Property Evaluation

Texture profile analysis (TPA): Bread was cooled for 2 h and sliced into pieces of 10 cm × 10 cm × 2 cm. Texture was measured using a TMS-PRO texture analyzer (Food Technology Corporation, Sterling, VA, USA) equipped with an APN/25N cylindrical probe. The test parameters were: pre-test speed 1.0 mm/s, test speed 1.0 mm/s, compression ratio 50%, interval time 5 s, and trigger force 0.01 N. Hardness, springiness, cohesiveness, chewiness, and adhesiveness were recorded. Three replicates were performed for each group. The control group consisted of bread prepared without the R-1 fermented lyophilized powder, while all other ingredients and processing steps remained identical to those of the experimental groups; samples from all groups were analyzed after the same cooling period and taken from the same position within the loaf.

Color measurement: The color of bread crumb was measured using a HunterLab colorimeter, calibrated with standard white and black plates. The parameters L* (brightness), a* (red–green value), and b* (yellow–blue value) were recorded, with six replicates per group. The control bread was measured at the same position and time as the experimental groups, with six readings per group. The browning index (BI) was calculated according to Equation (1):

$$BI = [100 \times (x - 0.31)]/0.17, \text{ where } x = (a^* + 1.75\,L)/(5.645\,L + a^* - 3.012\,b^*) \quad (1)$$

Volatile flavor compound analysis: Volatile compounds were determined using gas chromatography–ion mobility spectrometry (GC-IMS). Bread crumb (1.0 g) was placed in a 20 mL headspace vial and sealed for equilibration. The control group consisted of bread prepared without the R-1 fermented lyophilized powder and was processed and analyzed in the same manner as the experimental groups. The chromatographic conditions were as follows: a WA-MS polar capillary column (30 m × 0.32 mm × 0.25 μm); temperature program: held at 40 °C for 1 min, then increased to 100 °C at 2 °C/min and held for 31 min,

then increased to 180 °C at 5 °C/min and held for 62 min; IMS drift tube temperature: 45 °C; drift gas: high-purity nitrogen at 1.0 mL/min. Compound identification was performed using the NIST database combined with retention indices. Quantification was carried out using the peak area normalization method. Principal component analysis (PCA) and flavor profile analysis were performed using SIMCA software (version 15., Sweden).

2.5. Data Processing

All experiments were repeated at least three times. Data were processed and statistically analyzed using Excel 2021 and SPSS 23.0. Results were expressed as mean ± standard deviation. A significance level of $p < 0.05$ was considered statistically significant. Graphs were prepared using Origin 2024. GC-IMS data were analyzed using the instrument's built-in software VOCal (04.12) and reporter for the analysis of volatile compound profiles.

3. Results and Discussion

3.1. Optimization of Fermentation Conditions and Antimicrobial Activity of the R-1 Fermentate

3.1.1. Effects of Whey Protein Concentration on Fermentation pH and Antibacterial Activity

Across all whey protein concentrations (2–10%, *w*/*v*), the inoculated groups exhibited a pronounced biphasic acidification pattern (Figure 1A): pH declined rapidly from initial values of approximately 5.5 to 3.5–4.0 within the first 24 h, followed by a stable plateau through 48 h and 72 h. This exponential acidification phase coupled with subsequent stationary equilibrium is characteristic of typical LAB fermentation kinetics [22]. Despite minor variations in initial pH across different whey protein concentrations, both the magnitude of acidification and the terminal pH values remained comparable throughout the 2–10% range, suggesting that substrate availability supports robust microbial growth and acid production without inducing substrate inhibition. In contrast, the uninoculated control groups maintained stable pH values between 6.5 and 7.0 over the entire 72 h incubation period, confirming the absence of spontaneous acidification and establishing that the observed pH reduction should be exclusively attributable to the metabolic activity of the marine-derived R-1 strain.

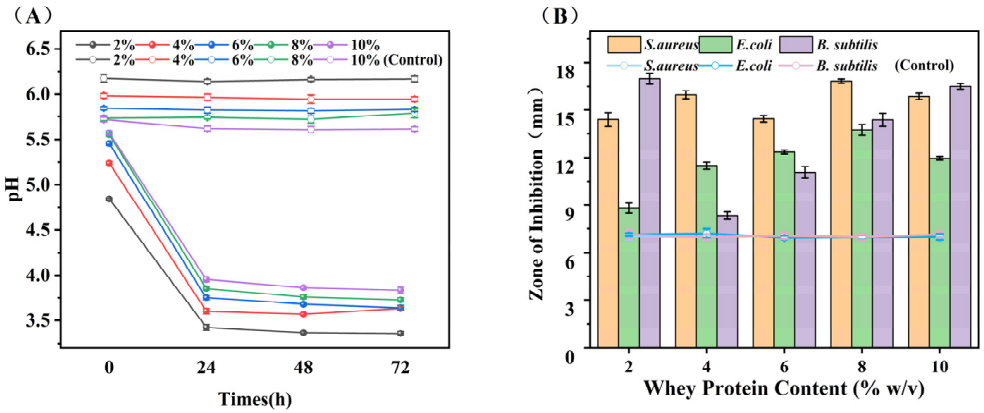

Figure 1. pH values (**A**) and antibacterial activity (**B**) of fermentation with different whey protein concentrations. The uninoculated control in (**A**) was the medium prepared by replacing the seed culture with an equal volume of sterile purified water and processed identically; the control in (**B**) was the uninoculated medium filtrate (UMF) applied in place of the fermentation supernatant.

Against *E. coli*, a clear substrate concentration dependency was observed (Figure 1B). The strongest inhibitory effects were obtained at 6% and 8% whey protein concentrations, with inhibition zones of 13.12 ± 0.27 mm and 13.74 ± 0.36 mm, respectively, significantly larger than that of the 2% group (11.23 ± 0.35 mm) ($p < 0.05$). This enhanced activity

at moderate substrate concentrations indicates that optimal production of low-molecular-weight antimicrobial compounds—particularly the synergistic combination of organic acids and antimicrobial peptides—occurred within this range, enabling effective penetration of the lipopolysaccharide barrier of Gram-negative bacteria [23]. This is consistent with the findings of García-Díez et al. [24], who reported that *Lactobacillus rhamnosus* cell-free supernatant combined with whey protein isolate exhibited potent activity against *E. coli*. Collectively, these observations suggest that the fermentation supernatant exerts its anti-Gram-negative activity primarily through the synergistic action of multiple antimicrobial metabolites, with substrate concentration serving as a critical determinant of their production efficiency.

The fermentation supernatant exhibited strong inhibitory activity against *B. subtilis*, a key spoilage microorganism responsible for ropy bread spoilage. The strongest effects were observed at 2%, 6%, and 8% whey protein concentrations, with inhibition zones of 14.65 ± 0.38, 14.89 ± 0.35, and 15.31 ± 0.42 mm, respectively—significantly larger than those of the 4% and 10% groups ($p < 0.05$). This non-monotonic, concentration-dependent pattern indicates an optimal substrate window for antimicrobial metabolite production rather than a simple positive correlation. Previous reports have demonstrated that antimicrobial peptides derived from LAB-fermented whey protein exert potent activity against Gram-positive bacteria, including *B. subtilis*, through complementary mechanisms of membrane disruption and cell-wall synthesis inhibition [25]. Together, these findings suggest that the observed activity against *Bacillus* species is associated with the antimicrobial metabolites produced by R-1 fermentation.

Against S. aureus, the fermentation supernatant produced inhibition zones of 13.82–15.97 mm across all whey protein concentrations, indicating stable and potent anti-staphylococcal activity. Inhibition was slightly weakened at 6% whey protein (14.43 mm) but reached its maximum at 8% (15.97 mm), which was higher than that against E. coli at the same whey protein concentration (13.74 mm). This preference for Gram-positive bacteria is consistent with the mode of action of Class IIa pediocin-like bacteriocins, which readily bind lipoteichoic acid receptors and insert into the membrane of Gram-positive cells lacking an outer membrane barrier, whereas the LPS-rich outer membrane of Gram-negative bacteria imposes both physical and electrostatic barriers [20,22]. To validate the specificity of the observed antibacterial activities against both Gram-negative and Gram-positive bacteria, control supernatants from uninoculated whey protein cultures were included in parallel. In all cases, these controls produced no measurable inhibition zones at any concentration tested, confirming that the antibacterial effects described above were entirely attributable to bioactive metabolites produced by strain R-1 during fermentation, rather than to the whey protein substrate itself. This finding is in full agreement with previous reports on LAB fermentation products [26] and further underscores that the concentration-dependent activity patterns observed for both *E. coli* and *B. subtilis* reflect genuine metabolic output of the marine-derived strain, rather than non-specific medium effects. Overall, these control experiments establish that the antimicrobial efficacy documented in this study is specifically derived from the fermentation metabolism of R-1, thereby reinforcing the central role of microbial biotransformation in generating the bioactive components responsible for the observed inhibition.

3.1.2. Effects of Sucrose Concentration on Fermentation pH and Antibacterial Activity

Across all sucrose concentrations (2–8%, *w*/*v*), the inoculated groups exhibited a characteristic biphasic acidification pattern (Figure 2A): pH declined rapidly from initial values of approximately 5.5 to 3.5–4.0 within the first 24 h, followed by stabilization through 48 h and 72 h. Notably, while the high-sucrose groups exhibited a slightly higher initial pH yet

a more pronounced early-stage pH drop, the final pH values converged after 24 h across all treatments, suggesting that sucrose concentration primarily modulates the acidification rate rather than the ultimate extent of pH reduction under the conditions tested. Similar results were also observed in other studies that initial sugar concentration governs acidification kinetics within non-limiting substrate ranges, whereas terminal pH is determined by intrinsic acid tolerance and buffer equilibrium [4]. In contrast, uninoculated controls maintained stable pH values between 6.5 and 7.0 throughout the incubation, confirming that the whey protein–sucrose matrix lacks spontaneous acidification capacity and that the observed pH reduction is exclusively attributable to the metabolic activity of marine-derived R-1. Collectively, sucrose concentrations of 2–8% all supported rapid establishment of a stable acidic environment (pH < 4.0), which not only exerts direct antimicrobial effects but also stabilizes antimicrobial peptides and promotes the accumulation of synergistic inhibitory factors such as acetic acid, phenyllactic acid, and hydrogen peroxide [22].

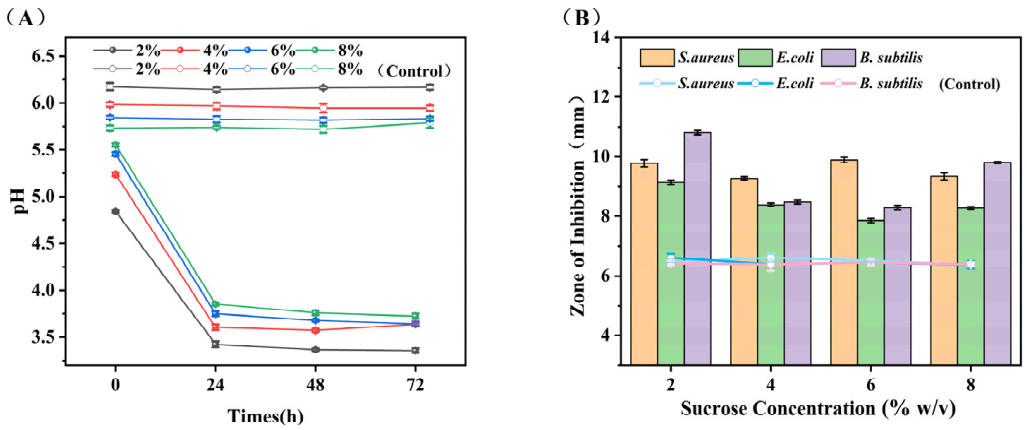

Figure 2. pH values (**A**) and antibacterial activity (**B**) of fermentation with different sucrose concentrations. The uninoculated control in (**A**) was the medium prepared by replacing the seed culture with an equal volume of sterile purified water and processed identically; the control in (**B**) was the uninoculated medium filtrate (UMF) applied in place of the fermentation supernatant.

The antibacterial activity of cell-free supernatants against the three indicator strains exhibited distinct sucrose-dependent patterns (Figure 2B). Against *S. aureus*, inhibition zones ranged from 9.12 to 9.88 mm, with no significant differences among treatment groups ($p > 0.05$), indicating that sucrose concentration within the 2–8% range had little impact on anti-staphylococcal activity. In contrast, against *E. coli*, the strongest inhibition was observed at 2% sucrose (9.21 ± 0.28 mm), which was significantly larger than that of the 6% group (7.86 ± 0.22 mm, $p < 0.05$), with the 4% and 8% groups yielding intermediate values. This non-linear response suggests that excessive sucrose may alter metabolic flux or the ratio of acid production to antimicrobial substance synthesis, thereby diminishing activity against Gram-negative bacteria [27].

Against *B. subtilis*, a non-monotonic trend was also observed: the 2% group exhibited the largest inhibition zone (10.78 ± 0.35 mm), significantly surpassing the 4%, 6%, and 8% groups ($p < 0.05$), indicating that a moderate carbon source level is more favorable for achieving effective inhibition against *Bacillus* species [28]. This sucrose-dependent antibacterial profile is consistent with carbon catabolite regulation of secondary metabolism [27], where optimal sugar availability promotes the biosynthesis of antimicrobial peptides and organic acids, whereas excess sucrose may redirect metabolic flux toward biomass accumulation or exopolysaccharide production at the expense of antimicrobial output. Control supernatants from uninoculated cultures produced no inhibition zones under any sucrose concentration, further confirming that the antibacterial activities observed were specifically

derived from R-1 fermentation metabolism. Taken together, these results demonstrate that low-to-moderate sucrose levels (particularly 2%) are most favorable for inhibiting both *E. coli* and *B. subtilis* under the conditions of 8% whey protein and 5% inoculum, providing a basis for selecting the optimal sucrose concentration in the fermentation formulation for bread preservation applications.

3.1.3. Effects of Inoculum Size on Fermentation pH and Antibacterial Activity

Across all inoculum sizes (2–5%, v/v), the pH of inoculated groups declined rapidly from initial values of approximately 4.8–5.8 to 3.5–3.8 within the first 24 h, followed by stabilization at 3.4–3.6 through 48 h and 72 h (Figure 3A), indicating that organic acid production quickly brought the system to a stable acidic phase. Notably, the 2% inoculum group exhibited a slightly greater pH drop during the first 24 h compared to higher inoculum sizes (3–5%). This seemingly counterintuitive observation can be attributed to the fact that a moderate initial cell density helps shorten the lag phase and accelerate organic acid accumulation [29], whereas excessively high inoculum sizes may cause rapid early-stage nutrient depletion and metabolic feedback inhibition, thereby limiting subsequent acidification potential.

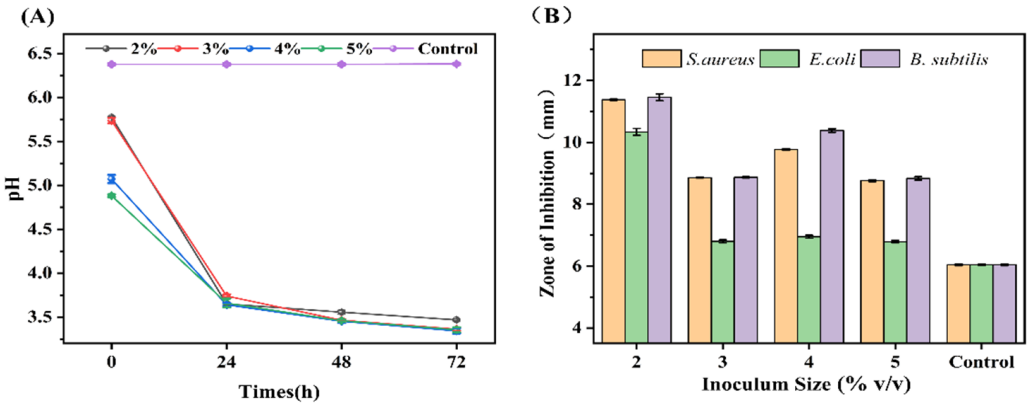

Figure 3. pH values (**A**) and antibacterial activity (**B**) of fermentation with different inoculum sizes. The uninoculated control in (**A**) was the medium prepared by replacing the seed culture with an equal volume of sterile purified water and processed identically; the control in (**B**) was the uninoculated medium filtrate (UMF) applied in place of the fermentation supernatant.

However, at 48 h and 72 h, the final pH values among all inoculated groups converged to comparable low levels, suggesting that the acid-producing potential of R-1 is primarily limited by the total amount of fermentable substrates and metabolic balance, rather than by inoculum size. Increasing inoculum size thus primarily accelerates the achievement of acidification rather than further lowering the final pH. This observation aligns with reports on *L. plantarum* in milk fermentation, where increasing inoculum size from 1% to 3–10% accelerated acid production yet terminal acidity converged, as high cell density resulted in rapid depletion of essential nutrients [29]. In contrast, uninoculated controls maintained a stable pH of approximately 6.3 throughout the 72 h period, confirming that acidification was exclusively attributable to the metabolic activity of R-1.

The antibacterial activity of cell-free supernatants against the three indicator strains exhibited clear inoculum-dependent patterns (Figure 3B). Against *S. aureus*, the strongest inhibition was observed at 2% inoculum (11.46 ± 0.33 mm), significantly higher than other inoculum groups ($p < 0.05$), while the 3–5% groups yielded inhibition zones of approximately 9.56–10.23 mm with no significant differences among them, indicating that increasing inoculum size beyond 2% does not enhance anti-staphylococcal activity. Against *E. coli*, a U-shaped trend was observed: the 2% group (8.45 ± 0.26 mm) showed

significantly stronger inhibition than the 3% and 4% groups ($p < 0.05$), while the 5% group (7.45 ± 0.23 mm) was not significantly different from the 2% group, suggesting that excessively high or low inoculum sizes may favor anti-Gram-negative activity through different metabolic regulation mechanisms.

Against *B. subtilis*, the 2% inoculum group again exhibited the largest inhibition zone (10.95 ± 0.31 mm), significantly surpassing the 3–5% groups ($p < 0.05$), which showed similar zones of approximately 9–10 mm with no significant differences. This consistent "low-inoculum superiority" across all three indicator strains aligns with the commonly observed cell density-dependent metabolic regulation in LAB fermentation, where antimicrobial substance accumulation is governed by the interplay of quorum sensing thresholds, nutrient availability, and metabolic feedback inhibition [30]. Control supernatants from uninoculated cultures produced no inhibition zones under any inoculum size, further confirming the specificity of the observed antibacterial activities.

Overall, integrating the pH and antibacterial results, an inoculum size of 2% was sufficient to drive R-1 to produce high levels of antimicrobial metabolites and achieve broad-spectrum inhibition against both Gram-positive (*S. aureus*, *B. subtilis*) and Gram-negative (*E. coli*) bacteria. This "low-inoculum high-efficiency" phenomenon is consistent with recent studies on cell density-dependent metabolic optimization in LAB fermentation [31]: moderate initial cell density helps maintain orderly activation of quorum sensing signaling systems, avoiding rapid nutrient depletion and metabolic feedback inhibition associated with excessively high density, thereby maximizing secondary metabolite production. For bread applications, an inoculum size of 2% was selected, as it yielded antibacterial effects comparable to or better than higher inoculum levels, while offering additional advantages in terms of reduced fermentation costs and simplified processing.

3.1.4. Antimicrobial and Antifungal Activity of the Optimized Fermentation Product

To validate the antimicrobial efficacy of the optimized fermentation product, the supernatant obtained under the combined optimal conditions (8% whey protein, 2% sucrose, and 2% inoculum) was evaluated against three bacterial indicators and one fungal indicator.

Against *S. aureus*, *E. coli*, and *B. subtilis*, clear and well-defined inhibition halos were observed around wells containing the fermented supernatant, whereas the matrix control (UMF) produced no inhibition zones) (Figure 4A). The sharp edges of the inhibition halos suggest that the active components diffused uniformly through the agar matrix and maintained stable concentrations above their respective minimum inhibitory concentration thresholds [3,30]. Beyond this qualitative confirmation, the antibacterial activity of the supernatant obtained under the combined optimal conditions was quantitatively compared with the best results from the individual single-factor optimizations. As shown in Table 1, the inhibition zone diameters against *S. aureus*, *E. coli*, and *B. subtilis* reached 15.38 ± 0.28 mm, 15.25 ± 0.31 mm, and 15.88 ± 0.35 mm, respectively. These values represent the highest or among the highest levels achieved across all single-factor experiments: for *S. aureus*, the value (15.38 mm) markedly exceeded the best inoculum size result (11.46 mm); for *E. coli*, it was comparable to the best whey protein result (13.74 mm); and for *B. subtilis*, it surpassed both the best whey protein result (15.31 mm) and the best sucrose result (10.78 mm). This enhanced and broad-spectrum efficacy, observed descriptively relative to the single-factor optima, indicates that the combined optimal conditions supported the concurrent production of antimicrobial metabolites effective against both Gram-positive and Gram-negative indicators, with the resulting activity matching or exceeding the best values obtained in the individual single-factor experiments. Because the present optimization used a single-factor-at-a-time design, a full factorial or response surface design would be required to rigorously test for non-additivity among the three factors, and this is a

direction for future work. These quantitative data are fully consistent with the qualitative inhibition halo observations, collectively confirming that the R-1 fermentate exerts potent and reproducible antibacterial activity against both Gram-positive (*S. aureus*, *B. subtilis*) and Gram-negative (*E. coli*) spoilage-associated bacteria.

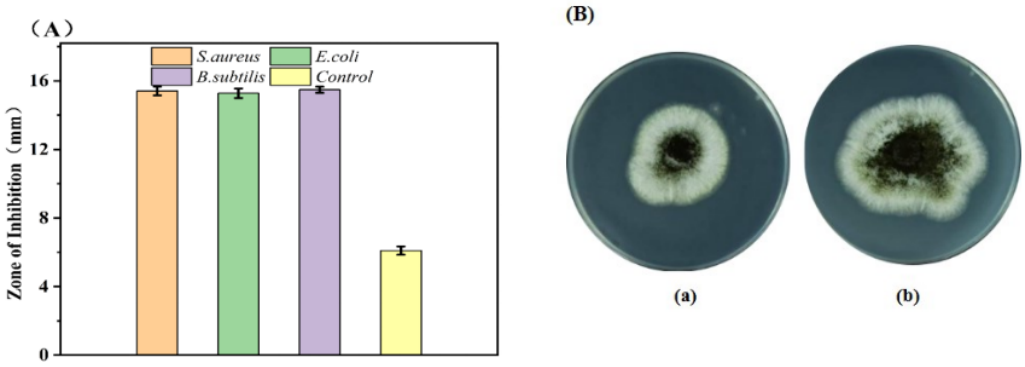

Figure 4. Inhibition zones of the fermentation supernatant against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* (**A**), and its inhibitory effect on *Aspergillus niger.* (**B**). Note: (**a**) fermented supernatant group; (**b**) matrix control (UMF, uninoculated medium filtrate) group.

Table 1. Antimicrobial activity of *Lacticaseibacillus casei R-1* fermented supernatant against indicator microorganisms.

Indicator Strain	Inhibition Zone Diameter (mm)	Inhibition Degree
Staphylococcus aureus	15.38 ± 0.28	++
Escherichia coli	15.25 ± 0.31	++
Bacillus subtilis	15.88 ± 0.35	+++
Aspergillus niger	15.42 ± 0.19	++

Note: The Oxford cup (well) diameter was 6.0 mm, and inhibition zone diameters are reported as the total diameter including the well. The degree of inhibition was graded according to the inhibition zone diameter: ++, 12.0–18.0 mm; +++, 18.0–24.0 mm. Values are the mean ± standard deviation ($n = 3$). CFS, cell-free supernatant.

Beyond its antibacterial effects, the optimized fermentation product also exhibited significant antifungal activity against *A. niger* (Figure 4B), a common mold contaminant in bakery products. Compared with the untreated control, the colony diameter of *A. niger* was markedly reduced upon exposure to the fermented supernatant, with suppressed mycelial growth and loss of the characteristic rapidly expanding villous morphology at the colony margin. This morphological alteration suggests that the fermentate interferes with fungal development in addition to its antibacterial action. Previous studies have reported that the antifungal activity of LAB fermentation products may involve multiple synergistic pathways: organic acids—particularly acetic acid and phenyllactic acid—can disrupt fungal cell membrane integrity and inhibit spore germination; certain cyclic dipeptide metabolites might interfere with chitin synthesis in the fungal cell wall; additionally, nutrient competition and biofilm formation could further restrict mold colonization [28]. Notably, the same organic acids and antimicrobial peptides implicated in antibacterial activity may also contribute to these antifungal effects, suggesting that the R-1 fermentate likely exerts its preservative function through a multi-target, broad-spectrum mode of action.

Collectively, these results demonstrate that the marine-derived *L. casei* R-1 fermented supernatant possesses stable and visible broad-spectrum antimicrobial activity, effectively inhibiting both bacterial spoilage organisms (*Bacillus* spp., *Staphylococcus* spp.) and fungal contaminants (*Aspergillus* spp.)—the two primary microbial threats to bread shelf life during ambient storage [9]. The enhanced activity under the combined optimal conditions underscores the importance of multi-factor optimization in maximizing the production of

bioactive antimicrobial components. This dual inhibitory capacity, achieved without the addition of chemical preservatives, provides direct experimental evidence for the potential application of the R-1 fermentate as a natural biopreservative in bread formulations and offers a practical pathway toward chemical-free baked products.

3.2. Analysis of Bioactive Components in the R-1 Fermentate

3.2.1. Changes in Bioactive Components Before and After Fermentation

To elucidate the biochemical basis underlying the antimicrobial activity of the R-1 fermentate, the contents of total phenolics, peptides, and free amino acids were determined before and after fermentation under the optimized conditions. The results are presented in Figure 5.

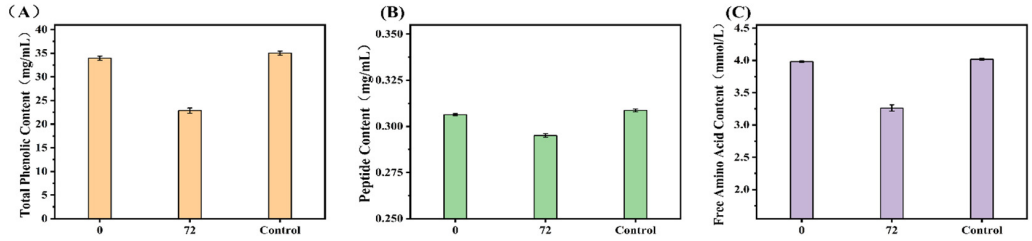

Figure 5. Changes in bioactive components before and after R-1 fermentation. (**A**) Total phenolic content; (**B**) Peptide content; (**C**) Free amino acid content.

Total phenolic content decreased significantly from 35.02 ± 0.71 mg·mL^{-1} in the uninoculated control to 22.86 ± 0.63 mg·mL^{-1} in the fermented group ($p < 0.05$). This reduction likely reflects multiple concurrent processes during fermentation: phenolic compounds may be assimilated by R-1 as carbon or energy sources [4]; others may undergo oxidative polymerization or form bound-state complexes with proteins and polysaccharides [29], thereby reducing the measurable soluble free phenolic fraction. Notably, such conjugation can alter the chromogenic intensity in Folin–Ciocalteu assays without necessarily indicating a loss of bioactivity [20]. Indeed, phenolic–peptide conjugates formed during LAB fermentation may exhibit enhanced membrane permeability and targeted bioactivity [21]. Collectively, these observations suggest that the decline in total phenolic content reflects a dynamic biotransformation process rather than a net loss, in which bound phenolic compounds are released as free forms and subsequently converted into other bioactive metabolites such as phenolic acid derivatives, potentially enhancing the bioaccessibility and antimicrobial functionality of the fermentation product.

Peptide content decreased slightly from 0.306 ± 0.012 mg·mL^{-1} before fermentation to 0.290 ± 0.010 mg·mL^{-1} after fermentation, though the difference was not statistically significant ($p > 0.05$). This modest change suggests that under the optimized fermentation conditions, the proteolytic activity of R-1 was limited, and substantial net accumulation of small peptides—as detectable by current methods—had not occurred within the 72 h fermentation period. Alternatively, a synthesis–degradation steady state may be operative: while some proteins are hydrolyzed by extracellular proteases to generate peptides, a portion of these peptides may be further degraded by peptidases into free amino acids or assimilated by R-1 as nitrogen sources, maintaining relatively stable total peptide levels. It is therefore plausible that the antibacterial effects of the fermentate are primarily mediated through acidification and the production of low-molecular-weight organic acids, rather than through massive peptide accumulation. Nevertheless, the absence of a significant change in total peptide content does not preclude qualitative shifts in peptide composition; specific peptides with antimicrobial activity may have been generated during fermentation, even though their net concentration remained unchanged. Future peptidomics studies are

warranted to characterize the peptide profile and identify potential bioactive sequences contributing to the observed antibacterial effects.

Free amino acid content decreased markedly from 2.54 ± 0.08 mmol/L before fermentation to 1.87 ± 0.06 mmol/L after fermentation ($p < 0.05$), indicating active nitrogen assimilation during R-1 growth. This decline reflects the utilization of free amino acids as nitrogen sources for cell proliferation and metabolic regulation, as well as their role as precursors in the biosynthesis of antimicrobial metabolites. Specifically, deamination of certain amino acids may generate glycolytic intermediates (e.g., pyruvate, α-ketoglutarate), which can be further converted into organic acids such as lactic and acetic acid, thereby contributing directly to antibacterial activity. In addition, amino acid decarboxylation may yield biogenic amines and other derivatives with potential antimicrobial effects, while specific amino acids—particularly glycine, serine, and cysteine—may serve as precursors for thiol-containing antimicrobial compounds or participate in post-translational modifications of bacteriocin-like peptides [30]. Collectively, these nitrogen-driven metabolic pathways are metabolically coupled with organic acid and bacteriocin biosynthesis, suggesting that modulation of nitrogen flux could further enhance the antimicrobial properties of the fermentation product, although this warrants future investigation.

3.2.2. Organic Acid Composition Analysis

R-1 fermentation significantly altered the organic acid composition and content in the fermentation supernatant (Figure 6, Table 2). Among all detected organic acids, lactic acid exhibited the most pronounced change, increasing approximately 3.6-fold from 1218.15 ± 18.32 mg·L^{-1} in the control to 4399.65 ± 25.41 mg·L^{-1} in the fermented group ($p < 0.05$). This substantial accumulation indicates that R-1 actively metabolizes carbohydrates via the glycolytic pathway [22], generating lactic acid as the primary fermentation product. The marked increase in lactic acid serves as the key driving force for the rapid pH decline and the establishment of an acidic environment post-fermentation, aligning closely with the pH reduction and enhanced inhibition zones documented in Section 3.1. Beyond its direct pH-lowering effect, high-level lactic acid also could enhance the antimicrobial efficacy of other weak organic acids that are co-produced during fermentation—namely acetic acid and butyric acid—through an acid-effect multi-layer synergy mechanism [26].

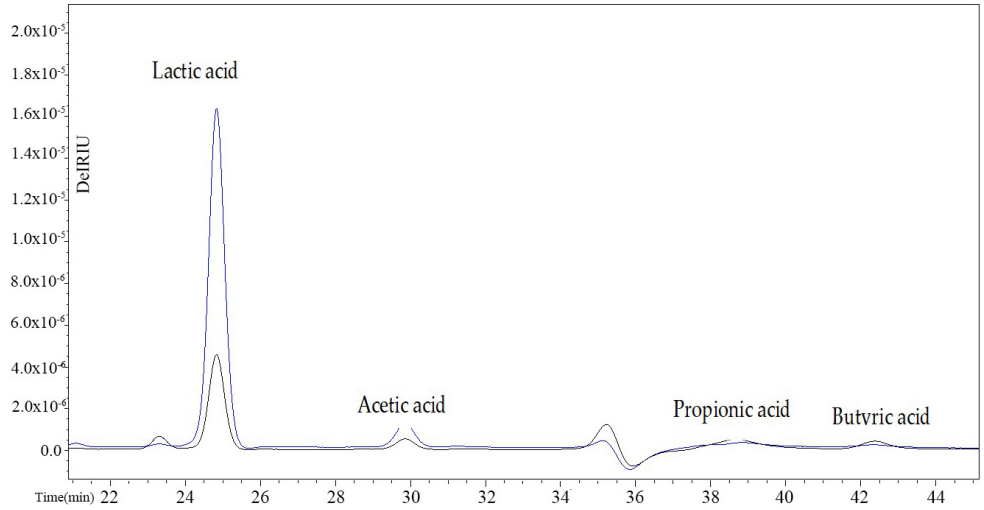

Figure 6. HPLC chromatogram of organic acids in the R-1 fermented supernatant.

Acetic acid content also increased significantly, from 378.28 ± 9.45 mg·L^{-1} to 692.31 ± 15.27 mg·L^{-1} ($p < 0.05$). As a volatile weak organic acid, acetic acid exhibits strong transmembrane diffusion and intracellular acidification capabilities [27], generally

providing more potent antibacterial and antifungal effects than lactic acid at equivalent pH levels. This is attributable to its higher proportion of undissociated molecules at a given pH, facilitating more efficient penetration of microbial cell membranes and intracellular proton release to disrupt the proton motive force [30]. The concurrent production of both lactic and acetic acids by R-1 suggests that this strain operates with a mixed metabolic flux, exhibiting characteristics of both homo- and heterofermentation. This metabolic flexibility results in a multi-organic-acid composite system that broadens the antimicrobial spectrum and enhances functional stability across diverse food matrices.

Table 2. Organic acid composition of whey protein supernatant before and after R-1 fermentation.

Organic Acid	Control ($mg \cdot L^{-1}$)	Fermented ($mg \cdot L^{-1}$)	Change
Lactic acid	1218.15 ± 18.32	4399.65 ± 25.41	↑ 3.6×
Acetic acid	378.28 ± 9.45	692.31 ± 15.27	↑
Propionic acid	957.05 ± 12.67	937.25 ± 14.23	-
Butyric acid	343.98 ± 8.56	425.18 ± 10.34	↑

Note: ↑ indicates an increase in the corresponding organic acid content after R-1 fermentation compared with that before fermentation.

Butyric acid content also increased moderately, from 343.98 ± 8.56 $mg \cdot L^{-1}$ to 425.18 ± 10.34 $mg \cdot L^{-1}$ ($p < 0.05$). Recognized for its synergistic inhibitory effects, butyric acid—particularly through its hydrophobic alkyl chain—can insert into microbial membrane lipid bilayers, increasing permeability and producing cooperative disruptive effects with lactic and acetic acids [22]. Its moderate accumulation likely contributes to the significant growth restriction of *A. niger* observed in Section 3.1.4. In contrast, propionic acid content showed no significant change (957.05 ± 12.67 $mg \cdot L^{-1}$ vs. 937.25 ± 14.23 $mg \cdot L^{-1}$, $p > 0.05$), indicating that R-1 metabolism does not involve the methylmalonyl-CoA pathway characteristic of propionibacteria. This absence of propionic acid production is noteworthy, as it avoids the undesirable pungent and rancid off-odors associated with this organic acid [21], which is particularly advantageous for food preservation applications where sensory quality is paramount.

Overall, the biochemical transformations observed during R-1 fermentation converge on a coherent antimicrobial mechanism centered on organic acid production. Lactic acid drives extracellular acidification, while acetic and butyric acids act through intracellular acidification, together establishing a dual-mode, multi-target antimicrobial system that reduces the likelihood of microbial resistance [22,30]. The absence of propionic acid production further avoids undesirable off-odors, a sensory advantage for food applications. Supporting this organic acid-centric core, the biotransformation of phenolics into potentially more bioavailable forms and the metabolic coupling of free amino acid consumption with antimicrobial metabolite biosynthesis further reinforce the functional synergy of the fermentate, while the stability of total peptide content suggests that peptide accumulation is not the primary contributor to the observed efficacy. This multi-component biochemical network—dominated by organic acids and complemented by phenolic conversion and nitrogen-driven metabolism—thus underlies the broad-spectrum preservative efficacy of the R-1 fermentate and supports its application as a natural, multi-target preservation system for bakery products.

3.3. Quality Evaluation of Bread Supplemented with the Optimized Fermented Supernatant

3.3.1. Effects of Different Supplementation Levels of Lyophilized Powder of Optimized Fermented Supernatant on the Physicochemical and Microbiological Properties of Bread

The pH profiles of bread supplemented with different levels of the optimized supernatant exhibited three distinct patterns (Figure 7A). In the low supplementation groups

(0.3–0.5%), pH dropped rapidly to approximately 5.04–5.12 within the first 3 days of storage, followed by a slight rebound. This "decrease-then-increase" pattern reflects an initial secondary fermentation driven by residual sugars, followed by depletion of fermentable substrates and the buffering action of proteins and starch [9]. In the medium supplementation groups (0.6–0.8%), pH remained relatively stable throughout storage, fluctuating within a narrow range of 5.06–5.21, indicating a dynamic equilibrium between acid production and matrix buffering capacity [32]. This pH range provides sufficient acidity to inhibit spoilage microorganisms (e.g., *Penicillium* spp. and *Aspergillus* spp.) while preventing excessive acidification that could compromise gluten network integrity and sensory attributes [7,9], thus representing an optimal processing window.

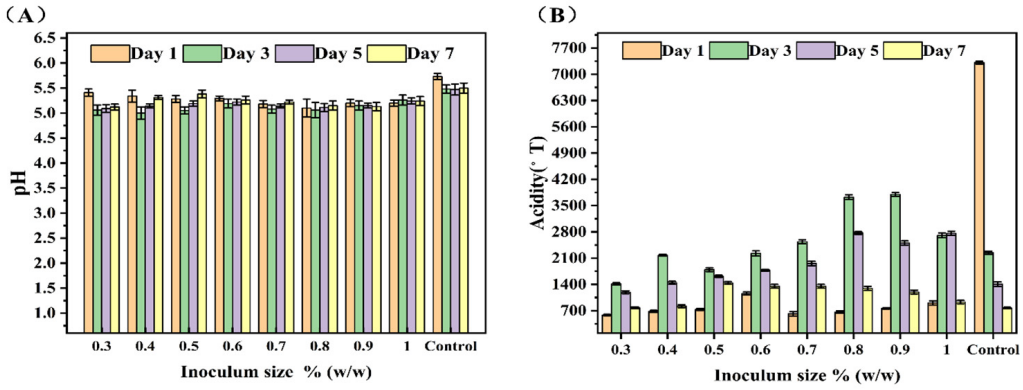

Figure 7. Changes in pH (**A**) and titratable acidity (**B**) of bread supplemented with R-1 fermentate during storage.

In contrast to the medium supplementation groups, the high supplementation groups (0.9–1.0%) exhibited a markedly different pH behavior. pH stabilized at 5.13–5.25 with negligible fluctuation, suggesting a product feedback inhibition phenomenon. This stabilization appears to result from a sequence of events initiated by the high organic acid concentrations at the early stage of storage. The initially high levels of lactic and acetic acids likely induced a rapid pH decline shortly after bread formulation, which in turn may have triggered an acid tolerance response in residual microbial cells. Through downregulation of key glycolytic enzymes such as phosphofructokinase, further acid production was suppressed, thereby preventing a significant pH decline during the later storage period [32]. This feedback inhibition phenomenon aligns with the report by Kawai et al. [33], who demonstrated that high concentrations of metabolites inhibit lactic acid synthesis.

Across all supplementation levels, pH exhibited a characteristic biphasic pattern—rapid decline during the first 3 days followed by stabilization after day 5–7—reflecting active acid production during early storage, succeeded by a plateau phase where nutrient depletion and metabolite accumulation curtail further acidification. Notably, the 0.6–0.8% range achieved effective acidification to pH < 5.2, reaching the mold inhibition threshold while avoiding excessive sourness. In contrast, the high supplementation groups (0.9–1.0%) displayed a product-inhibited steady state, where initial acid stress induced an acid tolerance response that suppressed glycolytic activity and prematurely terminated acid production [32]. Thus, the 0.6–0.8% range represents the optimal equilibrium between effective acidification and avoidance of feedback inhibition, whereas the 0.9–1.0% range reflects a metabolically constrained steady state governed by product inhibition and stress adaptation, rather than a simple extension of the acidification trend.

The titratable acidity trend was essentially inverse to that of pH (Figure 7B), exhibiting a clear dose-dependent pattern. At 0.3% supplementation, acidity increased only modestly over time, reflecting limited substrate availability and restricted metabolic activity [27]. In

the 0.4–0.7% range, acidity rose progressively and peaked on day 3, indicating efficient conversion of fermentable sugars into organic acids. The 0.8–0.9% groups achieved the highest peak acidity among all treatments, confirming this range as the optimal condition for metabolic acid production and nutrient utilization [34]. In contrast, the 1.0% group exhibited a significantly lower acidity peak with a delayed appearance (day 5), followed by a return to baseline levels—suggesting that excessive fermentate concentration exerted feedback inhibition on microbial metabolism. This inhibition likely involves downregulation of glycolytic enzymes (e.g., phosphofructokinase and lactate dehydrogenase) [4] and the induction of an acid tolerance response, which redirects metabolic flux from acid production toward cell maintenance [35]. Thus, the acidification efficacy of the R-1 fermentate is dose-dependent: too low (0.3%) results in limited metabolic activity, moderate levels (0.8–0.9%) support the highest acid production, while excessive supplementation (1.0%) paradoxically weakens acidification through metabolic feedback inhibition and stress adaptation.

The optimized supernatant exerted a pronounced dose-dependent and time-dependent inhibitory effect on both bacterial and fungal contamination (Figure 8, Table 3). By day 7, the total bacterial count in the control group had risen to 1.1×10^3 CFU/g, whereas the 0.8–1.0% supplementation groups maintained stable levels within the 10^1–10^2 CFU/g range—a reduction of 1–2 orders of magnitude; the 0.8% group also exhibited an 84.4% reduction in fungal load (2.5×10^2 CFU/g) compared to the control (1.6×10^3 CFU/g). As storage progressed to day 14, the control group showed exponential bacterial proliferation (1.67×10^6 CFU/g) accompanied by heavy mold growth and visible colonies, indicating complete loss of edibility. In contrast, the high supplementation groups (0.8–1.0%) maintained bacterial counts below 10^4 CFU/g and fungal counts within the 10^2–10^3 CFU/g range at day 14, with no visible mold observed throughout the entire storage period, effectively extending shelf life by 100% relative to the control.

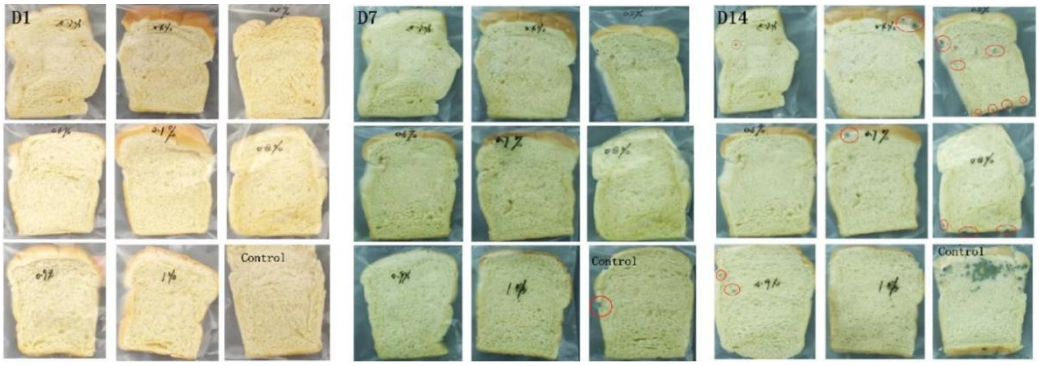

Figure 8. Visual appearance of mold inhibition in bread supplemented with different concentrations of R-1 fermentate after 14 days of storage. The red circles denote the mold growth rate and the associated morphological changes.

The low supplementation groups (0.3–0.6%), however, maintained bacterial counts around 10^5 CFU/g at day 14—an approximately 2-log reduction relative to the control, yet still above the critical threshold of 10^4 CFU/g, manifesting a bacteriostatic rather than bactericidal effect. This limited efficacy at low supplementation levels can be attributed to two factors: first, the acidification–buffering equilibrium was followed by a pH rebound, weakening the persistence of acid stress [9]; second, protease and peptidase activity in the bread matrix may degrade antimicrobial peptides, reducing their effective concentration below the minimum inhibitory concentration [36].

At ≥0.8% supplementation, the marked preservative efficacy is likely attributed to a multi-layered antimicrobial network established by the optimized supernatant. Based on the biochemical composition of the fermentate and previous reports, this network is

proposed to operate through at least three complementary mechanisms. First, organic acids—particularly the lactic acid–acetic acid system—continuously lower the environmental pH, creating sustained acid stress that may force contaminating microorganisms to activate the acid tolerance response (ATR). This process is thought to consume substantial ATP for proton efflux and stress-protective protein synthesis, ultimately leading to energy depletion [37]. Second, bacteriocins and other antimicrobial peptides, which accumulate to concentrations exceeding their minimum inhibitory concentrations, are believed to directly disrupt membrane integrity by binding to lipid II precursors, forming depolarizing pores or inhibiting peptidoglycan synthesis [38]. Third, LAB may competitively deplete essential trace nutrients such as manganese, potentially limiting the secondary germination and proliferation of residual cells and spores [39]. The proposed synergistic interplay of these complementary mechanisms—pH-mediated stress, membrane-targeting peptides, and nutrient competition—likely underpins the potent preservative efficacy observed at ≥0.8% supplementation, although further experimental validation is warranted to confirm their individual contributions.

Table 3. Microbial counts in bread supplemented with different concentrations of R-1 fermentate during storage.

Times	Addition Level (%)	Total Bacterial Count (Log CFU/g)	*Staphylococcus aureus* (Log CFU/g)	Total Fungal Count (Log CFU/g)
7 d	0 (control)	1.1×10^3	6.3×10^3	1.6×10^3
	0.3	8.8×10^2	5.7×10^2	9.9×10^2
	0.4	8.3×10^2	5.7×10^2	8.8×10^2
	0.5	9.1×10^2	6.0×10^2	6.7×10^2
	0.6	7.9×10^2	4.2×10^2	6.1×10^2
	0.7	6.1×10^2	3.8×10^2	4.7×10^2
	0.8	4.7×10^2	3.2×10^2	2.5×10^2
	0.9	3.2×10^2	2.7×10^2	1.0×10^2
	1.0	5.0×10^1	6.0×10^1	5.0×10^1
14 d	0 (control)	1.67×10^6	1.2×10^5	TNTC
	0.3	4.5×10^5	2.7×10^5	TNTC
	0.4	5.2×10^5	1.3×10^5	TNTC
	0.5	3.7×10^5	1.1×10^5	TNTC
	0.6	3.3×10^5	1.1×10^5	TNTC
	0.7	9.9×10^5	8.2×10^5	TNTC
	0.8	7.4×10^4	5.3×10^4	9.9×10^5
	0.9	5.8×10^4	4.3×10^4	9.3×10^5
	1.0	3.6×10^4	1.3×10^4	8.9×10^5

Note: Too numerous to count' indicates that the colony count exceeded 300 CFU/plate and could not be accurately enumerated.

As demonstrated above, the microbiological data reveal a clear dose-dependent transition from bacteriostatic to bactericidal activity, with the critical threshold occurring at 0.8% supplementation. Below this threshold (0.3–0.6%), the fermentate only delays microbial proliferation without eliminating contamination, whereas at or above 0.8%, the multi-layer synergy of organic acids, bacteriocins, and competitive exclusion becomes fully activated, achieving complete mold inhibition and 100% shelf-life extension. This dose-effect inflection point aligns closely with recent findings on LAB fermentates in bread, which demonstrated that a minimum effective concentration is required to establish an adequate inhibitory barrier [40]. When integrated with the pH and acidity profiles discussed above, the microbiological results further converge on the 0.6–0.8% range as the optimal supplementation window: within this range, pH (5.06–5.21) and acidity achieve a stable equilibrium that

effectively inhibits spoilage microorganisms without causing excessive sourness, while the antimicrobial components are present at sufficient concentrations to exert both bacteriostatic and bactericidal effects. Overall, the convergence of pH stability, optimal acid production, and maximal antimicrobial efficacy within the 0.6–0.8% range provides a microbiological safety-oriented quantitative basis for the precise regulation of fermentate dosage in chemical-free bread formulations.

3.3.2. Effects of Supplementation Level of the Optimized Fermented Lyophilized Powder on Bread Quality Properties

The incorporation of the optimized R-1 fermented lyophilized powder exerted a significant dose-dependent effect on bread quality, encompassing texture, color, and flavor profiles (Figures 9 and 10; Table 4). Given that consumer acceptance of bakery products is determined by the interplay of these multiple sensory attributes, a systematic evaluation across the entire supplementation range (0.3–1.0%) was essential to identify the optimal dosage that balances preservation efficacy with overall quality enhancement.

Texture profile analysis revealed a non-monotonic response in bread firmness with increasing supplementation (Figure 9). Hardness decreased progressively from the control (6.35 N) to reach its lowest values (4.12–4.58 N) at 0.6–0.8% supplementation—representing reductions of 27.8–35.1%—before rebounding at 0.9–1.0% (5.89 N). Springiness followed a similar trend, peaking at 0.92–0.95 in the 0.5–0.8% groups, while chewiness decreased synchronously. This biphasic behavior indicates that moderate acidification (pH 5.06–5.21) optimizes gluten network integrity through conformational changes in gluten proteins—increased net positive charge promoting moderate protein unfolding and enhanced hydrophobic interactions—whereas excessive acidity (pH < 5.0) compromises protein structure, diminishing the textural benefits [4,41]. Concurrently, exopolysaccharides and other LAB metabolites present in the optimized lyophilized powder may act as plasticizers, further improving crumb softness and delaying staling [40].

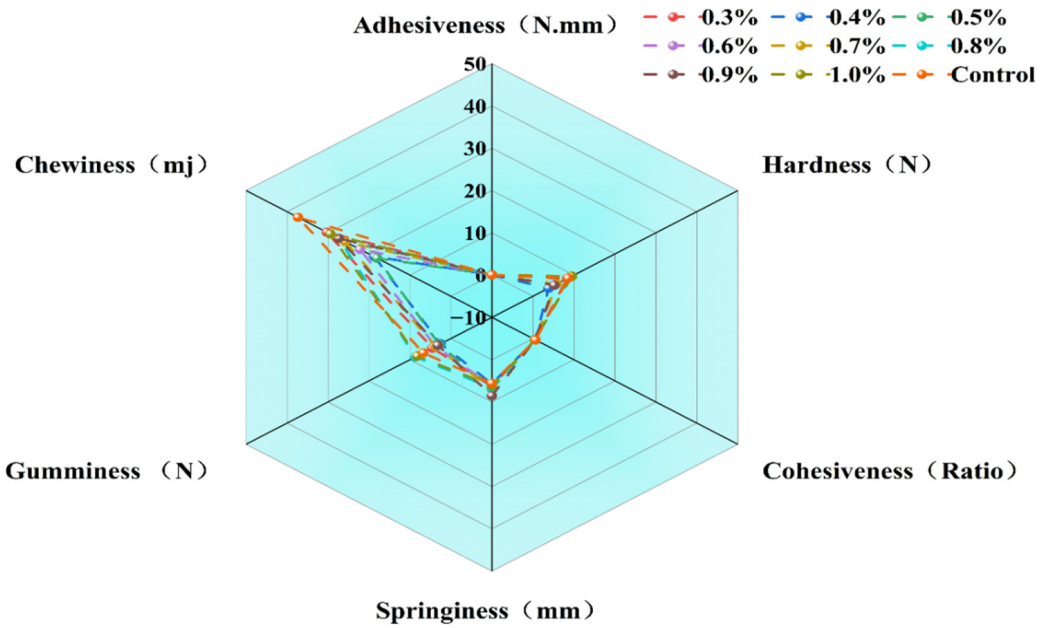

Figure 9. Texture profile analysis of bread supplemented with different concentrations of R-1 fermentate. Parameters measured: hardness, springiness, cohesiveness, chewiness, and adhesiveness.

Colorimetric analysis revealed that the 0.6–0.8% groups exhibited the highest browning index (BI: 861–863), representing a 3.5–3.7% increase over the control (BI: 832), whereas the 0.9–1.0% groups showed markedly lower BI values (BI: 769–849) (Table 4). This

concentration-dependent pattern reflects a balance between two opposing effects: moderate acidification activates endogenous amylases and proteases, increasing the supply of Maillard reaction precursors (reducing sugars and free amino acids) and promoting crust browning; excessive acidification, however, protonates amino groups, impairing nucleophilicity and inhibiting Schiff base formation and subsequent melanoidin accumulation [41,42]. Thus, the 0.6–0.8% range represents the optimal window for both texture and color, achieving the best balance between crumb softness and desirable crust coloration.

Table 4. Color characteristics of bread supplemented with different concentrations of R-1 fermentate.

Addition Level (%)	L*	a*	b*	BI
0 (control)	17.34 ± 0.02	9.33 ± 0.04	27.96 ± 0.06	832.21 ± 7.51
0.3	7.66 ± 0.02 [a]	8.97 ± 0.08 [a]	25.39 ± 0.07 [a]	795.92 ± 10.59 [a]
0.4	15.60 ± 0.01 [a]	8.97 ± 0.08 [a]	25.39 ± 0.07 [a]	856.61 ± 13.68 [b]
0.5	10.53 ± 0.03 [a]	9.57 ± 0.06 [a]	17.62 ± 0.10 [a]	849.53 ± 13.89 [c]
0.6	10.37 ± 0.03 [a]	8.80 ± 0.06 [a]	17.31 ± 0.08 [a]	861.10 ± 16.52 [b]
0.7	11.74 ± 0.01 [a]	8.65 ± 0.05 [a]	19.43 ± 0.05 [a]	863.03 ± 11.27 [a]
0.8	10.44 ± 0.02 [a]	9.59 ± 0.08 [a]	19.43 ± 0.05 [a]	852.63 ± 9.85 [b]
0.9	8.64 ± 0.07 [a]	6.71 ± 0.09 [a]	13.93 ± 0.12 [a]	769.43 ± 23.04 [a]
1.0	13.83 ± 0.02 [a]	9.46 ± 0.07 [b]	22.68 ± 0.07 [a]	848.62 ± 14.49 [c]

Note: Data are expressed as the mean ± standard deviation ($n = 3$). Different lowercase letters within the same column indicate significant differences ($p < 0.05$). Different lowercase letters ([a], [b], [c]) indicate significant differences ($p < 0.05$).

Principal component analysis of volatile compounds revealed that the 0.5% supplementation group occupied a unique position in the flavor chemical space, distinctly separated from both lower (0.3%, 0.4%) and higher (0.6–1.0%) concentration groups (Figure 10A), suggesting that this level represents a critical inflection point for flavor composition. The control group (0%) was distributed in the negative PC1 region, while supplemented groups showed gradient distribution along the positive PC1 direction with increasing concentration, confirming that PC1 primarily captured flavor differences associated with lyophilized powder addition. This distinct clustering aligns with Dong et al. [43], who observed that excessive fermentate concentrations led to decreased volatile compound diversity and simplified flavor characteristics.

GC-IMS analysis identified 45 volatile compounds across all samples, including 5 aldehydes, 13 alcohols, 11 esters, 4 ketones, 1 acid, and 11 others (Figure 10B). Esters were the most abundant category, consistent with LAB esterase activity promoting ester synthesis during fermentation [6,27]. At 0.5% supplementation, a distinctive flavor balance was achieved: C5 unsaturated alcohols (e.g., (Z)-2-penten-1-ol) contributed fresh green and fruity notes, while 3-(methylthio)propanal—a sulfur-containing aldehyde generated via Strecker degradation during the Maillard reaction—provided characteristic crusty and cereal-like aromas [42]. This unique combination was not observed at other concentrations. At lower levels (0.3–0.4%), green and medicinal notes predominated; at higher levels (0.6–1.0%), creamy and roasted coffee-like notes became more prominent, with 2,3-pentanedione (buttery) accumulating significantly at 0.9–1.0% [8,43]. The independent clustering of the 0.5% group in PCA, coupled with the specific enrichment of 3-(methylthio)propanal, validates 0.5% as the optimal point for flavor chemical balance—achieving a harmonious integration of green, baked, and buttery notes [44].

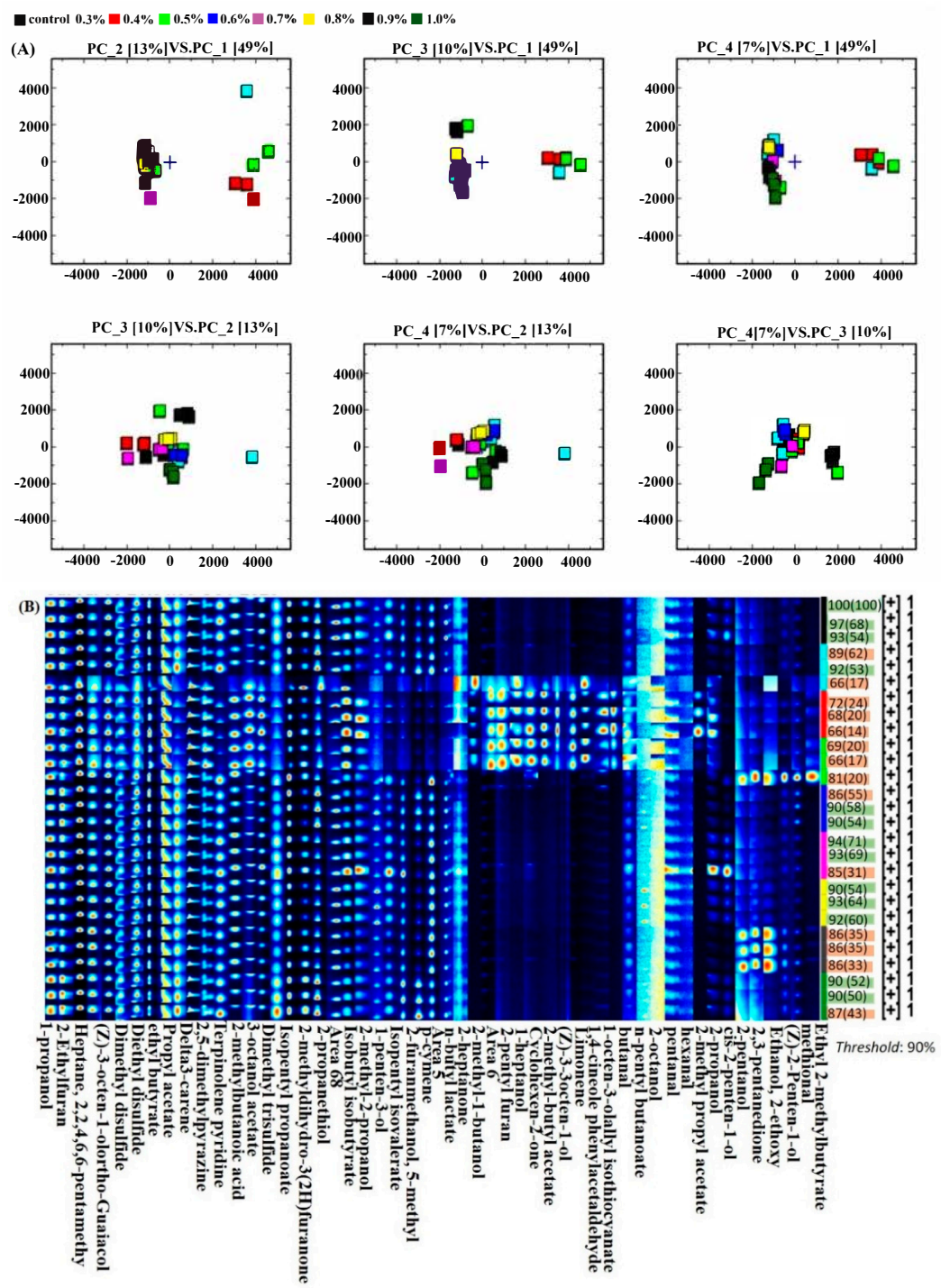

Figure 10. Volatile flavor compound analysis of bread supplemented with different concentrations of R-1 fermentate. (**A**) PCA score plot showing separation of samples based on volatile compound profiles; (**B**) Heatmap of relative abundances of key flavor compounds across treatment groups. Color intensity indicates normalized peak area (blue, low; yellow, high).

The texture, color, and flavor analyses reveal that different quality attributes respond optimally to distinct supplementation ranges: 0.6–0.8% for texture and color, and 0.5% for flavor. However, when these sensory findings are considered alongside the microbiological and physicochemical data discussed in Section 3.3.1, the 0.6–0.8% range emerges as the most practical compromise—effectively balancing preservation efficacy (≥0.8%) with overall quality enhancement. Within this range, bread maintained optimal texture and desirable crust color while retaining a flavor profile sufficiently close to the 0.5% optimum

for consumer acceptance. Thus, the convergence of pH stability, optimal acid production, maximal antimicrobial efficacy, and superior sensory attributes within the 0.6–0.8% range demonstrates that the R-1 lyophilized powder achieves the objective of preservation without compromising, and even improving, quality. This integrated assessment underscores the importance of a holistic approach to dosage optimization, where preservation, texture, color, and flavor must be considered collectively rather than in isolation.

Nevertheless, the 0.6–0.8% range was identified as the most practical compromise based on the parameters measured in this study; several technological and validation aspects remain to be addressed before industrial application. Beyond quantifying key bioactive metabolites, future work should assess additional bread physicochemical parameters, including specific loaf volume (mL/g), crumb density (g/cm^3), porosity, and moisture distribution, to fully define the technological performance of the fermentate. In addition, the antifungal efficacy of the fermentate should be further validated against both Penicillium spp. (e.g., *P. roqueforti* and *P. chrysogenum*) and *Aspergillus niger*. Although *L. casei* R-1 showed no detectable amino acid decarboxylase activity toward histidine, tyrosine, lysine, or ornithine under the tested conditions and is therefore considered non-aminogenic, pilot-scale baking trials across diverse flour matrices and storage conditions are still needed to confirm industrial feasibility and scalability. Future studies should also evaluate consumer sensory acceptance and staling behavior during storage to further support practical application.

4. Conclusions

This study demonstrated that fermentation of the marine-derived strain *Lacticaseibacillus casei* R-1 produces a bioactive fermentate with potent antimicrobial activity and quality-enhancing properties for bread applications. The optimized fermentation conditions yielded a LAB-based fermentate enriched with organic acids, which exert broad-spectrum inhibitory effects against bacterial and fungal spoilage organisms. The combination of the three optimized factors supported concurrent production of these antimicrobial components, although formal synergy was not tested in the present single-factor design. The 0.6–0.8% supplementation range represents the most practical compromise, effectively balancing preservation efficacy with overall quality enhancement. This strategy offers a scalable and clean-label solution for bread preservation while addressing the underexplored potential of marine-derived LAB fermentates in bakery products. Future work should focus on quantitative characterization of key bioactive metabolites and validation across diverse production matrices.

Author Contributions: L.W., Conceptualization, Methodology, Formal analysis, Data curation, Writing—original draft, Visualization and Writing—review and editing. A.L., Conceptualization, Methodology, Formal analysis, Data curation, Writing—original draft, Visualization and Writing—review and editing. M.L., Methodology, Formal analysis, Writing—original draft, and Writing—review and editing. P.W., Data curation, Writing—original draft, Writing—review and editing, Supervision, Project administration, Funding acquisition, and Conceptualization. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the "Xiamen Ocean Research and Development Institute Co-construction Project (K260103)", "Fund of the Technology Innovation Center for Exploitation of Marine Biological Resources, MNR, (TICMBR202402)", "Open Fund of the Marine Biological Resources Development and Utilization Engineering Technology Innovation Center of the Ministry of Natural Resources (TICMBR202402)", "Innovation Research and Development Special Funds of the Municipality-province-ministry Co-constructed (grant number: GJZX-HYSW-2024-09)", "Fujian Science and Technology Program Guiding Project (2025Y0055)".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: We would like to express our gratitude to Xu Tang, Xiaofeng Chen, and Zhiwen Wu for their valuable contributions to the preliminary research for this article. We also extend our thanks to Wenbin Yang and Jian Chen from Jinlezi (Fujian) Food Technology Co., Ltd., Zhangzhou 363000, China, for providing the conceptualization and study design of the baking in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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