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Inhibitory efficacy, production dynamics, and characterization of postbiotics of lactic acid bacteria

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Abstract

Antimicrobial resistance (AMR) poses a significant threat to human health and food safety. Lactic acid bacteria (LAB) produce bioactive compounds, known as postbiotics, that act as promising natural preservatives with broad-spectrum antimicrobial activity. This study aimed to evaluate the antimicrobial spectrum, production dynamics, and physicochemical properties of postbiotics derived from five LAB strains: *Lactobacillus plantarum* NBRC 3070, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393, *Lactobacillus rhamnosus* GG ATCC 53103, and *Bifidobacterium animalis* subsp. *lactis* ATCC 27673. The antimicrobial activity of these postbiotics was assessed against several Gram-positive and Gram-negative pathogens. A crude bacteriocin-like inhibitory substance (BLIS), a postbiotic component, was partially purified using ammonium sulfate purification and characterized enzymatically. Its molecular weight was estimated by SDS-PAGE. The results showed that postbiotics, particularly those from *L. plantarum* and *L. acidophilus*, exhibited strong antimicrobial activity. The inhibitory effect was most pronounced against *Escherichia coli, Salmonella Typhimurium,* and *Staphylococcus aureus* after a 16-h exposure. The postbiotics production peaked between 24 and 36 h of incubation, achieving 85.71–89.28% inhibition. These postbiotics remained stable at high temperatures (up to 121 °C), across a wide pH range (3–5 and 9–11), and under varying salt concentrations. Neutralized cell-free supernatants from *L. plantarum, L. acidophilus, L. casei,* and *L. rhamnosus* GG retained antimicrobial activity, and enzyme treatments confirmed the proteinaceous nature of the BLIS. SDS-PAGE revealed diffuse protein bands between < 3.3 and 6.5 kDa. Lyophilization enhanced the concentration and stability of antibacterial compounds by reducing water content. In addition to BLIS, LAB strains produced other antimicrobial metabolites, including lactic acid, acetic acid, hydrogen peroxide, fatty acids, and notably, oleic acid. These postbiotic components remained effective after one month of storage at 4 °C and 20 °C for one month. The novelty of this study lies in its comprehensive characterization of postbiotics from well-established LAB strains across multiple functional parameters. Overall, the findings suggest that these LAB-derived postbiotics are stable, effective, and hold potential as natural antimicrobial agents in food preservation.

Keywords Antimicrobial substance, Bacteriocins-like inhibitory substances, Postbiotics, Lactic acid bacteria, Storage stability, SDS-PAGE

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Introduction

Foodborne pathogens and spoilage microorganisms threaten human health and food quality, leading to global public health concerns [1]. Notable pathogens such as *Escherichia coli, Salmonella Typhimurium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Serratia marcescens*, *Listeria monocytes, Campylobacter jejuni,* and *Bacillus cereus*, responsible for severe illness and fatalities worldwide [2]. These risks underscore the urgent need for effective agents that enhance food safety and extend shelf life [3]. As consumers demand shift away from chemical preservatives due to health concerns [4], to natural preservation strategies, particularly bio-preservation, have gained momentum [5–8]. Compounding these challenges is the global rise of antimicrobial resistance (AMR) and multi-drug resistance (MDR), which limits the efficacy of conventional antibiotics and preservatives [9]. In response, lactic acid bacteria (LAB) and their metabolites are being explored as natural, broad-spectrum antimicrobial agents with potential to counter AMR in food systems [10]. These bio-preservatives align with the food industries, increasing focus on safety and consumer health [11]. Consequently, recent research emphasizes screening microorganisms capable of producing active biomolecules and characterizing their antibacterial compounds [12].

LAB produces a variety of antimicrobial metabolites, including organic acids, hydrogen peroxide (H_2O_2), carbon dioxide, bacteriocins, and bacteriocin-like inhibitory substances (BLIS), collectively referred to as postbiotics [13]. International Scientific Association for Probiotics and Prebiotics (ISAPP) defines postbiotics as"a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host"[14]. LAB strains such as *Lactobacillus* and *Bifidobacterium* release these compounds into their cell-free supernatant (CFS) during growth [15], thereby contributing to pathogen inhibition [16, 17]. Bacteriocins and BLIS are ribosomally synthesized peptides or proteins classified as "Generally Regarded As Safe" (GRAS) [18]. They exhibit either broad- or narrow-spectrum bactericidal and bacteriostatic activity against foodborne pathogens and spoilage bacteria [19]. Due to their low toxicity, high thermal and pH stability, salt tolerance, lack of resistance development and immunogenicity, and susceptibility to digestive proteases, these compounds are ideal candidates for safe food preservatives [20–23]. The term BLIS is typically used when bacteriocin has not yet been fully characterized at the molecular level [24]. Derived from various LAB species and strains, BLIS shows significant antimicrobial activity against both Gram-positive and Gram-negative bacteria and remains stable across varying environmental conditions [25, 26]. Analytical techniques such as SDS-PAGE and high performance liquid chromatography (HPLC) commonly identify and characterize BLIS in postbiotics [27]. However, challenges to commercialization remain, including the need for thorough physicochemical characterization and stability testing. The production and effectiveness of postbiotics, including BLIS, depend on multiple factors, such as bacterial species, growth conditions, media types and composition, and production strategies [28]. Therefore, optimizing these parameters is crucial for maximizing the efficacy and the industrial applications of postbiotics in meat and food preservation [29].

This study focuses on five commercial probiotic LAB strains: *Lactobacillus plantarum* NBRC 3070, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393, *Lactobacillus rhamnosus* GG ATCC 53103, and *Bifidobacterium animalis* subsp. *lactis* ATCC 27673. While previous research has confirmed these strains' ability to produce antimicrobial substances, such as organic acids, H_2O_2, bacteriocins, and BLIS, their full antimicrobial and physicochemical potential, particularly strains ATCC 4356, ATCC 393, ATCC 53103, and ATCC 27673, remains underexplored [30–34]. Despite increasing interest in LAB-derived postbiotics in the last decades, key knowledge gaps persist in areas such as physicochemical properties, enzyme sensitivity, cultivation conditions (temperature, pH, and salt), inhibition profile, and storage stability of their postbiotic metabolites. Moreover, limited data exist on the partial characterization of BLIS and chemically characterized the CFS from these strains in terms of protein, acetic acid, lactic acid, and FA contributions to antibacterial activity. Most existing literature focuses primarily on the inhibitory effects of bacteriocins or BLIS against specific pathogens like *E. coli, Salm.* Typhi, *and Staph. aureus aureus* [2, 35, 36].

To address these gaps, this study examines the antimicrobial efficacy of LAB-derived postbiotics against a broad range of foodborne pathogens, including *E. coli, Salmonella Typhimurium, Staphylococcus aureus, Shigella sonnei, Pseudomonas aeruginosa, Serratia marcescens, and Bacillus cereus.* Our approach is novel in its integration of production profiling, environmental stability testing, storage assessments, and in-depth chemical analysis of the CFS components. Furthermore, we screened the strains to determine optimal growth conditions (pH and biomass density) for active postbiotic production. Enzymatic treatments were used to confirm the pretentious nature of the active compounds, and semi-purified BLIS was further characterized using ammonium sulfate precipitation and SDS-PAGE analysis. These findings of the study have significant industrial and commercial implications, offering insights into the development of stable, natural preservatives for improving food safety and shelf life while contributing to the broader fight against AMR.

Material and methods

Bacterial strains, media, and cultivation conditions

This study evaluated the antibacterial potency of five commercially available probiotic lactic acid bacteria (LABs) strains: *Lactiplantibacillus plantarum* NBRC 3070 (LP 3070), *Lactobacillus acidophilus* ATCC 4356 (LA 4356), *Lacticaseibacillus casei* ATCC 393 (LC 393), *Lacticaseibacillus rhamnosus* GG ATCC 53103 (LGG 53103), and *Bifidobacterium animalis* subsp. *lactis* ATCC 27673 (BAL 27673). These strains were selected based on their established probiotic status and reported ability to inhibit foodborne and spoilage microorganisms. Antimicrobial activity was tested against seven Gram-positive and Gram-negative indicator strains: *Escherichia coli* ATCC 25922, *Salmonella Typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 43300, *Shigella sonnei* ATCC 25931, *Pseudomonas aeruginosa* ATCC 10145, *Serratia marcescens* ATCC 14756, and *Bacillus cereus* ATCC 14579. These indicators were chosen for their susceptibility to probiotic action, relevance in food spoilage, and availability. All bacterial strains were kindly provided as liquid stocks by the Faculty of Applied Science, School of Biology at Universiti of Technology MARA (UiTM), Malaysia. LAB strains were revived in de Man, Rogosa, and Sharpe (MRS) broth (Condalab, Spain) under anaerobic conditions using RS Biotech, Galaxy S Incubator (UK) at 37 °C for 24 h. Indicator strains were cultured in Luria Bertani (LB) broth (Condalab, Spain) at 28–37 °C for 24 h (Supplementary Table S1). All final cultures were stored at – 80 °C in 40% glycerol (Sigma, EU) using an ultra-low temperature freezer (SANYO, Japan) for further use.

Physical characterizations of postbiotics

Antimicrobial spectrum

The antibacterial spectrum of the cell-free supernatant (CFS) of LAB, containing postbiotics, was assessed via broth microdilution in 96-well plates [37]. LAB strains were inoculated at 2% (v/v) into 50 ml MRS broth and incubated anaerobically at 37 °C for 18 h to produce antimicrobial substances. CFS was collected at the end of the logarithmic phase (OD_{600nm}: 0.52–0.68, ~ 10^8 CFU/mL) using a UV–Vis Spectrophotometer (Thermo Scientific, Multiskan GO, Finland). Cultures were centrifuged (10,000 rpm, 20 min, 4 °C, Eppendorf 5430 R), heated at 80 °C for 20 min (WTB15, Memmert GmbH, Germany), pH-adjusted to 6.0–6.5 with 5 M NaOH (Sigma, Germany), sterile-filtered (0.22 µm Millex PVDF Syringe filter, Merck), and lyophilized (Labconco™, Thermo Fisher Scientific). Lyophilized neutralized CFS (nCFS) was for storage at 4 °C [38]. As per Yi & Kim [37] 40 µL of untreated CFS (rCFS) was poured into 96-well plates (SPL, Korea), followed by 10 µL of overnight indicator cultures (OD_{600nm}: 0.120–0.18) and adjusted to 200 µL with LB broth. MRS broth (pH 6.5) served as the negative control, while cultures without rCFS served as growth controls.

Time-dependent antimicrobial effectiveness

The effect of rCFS exposure time on pathogen inhibition was evaluated via agar well diffusion after 16 and 24 h, following Ohaegbu [39]. LB agar plates were swabbed with indicator inoculum (OD_{600nm}:0.14–0.18). After 1 h, 6-mm wells were pounced using the reverse end of sterile pipette tips filled with 100 µL of rCFS. Plates were allowed to diffuse for 30 min at room temperature, with MRS broth and 0.02% acetic acid used as negative and positive controls, respectively. Inhibition zones were measured using a vernier caliper after incubation.

Evaluation of BLIS

Neutralized, heat-treated CFS (nCFS) retaining antagonistic activity was assumed to contain BLIS. To test this, two-fold serial dilutions of lyophilized nCFS (cCFS) were mixed indicator cultures in 96-well plates, based on Bajpai [40]. Each well received 50 µL of LB broth, 50 µL of cCFS, and indicator inoculum (OD_{600nm}: 0.10–0.14). MRS broth and indicator-only wells served as controls. OD_{600nm} was measured after 24 h of incubation at 37 °C. Inhibition (%) was calculated using Eq. 1:

$$I\%, = [(\mathrm{OD}_{600nm}\mathrm{control} - \mathrm{OD}_{600nm}\mathrm{sample})/\mathrm{OD}_{600nm}\mathrm{control}]\ \mathrm{X}\ 100 \quad (1)$$

All tests were performed in triplicate ($n = 3$), and the results were summarized.

Postbiotics production dynamics

Growth (OD_{600nm}), pH change, and antimicrobial production dynamics of each LAB strain were studied by inoculating 1% (v/v, 10 mL) into 1 L MRS broth (initial pH of 6.20 ± 0.2), incubated at 37 °C for 48 h. At 4-h intervals, samples were taken (10 mL) to measure OD_{600nm} using a spectrophotometer and pH meter using a pH meter (Mettler Toledo SevenCompact S220, Schwiez). The antimicrobial activity of harvested CFS was evaluated using a 96-well broth microdilution using *E. coli* ATCC 25922 as the test strain [41]. A Box-Behnken response surface methodology (RSM) model was used to analyze the effects of incubation time (0–48 h), OD values (01.20), and pH (3.8–6.5) on antimicrobial activity. The RSM model was defined as: $Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$, where Y = antimicrobial activity, X_i = independent variables (time, pH, OD), and β terms as regression coefficients for linear, quadratic, and interaction effects. All tests were conducted in triplicate to ensure the reliability of the results.

Susceptibility of postbiotics to temperature, pH, and salt concentrations

Thermal stability of rCFS was determined by heating 15 mL aliquots at 40℃ 60℃, 80℃, or 100℃ for 30 min in a water bath, autoclaving at 121℃ for 15 min (STURDY SA-300H, Taiwan). pH tolerance was tested by adjusting rCFS to pH 3.0, 5.0, 7.0, 9.0, and 11.0 with 5 M NaOH or HCl, followed by a 2-h incubation at room temperature, and readjusted to pH 6.5 before testing. Salt tolerance was evaluated by supplementing rCFS with 2–10%NaCl and incubating at 37 °C for 2 h. The antimicrobial activity was tested against *E. coli* ATCC 25922 and *Staph. aureus* ATCC 43300 in 96-well plates by mixing treated rCFS with LB broth (1:1) inoculated with 0.05% (v/v) bacterial culture. The assay was performed in 96-well microplates using the broth microdilution method. Untreated rCFS and MRS broth were used as positive and growth controls, respectively. All assays were carried out in triplicate ($n = 3$).

Enzymatic susceptibility of BLIS

To determine the proteinaceous nature of the BLIS, nCFS was treated with proteolytic enzymes: pepsin (pH 3.0, 37 ∘C, 0.1 M HCl), trypsin, and papain (both in pH 7.5, 37 ∘C, 10 mM PBS) at 1.0 mg/mL [26, 42]. Enzymes (Bendosen, Norway) were incubated with nCFS at 37 °C for 4 h, then inactivated by heating at 100 °C for 5 min. Antibacterial activity was assessed via broth microdilution against *E. coli* ATCC 25922, *Salm. Typhimurium* ATCC 14028, *Staph. aureus* ATCC 43300, and *Ps. aeruginosa* ATCC 10145.

Partial purification and characterization of crud BLIS

The study aimed to extract and partially purify BLIS from five LAB strains following the method of [43] with slight modifications. Briefly, 10 mL of LAB strains were cultured in 1 L of MRS broth (2%, v/v) at 37 °C for 24 h under anaerobic conditions in a shaking incubator (BS-1E, Zenith Lab, China) at 125 rpm. The cultures were centrifuged at 10,000 rpm for 20 min at 4 °C, and the pH of the resulting supernatant was adjusted to 6.0–6.5 using 5 M NaOH. The nCFS was then filtered through a 0.22 µm membrane to obtain crude BLIS [44], which was stored at 4 °C for subsequent use. For protein precipitation, 350 g of ammonium sulfate (Duksan, Korea) was gradually added to 500 mL of BLIS (70% saturation) at 4 °C, gently stirring, and incubated overnight. The precipitated proteins were recovered by centrifugation (10,000 rpm, 30 min, 4 °C), dissolved in PBS (0.1 M pH 7.4), and dialyzed overnight using a 3000 Da molecular weight cut-off dialysis Membrane (Thermo Fisher Scientific, USA). This dialyzed filtrate, referred to as'semi-purified BLIS'[45], was assessed for antimicrobial activity against *Salm. Typhimurium* ATCC 14028 before freeze-drying.

The molecular weight of semi-purified BLIS was determined using 12% SDS-PAGE (Bio-Rad, USA), following the procedure described by Islam et al. [46]. Protein bands were visualized with Coomassie Brilliant Blue (CBB) R-250 (Thermo Scientific, USA), and molecular weight was estimated using a pre-stained protein marker (Bio-Rad, USA) ranging from 3.3–31 kDa.

Chemical characterization of cell-free supernatants

Protein analysis

Protein concentrations in rCFS, nCFS, and cCFS from LAB strains were quantified using the Bradford method [47] with modifications. A 5 µL aliquot of each CFS sample was mixed with 250 µL of Bradford reagent and incubated at room temperature for 5 min. Absorbance was measured at 595 nm (OD_{596nm}). A standard curve was generated using serial dilutions of bovine serum albumin (BSA, Thermo-Fisher Scientific, USA), and the protein concentration (mg/mL) was calculated using the following equation: $y = 0.0012x + 0.3572$ ($R^2 = 0.998$), where 'y' is the absorbance measured at OD_{595nm}, '0.0012' is the slop of the standard curve, 'x' is the protein concentration in mg/mL, and '0.3572' stands for the intercept of the standard curve (Supplementary Fig. S1).

Organic acid analysis

To quantify lactic and acetic acid production, 10 mL of the homogenized sample was titrated with 0.25 mol/L NaOH using 1 mL of phenolphthalein (0.5% in 50% alcohol) as an indicator. Titratable acidity was expressed as mg/mL of lactic acid and acetic acid, calculated from their molar equivalents. One milliliter of 1 N NaOH was assumed to neutralize 9.008 mg of lactic acid or 6.005 mg of acetic acid [48].

Hydrogen peroxide analysis

Hydrogen peroxide (H_2O_2) levels were determined by titrating approximately 25 mL of each fermenting sample with 0.1 N potassium permanganate, in the presence of 20 mL of diluted sulfuric acid (H_2SO_4) [48]. Results were expressed in mg, assuming that 1 mL of titrant corresponded to 1.70 mg of H_2O_2.

Fatty acid methyl esters

Fat and fatty acids (FAs) were extracted from cCFS using the hydrolytic method and methylated with methanol and HCl for form fatty acid methyl esters (FAMEs). The FAMEs were analyzed via gas chromatography-mass spectrometry (GC–MS, Shimadzu GC-2010-Plus) equipped with a flame ionization detector (FID) and Supelco SP™–2560 capillary column. Injector and detector temperatures were maintained at 225 °C and 285 °C,

respectively. The oven temperature program was as follows: initial hold at 100 °C for 4 min, ramped at 3 °C/min to 208 °C, and then held for 15 min at 244 °C. Helium was used as the carrier gas at a flow rate of 0.75 mL/min with a split ratio of 200:1. A 3 µL injection volume was used. FAMEs were identified and quantified using the Supelco 37 Component FAME Mix (Sigma-Aldrich, USA).

Storage stability of postbiotics

The rCFS containing antimicrobial substances from five LAB strains was prepared as described earlier and stored at 4 °C and – 20 °C for 15 and 30 days. Antimicrobial activity against *E. coli* ATCC 25922, *Salm. Typhimurium* ATCC 14028, *Staph. aureus* ATCC 43300, and *Ps. aeruginosa* ATCC 10145 were evaluated using the agar well diffusion method. Changes in pH were also analyzed, and the antimicrobial activity of stored samples was compared with freshly prepared rCFS following the method of Silva et al. [49].

Statistical analysis

All experiments were conducted in a completely randomized design (CRD), with LAB strains and indicator pathogens treated as independent variables. Data was analyzed using SPSS software version 25 (IBM Corp., USA, 2023). The parametric test, including one-way analysis of variance (ANOVA), t-test, and repeated measure ANOVA, were performed under the general linear model (GLM) to investigate significant variations among sample means (confidence level: 95%). Duncan's Multiple Range Test (DMRT) was used for post-hoc comparisons. Differences in antibacterial activity among LAB strains and storage times were examined using univariate GLM analysis. Sensitivity differences among pathogenic bacteria were analyzed using one-way ANOVA followed by Duncan's test. The 3D surface plot was constructed using SigmaPlot version 15. Each experiment was replicated in triplicates ($n = 3$), and the data was expressed as mean value ± standard deviation.

Results and discussions

Antimicrobial screening of postbiotics

The global rise of antimicrobial resistance (AMR) has rendered many conventional antibiotics less effective, highlighting the urgent need for alternative antimicrobial agents [50]. In this context, lactic acid bacteria (LAB)-derived postbiotics are gaining attention as natural preservatives capable of inhibiting multi-drug-resistant (MDR) microorganisms while ensuring food safety [3]. Five LAB strains were screened for postbiotics production and evaluated for antibacterial activity using a broth microdilution assay in 96-well plates. The percentage of inhibitory activity against seven indicator strains is presented in Fig. 1 and detailed in supplementary Table S2.

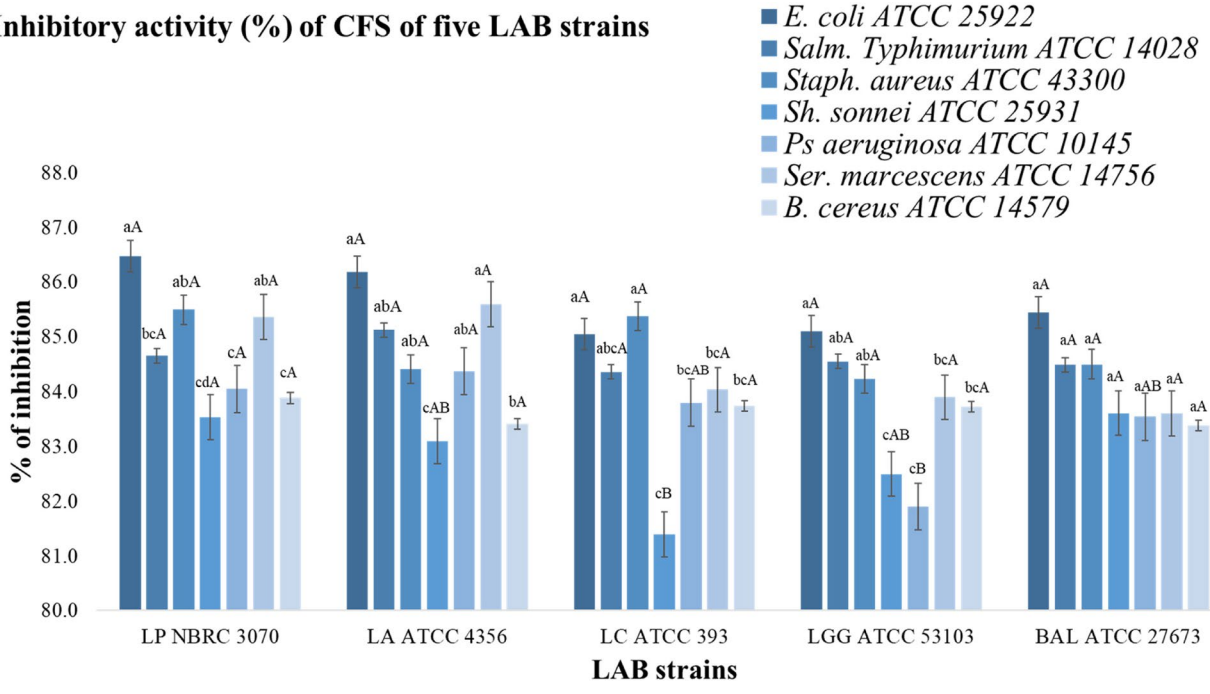

Fig. 1 Inhibitory activity (%) of untreated cell-free supernatant (rCFS) of LAB-containing antimicrobial substances. The inhibition was determined against seven indicator microorganisms assessed by broth microdilution techniques. The error bars indicate the standard error of the mean ($n = 3$). **a**-**c** Represents statistically significant differences among different indicator strains within each LAB strain ($n = 3$, $p < 0.05$, Duncan's test). **A** and **B** Represents statistically significant differences among different LAB strains within each indicator strain ($n = 3$, $p < 0.05$, Duncan's test). LP, *L. plantarum* NBRC 3070; LA, *L. acidophilus* ATCC 4356; LC, *L. casei* ATCC 393; LGG, *L. rhamnosus* GG ATCC 53103, and BAL, *B. animalis* subsp. *lactis* ATCC 27673

Except for *Bifidobacterium animalis* subsp. *lactis* ATCC 27673 (BAL 27673, $p = 0.620$), postbiotics from all LAB strains exhibited significant variability ($p < 0.01$), with inhibition ranging from 81.39 to 86.47%. This suggests a broad-spectrum efficacy against both Gram-positive and Gram-negative pathogens [51–53]. Statistically significant inhibition ($p > 0.05$) was observed only against *Ps. aeruginosa* ($p = 0.023$), while inhibition of other strains was not significant. Notably, LAB postbiotics were most effective against *E. coli* (85.10–86.47%), *Salm. Typhimurium* (86.52–85.29%), and *Staph. aureus* (84.23–85.49%), aligning with previous findings [52]. Research by Qiao et al. [54] Prabhurajeshowar and Chandrakanth [55], and Rouhi et al. [56] demonstrated that postbiotics from various *Lactobacillus* strains significantly inhibit common pathogens including, *E. coli, Salm. enterica, Salm. typhi, Ps. aeruginosa, Staph. aureus, L. monocytogenes* and *Shigella* spp. The inhibitory effect likely stems from cell membrane-disrupting compounds such as organic acids (e.g., lactic and acetic acid) and bacteriocins [16]. Among the strains, *Lactiplantibacillus plantarum* NBRC 3070 (LP 3070) and *Lactobacillus acidophilus* ATCC 4356 (LA 4356) postbiotics exhibited the highest inhibitory activity (83.53–86.47%, $p = 0.000$ and 83.09–86.18%, $p = 0.005$, respectively). *Lacticaseibacillus rhamnosus* GG ATCC 53103 (LGG 53103) postbiotics maintained consistent activity against *E. coli*, *Salm. Typhimurium*, and *Staph. aureus*. In contrast, *Lacticaseibacillus casei* ATCC 393 (LC 393) showed moderate activity, and both LC 393 and LGG 53103 were less effective against *Sh. sonnei* and *Ps. aeruginosa*, with inhibition rates not exceeding 82%. Such strain-specific differences may result from variable concentrations of postbiotics, detection methods, and inherent resistance of specific pathogens [57].

Time-dependent antimicrobial effectiveness

Agar well diffusion assay was used to assess the time-dependent antimicrobial activity of postbiotics at 16-h and 24-h. Statistically significant differences were observed between LAB strains and exposure time ($p < 0.05$) (Supplementary Table S3). As shown in Fig. 2, postbiotics were more effective after 16 h than 24 h, with activity declined by 1.73–14.21%. This decline aligns with previous findings showing that bioactive compounds can degrade or lose synergistic effects beyond 16–18 h [40, 58–60]. Prolonged exposure may also enable pathogens to adapt to acidic environments or active resistance mechanisms [61, 62]. Postbiotics from LC 393 and LGG showed the greatest decline in activity over 24 h

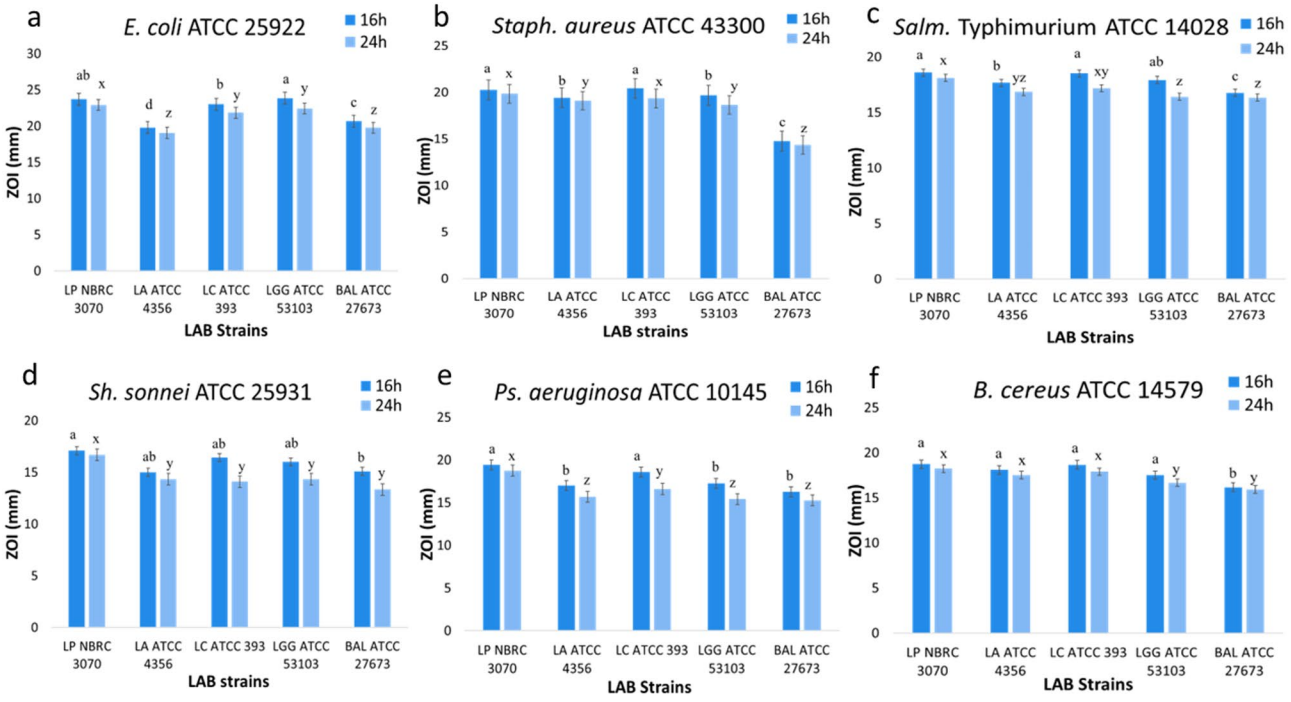

Fig. 2 Inhibition zone of untreated cell-free supernatants from LAB strains incubated for 16 and 24 h. The substances'antimicrobial activity was determined when they were exposed to pathogens of *E. coli* ATCC 25922 (**a**), *Staph. aureus* ATCC 43300 (**b**), *Salm. Typhimurium* ATCC 14028 (**c**), *Sh. sonnei* ATCC 25931 (**d**), *Ps. aeruginosa* ATCC 10145 (**e**), and *B. cereus* ATCC 14579 (**f**). The error bars indicate the standard error of the mean ($n = 3$). CFS, Cell-free supernatant. **a**-**c** Represents statistically significant differences among different CFS of LAB strains exposed to respective pathogens tested for 16 h ($n = 3$, $p < 0.05$, Duncan's test). $^{x-z}$ Represents statistically significant differences among different CFS of LAB strains exposed to respective pathogens tested for 24 h. ($n = 3$, $p < 0.05$, Duncan's test). LP, CFS obtained from *L. plantarum* NBRC 3070; LA, CFS obtained from *L. acidophilus* ATCC 4356; LC, CFS obtained from *L. casei* ATCC 393; LGG, CFS obtained from *L. rhamnosus* GG ATCC 53103, and BAL, CFS obtained from *B. animalis* subsp. *lactis* ATCC 27673

(4.15–14.21% and 4.76–10.64%, respectively), especially against *Sh. sonnei* and *Ps. aeruginosa* (Supplementary Table S4). These findings suggest the potential of LAB postbiotics in short-term applications, such as fresh meat preservation or rapid antimicrobial interventions.

Evaluation of Bacteriocin-like inhibitory substances (BLIS)

To confirm the presence of bacteriocin-like inhibitory substances (BLIS) as active components, heat-treated, pH-neutralized, and lyophilized CFS (cCFS) samples were analyzed. Earlier studies suggest that the lyophilization process itself and the MRS medium reduced oxygen metabolites and H_2O_2 activity [63]. As illustrated in supplementary Fig. S2, all strains except BAL 27673 retained 11.84–44.72% antimicrobial activity, indicating the presence of heat-stable and pH-resistant postbiotic components, such as BLIS [19, 24, 64]. BLIS of LP 3070 and LA 4356 were effective against all tested pathogens, while LC 393 and LGG 53103 were only active against *Salm. Typhimurium* and *Sh. sonnei*, consistent with earlier studies [65–67]. The absence of BLIS activity in BAL 27673 suggests that its antimicrobial activity primarily results from organic acid [68]. These results further confirm that BLIS production is strain-specific ($p < 0.01$).

Dynamics of postbiotics production

The optimum incubation time and temperature are crucial for LAB growth and metabolite production [69]. To see the dynamics of postbiotics production, in this study, LAB strains were cultivated in MRS broth under optimum conditions. Postbiotic production began in the early logarithmic phase (4 h) with inhibition ranging from 17.40–24.74%, at a pH of 5.21–5.27 and OD_{600nm} of 0.23–0.27 (Fig. 3a, b, c). This result explained that LAB strains effectively produce bioactive metabolites for their survival in the early logarithmic phase [70]. Average peak antimicrobial activity (87.50%) occurred during the stationary phase (32 h), corroborating the OD_{600} value of 1.21–1.35 and a pH drop of 3.76–3.84. Postbiotic levels slightly declined after peak production (87.50% to 82.45%, as depicted in Fig. 3), likely due to nutrient depletion, increased organic acid production, and reduced metabolic activity [71, 72]. Previous studies [73–77] indicate that maximum postbiotic levels are usually produced during the mid-to-late exponential or early stationary phase, with peak activity occurring after 24–48 h of incubation [78–80]. LC 393 produced postbiotics with the highest activity during the exponential (16–20 h) and early stationary (24–32 h) phases, followed by LGG 53103. LP 3070 and LA 4356 reached half-maximal activity at 24 h, compared to 16 h for LC 393 and LGG 53103. Response surface plots (Fig. 4) revealed optimal postbiotic production (> 90% activity) conditions of 28–31 h incubation, OD_{600nm} 0.7–1.1, and pH 3.8–4.9. LP 3070 reached maximum activity earlier (28 h), while LC 393 required a higher cell density. LGG 53103 showed the highest activity at the lower pH (3.90), reflecting strain-specific metabolic differences in selection for their industrial and preservative applications.

Susceptibility of postbiotics to temperature, pH, and salt concentrations

The stability of postbiotics under extreme conditions like thermal stability, pH tolerance, and salt tolerance holds great promise for food bio-preservation [81]. For instance, they can be easily incorporated into food formulations without significant alterations to existing processing or can withstand thermal processing (pasteurization), making them practical alternatives to synthetic preservations [82, 83]. Therefore, in this study, thermal stability, pH, and salt tolerance of postbiotics were investigated. As shown in supplementary Table S5, postbiotics retained > 95% of their antimicrobial activity after heat treatment (40–100 °C for 30 min). Even at 121 °C for 15 min, high activity persisted, with only a slight reduction in LP 3070 postbiotics, consistent with reports on bacteriocin from *Lactobacillus* spp. and *Weissella cibaria* [84, 85]. Postbiotics remained active across a wide pH range of 3–11, showing peak activity (≥ 96%) at pH 3–5, supporting their suitability for food preservation in acidic environments [42, 86, 87]. Although activity declined under basic conditions (pH 9–11), residual efficacy (19.75–44.48%) was observed. In this study, LP 3070 and LA 4356 showed diminished activity at neutral pH, while LC 393 and LGG 53103 retained 23.04–26.46% activity. Postbiotics were also found to be salt tolerant, retaining > 97% activity at NaCl concentrations of 2–10%, agreeing with the findings of Piazentin et al. [88] and surpassing the salt stability previously reported by Afrin et al. [43]. They observed activity loss in LAB bacteriocins of *L. plantarum, L. rhamnosus,* and *L. casei,* with increasing salt levels of 1–7%. These characteristics confirm the robustness of postbiotics in various meat and other animal-based food product processing environments, contributing to enhanced microbial safety and extending shelf-life without synthetic additives.

Susceptibility of BLIS to enzymes

The proteinaceous nature of BLIS was confirmed by enzymes susceptibility testing. As shown in Fig. 5, antibacterial activity of cCFS against *E. coli*, *Salm. Typhimurium, Staph. aureus*, and *Ps. aeruginosa* decreased ($p < 0.01$) by 1.28–10.97% following treatment with proteolytic enzymes such as trypsin (Fig. 5a), pepsin (Fig. 5b), and papain (Fig. 5c). This enzyme sensitivity underscores the role of peptide-based BLIS in antimicrobial activity, corroborating prior studies [25, 89]. Ghanbari et al. [90] found that treating *L. casei* AP8 and *L.*

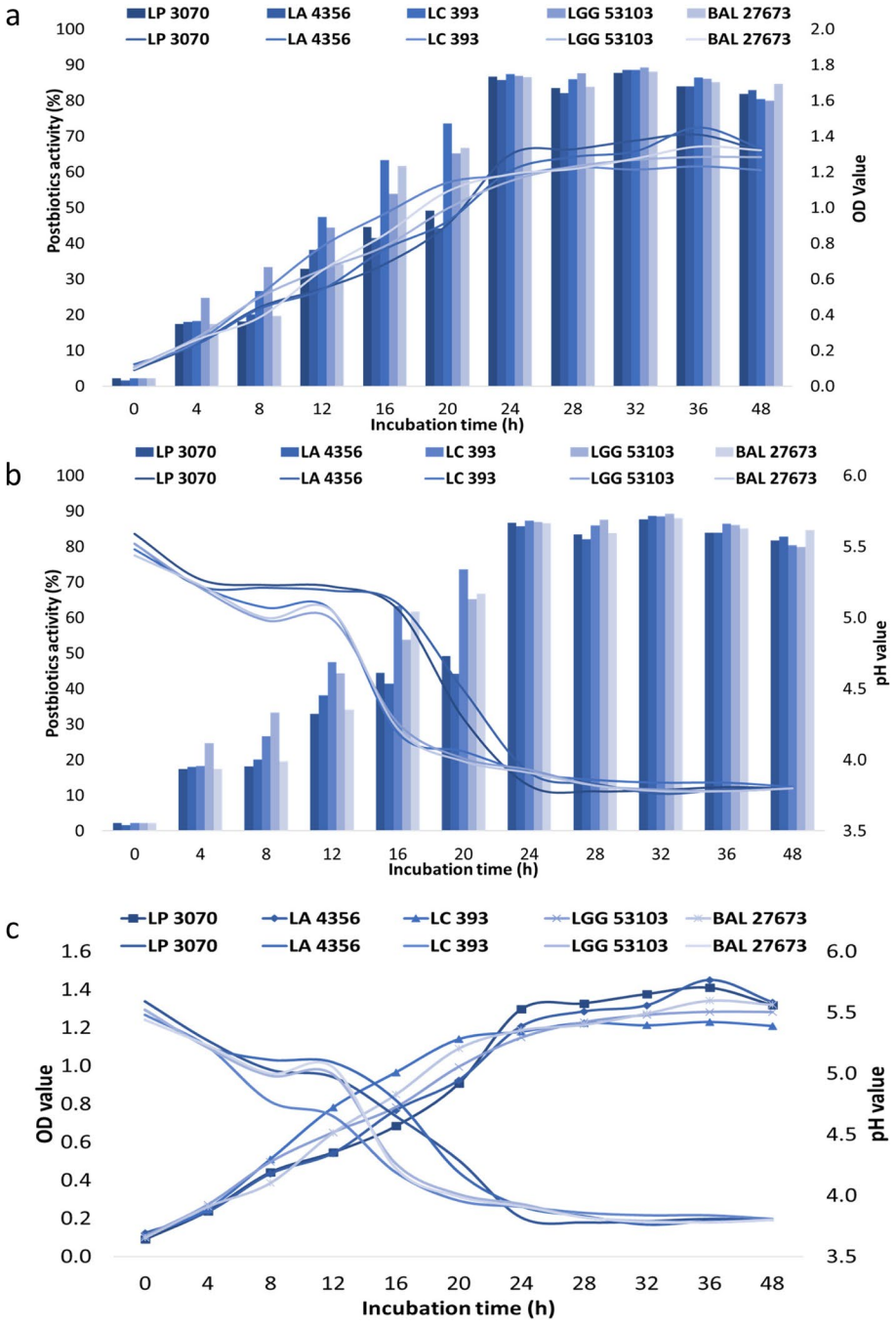

Fig. 3 Effect of OD value and pH changes on the postbiotics activity of LAB strains. The LAB strains were incubated for 48 h at 37 °C, and the inhibitory activity of these postbiotics against *E. coli* ATCC 25922 was influenced by (**a**) cell density (OD value), (**b**) pH values, and (**c**) OD and pH values. The bar graphs represent the antimicrobial activity of postbiotics, and the line graphs represent OD and pH values, respectively. OD values and optical density are measured at OD_{600nm}. LP: OD and pH values from *L. plantarum* NBRC 3070; LA: OD and pH values from *L. acidophilus* ATCC 4356; LC: OD and pH values from *L. casei* ATCC 393; LGG: OD and pH values from *L. rhamnosus* GG ATCC 53103

plantarum H5 CFSs with proteolytic enzymes eliminated their antimicrobial effect, indicating that peptides are the inhibitory components. These enzymes break down proteins into smaller peptides, supporting the idea that BLIS are bacteriocins [91]. As shown in Fig. 5, pepsin, more effective in acidic conditions, cleaves BLIS more efficiently than trypsin and papain. Some remaining activity of BLIS shows it is partially resistant to enzyme breakdown. However, its overall inactivity means that enzymes damage the structure and function of BLIS [92]. This leads to a loss of their ability to disrupt the membranes of harmful bacteria. Thus, the results confirm

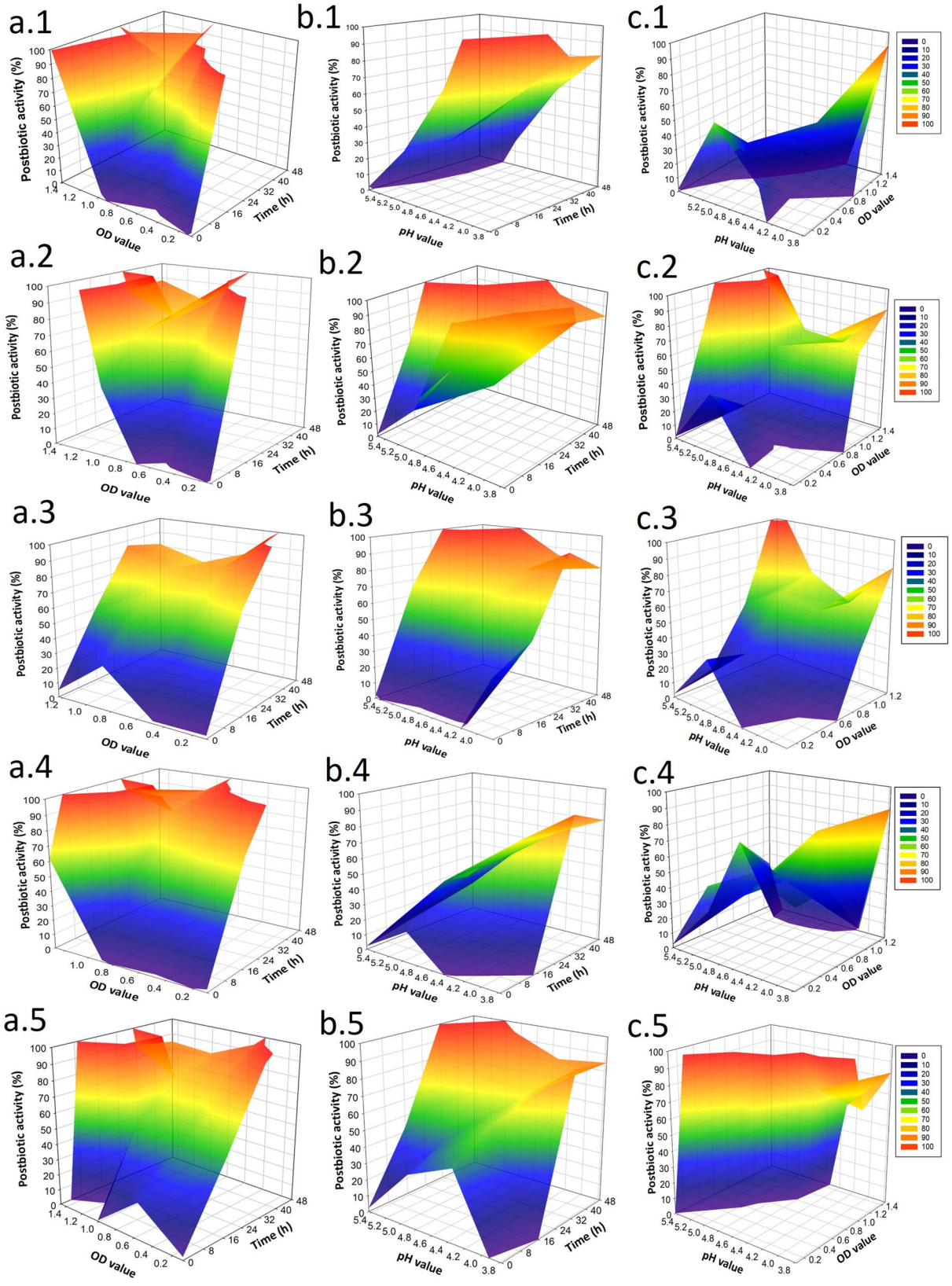

Fig. 4 (See legend on next page.)

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Fig. 4 Response surface plot of postbiotics activity. The Box-Behnken designs of the surface plot addressing parameters as independent variables in the axis of (**a**) incubation time (0-48 h) and OD_{600nm} value (0-1.2), (**b**) incubation time (0-48 h) and pH value (3.8-6.5), and (**c**) OD_{600nm} (0-1.2) and pH values (3.8-6.5). The combined effect of these independent variables changes the response or dependent variables of the antimicrobial activity of postbiotics. They were obtained from *L. plantarum* NBRC 3070 (**1**), *L. acidophilus* ATCC 4356 (**2**), *L. casei* ATCC 393 (**3**), *L. rhamnosus* GG ATCC 53103 (**4**), and (**5**) *B. animalis* subsp. *lactis*

that the active antibacterial components in BLIS are protein-based.

Partial purification and characterization of BLIS

Ammonium sulfate precipitation significantly enhanced the antimicrobial activity of BLIS by enriching bioactive peptides and removing impurities from crude BLIS [93]. In the present study, 70% ammonium sulfate was used to concentrate CFS from four LAB strains, to enhance their activity against *Salm. Typhimurium*. The activity, total protein, yield, volume, and purification fold of the ammonium salt purification are presented in Table 1. Following precipitation, the antimicrobial activity (AU/mL) of the semi-purified BLIS increased substantially, ranging from 6,827 to 10,240 AU/mL (Supplementary Fig. S3), compared to the crude BLIS to 1,067–1,280 AU/mL. The purification process also resulted in a marked increase in specific activity, from 16.29–21.30 AU/mg in crude BLIS to 617.27–876.71 AU/mg in the purified fractions. The purification folds ranged from 32.99–41.15, with recovery yields between 12.80% and 16.00%, indicating successful concentration of bioactive compounds. These findings demonstrated that ammonium sulfate precipitation effectively concentrates and enhances the potency of BLIS, consistent with previous reports showing purification folds between 1.65 and 106.7 for bacteriocins of *Lactobacillus* spp. [94–96].

SDS-PAGE analysis further characterized the molecular sizes of the active components. Coomassie Brilliant Blue (CBB) staining visualized distinct protein bands for BLIS from LP 3070, LA 4356, and LGG 53103, with estimated molecular weights ranging from < 3.3 to 6.5 kDa (Fig. 6). These findings align with the known size range of bacteriocins, or BLIS, which typically range from 0.14 to 8 kDa [94, 97–100], classified into Class I (< 5 kDa) and Class II (< 10 kDa) bacteriocins [19, 101]. Specifically, *L. plantarum* has been reported to produce several low-molecular-weight bacteriocins, such as PNMGL2 (7.62 KDa), M1-UVs300 (3.4 kDa), plantaricin W (2.3 kDa), KL-1Y (3.5 kDa), Z057, Bacteriocin GA15, which are effective in inhibiting foodborne pathogens [1, 102–105]. Similarly, *L. acidophilus* strains have been shown to produce a broader range of bacteriocins, including both smaller (e.g., 30SC, 3.50 kDA; LaKS400, 7.5 kDa) and larger bacteriocins with 37–68 kDa [30, 87, 94, 106]. These may correspond to Class II and Class III bacteriocins depending on their size and structure [30]. On the contrary, *L. rhamnosus* strains in various fermented foods have also been shown to produce bacteriocins with lower molecular weight, such as BCN 1 (0.1427 kDa), BCN 2 (0.605 kDa), and A5 (< 14 kDa) [95, 99, 100, 107]. On the other hand, no detectable protein bands were observed for LC 393 on 12% SDS-PAGE gel, possibly due to low concentrations of antimicrobial peptides or the presence of proteins smaller than the detectable limit (> 3.3 kDa). Small bacteriocins, particularly those below 3 kDa, can be difficult to visualize effectively using standard coomassie-stained DS-PAGE gel [108]. The term "BLIS" is used when the amino acid sequence or gene identity of the antimicrobial compound is unknown [24]. This designation remains appropriate in the current study, where the precise identity of the active peptides remains underdetermined.

Organic acid, protein, and hydrogen-peroxide content in CFS

Postbiotics are increasingly recognized as natural antimicrobial alternatives to mitigate the overuse of antibiotics and address rising AMR, which poses public health and economic challenge [109]. Among these, naturally derived preservatives such as organic acids, H_2O_2, bacteriocins, BLIS, and FAs from LAB are showing antimicrobial potentialities [109]. More specifically, organic acids lower pH levels, creating conditions that inhibit the growth of harmful microorganisms, demonstrating their potential as natural preservatives for food safety [83, 110]. LAB strains are known to produce over 90% of lactic and acetic acid via microbial fermentation [111, 112] This study analyzed the chemical profiles of CFSs from five LAB strains across three forms: untreated (rCFS), neutralized (nCFS), and lyophilized (cCFS). Statistically significant variations ($p < 0.05$) were observed in the level of organic acids, proteins, and H_2O_2 among the strains and CFS forms (Table 2). In rCFS, lactic acid levels ranged from 3.11 to 4.12 mg/mL, with LA 4356 producing the highest amount (4.12 mg/mL). The cCFS demonstrated an increased concentration, especially LA 4356 (6.01 mg/mL) and LP 3070 at 5.81 mg/mL. LC 393 showed moderate levels (3.39 mg/mL in rCFS and 5.08 mg/mL in cCFS). These differences likely reflect strain-specific metabolism and fermentation characteristics [113, 114], with LA 4356 showing enhanced lactic acid production due to its homofermentative nature and acid tolerance [115, 116]. The concentration effect from lyophilization, which removes water and increases the molecular density, further explains the elevated acid levels, consistent

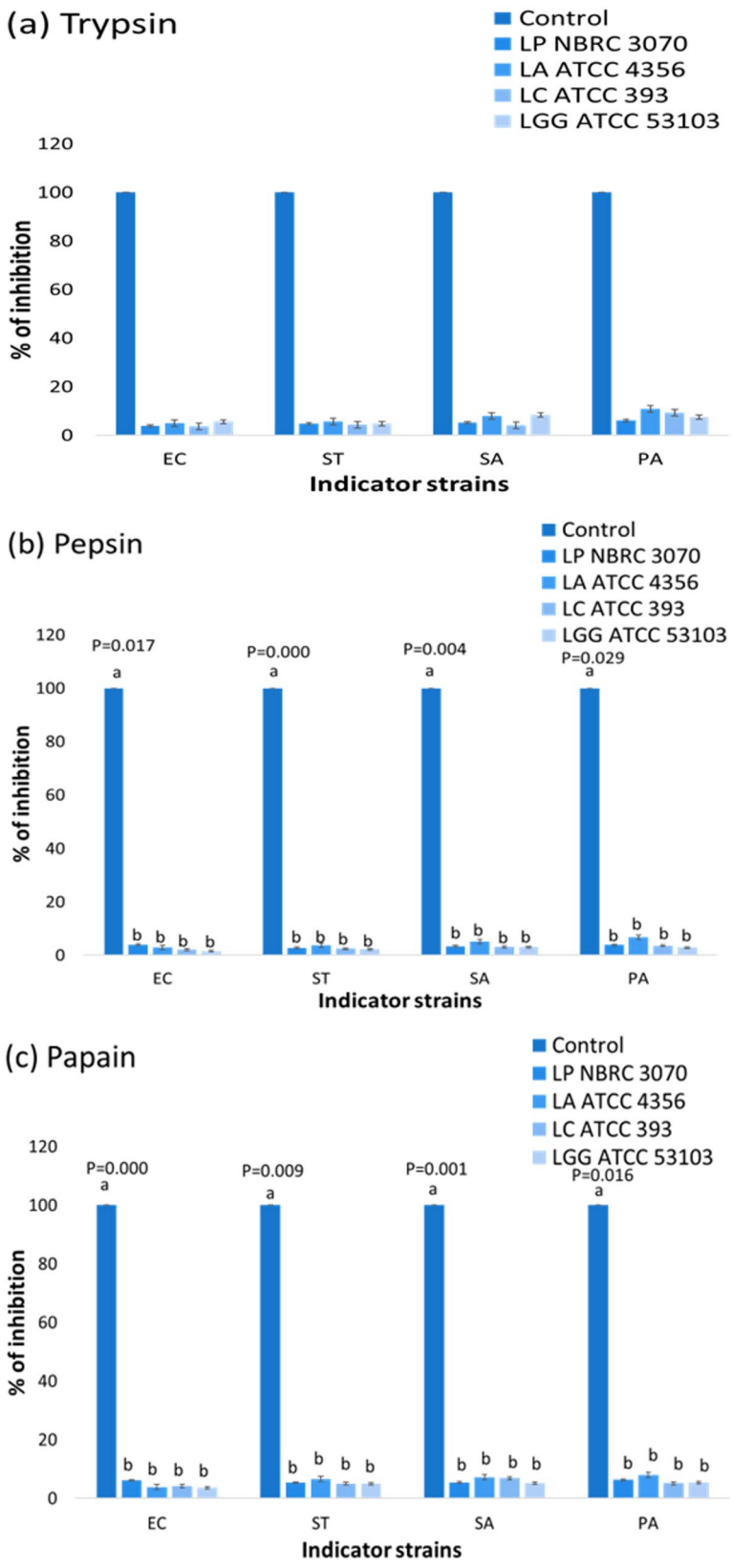

Fig. 5 (See legend on next page.)

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Fig. 5 Inhibitory activity (%) obtained from neutralized cell-free supernatants (nCFS) of four LAB strains. The nCFSs were treated with (**a**) trypsin, (**b**) pepsin, and (**c**) papain in broth microdilution assay against *E. coli* ATCC 25922 (EC), *Salm. Typhimurium* ATCC 14028 (ST), *Staph. aureus* ATCC 43300 (SA), and *Ps. aeruginosa* ATCC 10145 (PA) for 24 h aerobically. The inhibition activity of enzyme-treated nCFS against pathogenic bacteria strains was compared with positive control (100% activity of CFS) cultures with three replications. The error bars indicate the standard error of the mean ($n = 3$). [a] and [b] Represent statistically significant differences within each indicator strain among the BLIS of the LAB strain ($n = 3$, $p < 0.05$, Duncan's test)

Table 1 Partial purification and activity of BLIS from four LAB strains using 70% ammonium sulfate precipitation

LAB Strains	Purification stage	Volume (mL)	Activity (AU/mL)	Protein (mg/mL)	Total protein (mg)	Activity (AU)	Specific activity (AU/mg)	Purification	Yield (%)
LP	CFS	1000	1067	65.49	65,490	1,067,000	16.29	1	100
	$(NH4)_2SO_4$ precipitated CFS	20	8536	13.38	267.6	170,720	637.97	39.16	16.00
LA	CFS	100	1067	54.36	5436	106,700	19.63	1	100
	$(NH4)_2SO_4$ precipitated CFS	2	8536	10.73	21.46	17,072	795.53	40.53	16.00
LC	CFS	100	1067	57.03	5703	106,700	18.71	1	100
	$(NH4)_2SO_4$ precipitated CFS	2	6827	11.06	22.12	13,654	617.27	32.99	12.80
LGG	CFS	100	1280	60.08	6008	128,000	21.30	1	100
	$(NH4)_2SO_4$ precipitated CFS	2	10,240	11.68	23.36	20,480	876.71	41.15	16.00

All yield (%) values are expressed as activity units (AU) in the CFSs, with 100% representing the highest value; Purification folds are expressed as specific activities (AU/mg) in the CFS, with 1 representing the highest value; CFS, Cell-free supernatant; LP, CFS obtained from *L. plantarum* NBRC 3070; LA, CFS obtained from *L. acidophilus* ATCC 4356; LC, CFS obtained from *L. casei* ATCC 393; LGG, CFS obtained from *L. rhamnosus* GG ATCC 53103

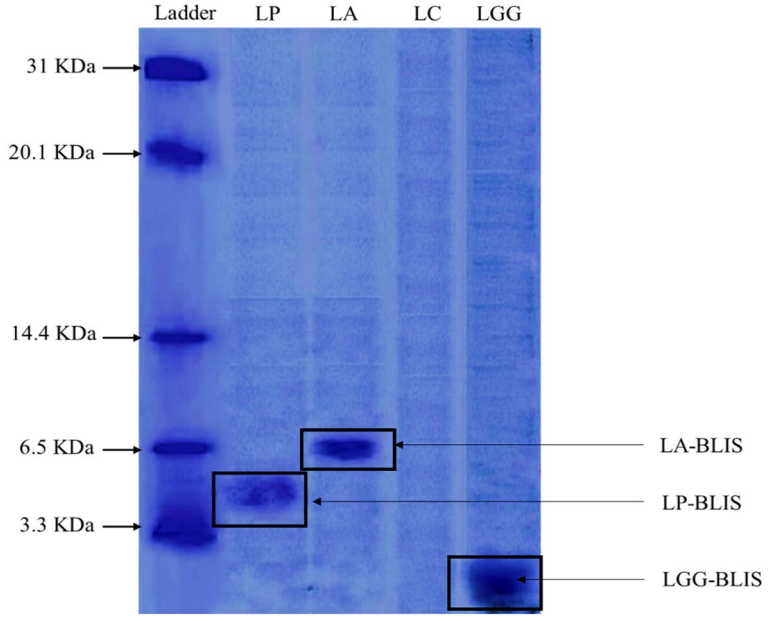

Fig. 6 Molecular weight of extracted bacteriocin-like inhibitory substances (BLIS) by SDS-PAGE analysis. The crude BLIS was obtained through ammonium sulfate purification from the cell-free supernatant of LAB strains. Ladder: pre-stained SDS-PAGE protein markers (Bio-Rad, USA) with a range of 3.3–31 kDa; LP: semi-purified BLIS from *L. plantarum* NBRC 3070; LA: semi-purified BLIS from *L. acidophilus* ATCC 4356; LC: semi-purified BLIS from *L. casei* ATCC 393, and LGG: semi-purified BLIS from *L. rhamnosus* GG ATCC 53103

with previous studies [63]. Lactic acid levels in this study align with the earlier reports [115] from the strains of *L. plantarum* (3.4–73.2), *L. acidophilus* (8.6–14), *L. casei* (6–162), *L. rhamnosus* (4–68), and *B. animalis* (0.32–1.02 mg/mL), though absolute concentrations may vary based on strains, substrates, and fermentation pH [117]. The acetic acid concentrations followed a similar trend. In cCFS, values ranged from 2.12 to 3.08 mg/mL, with BAL 27673 showing the highest production (3.08 mg/mL). In rCFS, acetic acid levels ranged from 1.36 to 1.92 mg/mL, again with BAL 27673 leading (1.92 mg/mL). Generally, the LAB strains produce more lactic acid

Table 2 Concentration of metabolites (mg/mL) in the CFSs (rCFS, nCFS, and cCFS) of five LAB strains

Metabolites	LAB rCFS				
	LP	LA	LC	LGG	BAL
Lactic acid	3.98^{ab}	4.12^{a}	3.39^{b}	3.22^{c}	3.11^{d}
Acetic acid	1.50^{ab}	1.36^{b}	1.48^{ab}	1.46^{ab}	1.92^{a}
Protein	0.65^{a}	0.54^{c}	0.57^{bc}	0.60^{b}	0.57^{bc}
H_2O_2	0.02^{c}	0.03^{b}	0.04^{a}	0.03^{b}	0.04^{a}
	LAB nCFS				
	LP	**LA**	**LC**	**LGG**	**BAL**
Lactic acid	ND	0.02	ND	0.01	ND
Acetic acid	ND	ND	ND	ND	ND
Protein	0.63^{a}	0.49^{c}	0.40^{d}	0.57^{b}	0.57^{b}
H_2O_2	ND	ND	ND	ND	0.003
	LAB cCFS				
	LP	**LA**	**LC**	**LGG**	**BAL**
Lactic acid	5.81^{b}	6.01^{a}	5.08^{c}	4.88^{d}	4.80^{d}
Acetic acid	2.24^{b}	2.12^{c}	2.20^{b}	2.18^{bc}	3.08^{a}
Protein	0.98^{a}	0.84^{b}	0.87^{b}	0.90^{ab}	0.92^{ab}
H_2O_2	ND	ND	ND	ND	ND

[a–d]Represent statistically significant differences among different LAB strains for each metabolite: *rCFS* untreated cell-free supernatant, *nCFS* neutralized cell-free supernatant, *cCFS* lyophilized cell-free supernatant, *ND* not detected. *LP L. plantarum* NBRC 3070; LA, *L. acidophilus* ATCC 4356; LC, *L. casei* ATCC 393; LGG, *L. rhamnosus* GG ATCC 53103; and BAL *B. animalis* subsp. *lactis* ATCC 27673

than acetic acid [118, 119], as supported by the current findings. Previous studies [114, 120–123] indicated that *L. plantarum, L. acidophilus, L. casei, L. rhamnosus,* and *B. animalis* produce acetic acid in the following ranges: 0.7–4.9, 0.822–2.52, 0.2–8.44, 0.4–5.80, and 0.69–0.79 mg/mL, respectively. Although *B. animalis* often produces more acetic acid than lactic acid due to the bifid shunt [124], lactic acid still dominated in this study, likely influenced by the glucose-rich MRS medium and a 48-h fermentation period. The lactic-to-acetic ratio in *B. animals* falls within the optimal 1.5–2.5 range for anaerobic energy efficiency [125]. In nCFS, lactic and acetic acids were barely detectable (≤ 0.02 mg/mL), suggesting effective neutralization during pH adjustment [53].

Protein content was highest in LP 3070 across all CFS forms, reaching 0.98 mg/mL in cCFS. Protein content increased significantly ($p > 0.05$) after lyophilization in all strains (0.84–0.98 mg/ml), likely due to solute following water removal [126]. LA 4356 consistently produces the lowest protein levels (0.54, 0.49, and 0.84 mg/mL for rCFS, nCFS, and cCFS, respectively). These results mirror earlier studies [63], which also reported higher protein concentrations in *L. plantarum* (0.492–0.617 mg/ml) than *L. acidophilus* (0.372–0.523 mg/ml). Regarding H_2O_2, the highest concentrations were observed in rCFS of LC 393 and BAL 27673 (0.04 mg/mL). In contrast, no H_2O_2 was detected in cCFS, and only trace amounts (0.003 mg/mL) were observed in nCFS of BAL 27673. This indicates that H_2O_2 is unstable in neutral or lyophilized conditions and requires an acidic pH for stability. Its production is closely tied to pH and NADH-oxidase activity [127], with degradation likely accelerated during processing. These results suggest that acidification and enzymatic oxidase activity may contribute to higher H_2O_2 levels in specific strains such as LC 393 and BAL 27673. Overall, cCFS samples exhibited the highest levels of lactic acid, acetic acid, and proteins, affirming their suitability for potential postbiotic applications. These findings are consistent with previous findings [128], and chemical profiles of LP 3070 and LA 4356 CFSs are comparable to other widely studied LAB strains like *L. plantarum*, *L. acidophilus,* and *L. casei* [128, 129], though some quantitative differences were observed.

Fatty acid methyl esters composition in CFS

Fatty acids (FA), particularly those incorporated into bacterial membrane structures, play a vital role in the antimicrobial functionality of LAB-derived postbiotics. The membrane lipid profile, including saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA), contributed to both cell membrane fluidity and antimicrobial mechanisms of LAB [130, 131]. In this study, 22 out of 36 fatty acid methyl esters (FAME) were identified in the CFS of five LAB strains using GC–MS (Table 3). These included various SFAs, MUFAs, PUFAs, further classified by chain length and position (n-3, n-6, and n-9). Comparatively, Wong et al. [119] identified 16 FAs *L. plantarum* CFS from 36 FAME, confirming the strain-and condition-specific nature of the FA profile. Among the strains, LC 393 showed the most diverse FA profile ($P < 0.05$), with 21 different FAs detected, followed by LGG 53103 and BAL 27673 (20 FAs each), and LA 4356 and LP 3070 with 13 and 12 FAs, respectively (Fig. 7). Among the eleven SFAs identified, palmitic acid (C16:0) and stearic acid (C18:0) were prevalent, while short-chain SFAs (C6:0 to C18:0) were either low in concentrations or undetectable. Notably, LA 4356 showed a higher level of arachidic acid (C20:0), although its quantity varied across strains. These results corroborated the findings of Lim et al. [132], who identified oleic (C18:1n9c) and palmitic acid (C16:0) as dominant in the CFS of *Weissella cibaria*, CMU. Similarly, Wong et al. [119] reported higher levels of palmitic and stearic acids than oleic acids in *L. plantarum* strains. In this study, six MUFAs were detected, with oleic acid being consistently present across all strains. Cis-11-eicosenoic acid (C20:1) was also observed in some strains, contributing to membrane fluidity and potential antimicrobial functions [133]. Among the five PUFAs, linolenic acid (C18:3n3) was detected in all strains, along with linolelaidic acid (C18:2n6t) and linoleic acid (C18:2n6c), supporting their proposed role in antimicrobial action. LP 3070 and LC 393 had moderate levels of both SFA and PUFA, with

Table 3 Fatty acids composition (%) of lyophilized cell-free supernatant (cCFS) from five strains of lactic acid bacteria

Fatty acid name	Carbon structure	LAB stains (± SEM)				
		LP	LA	LC	LGG	BAL
Caproic acid	C6:0	Nd	Nd	0.193 ± 0.003^{iA}	0.109 ± 0.003^{iB}	Nd
Caprylic acid	C8:0	Nd	Nd	0.371 ± 0.003^{fA}	0.164 ± 0.158^{iB}	0.413 ± 0.002^{efgA}
Capric acid	C10:0	0.110 ± 0.003^{iE}	0.250 ± 0.003^{fD}	0.575 ± 0.007^{fghB}	0.322 ± 0.005^{hiC}	0.615 ± 0.004^{deA}
Undecanoic acid	C11:0	Nd	Nd	0.621 ± 0.004^{fghA}	0.414 ± 0.003^{ghiB}	0.175 ± 0.002^{ghC}
Lauric acid	C12:0	0.150 ± 0.006^{hiD}	0.615 ± 0.008^{fA}	0.343 ± 0.004^{hiB}	0.232 ± 0.005^{iC}	0.355 ± 0.005^{efghB}
Tridecanoic acid	C13:0	Nd	Nd	0.551 ± 0.006^{fghA}	0.367 ± 0.002^{ghiB}	0.159 ± 0.006^{ghC}
Myristic acid	C14:0	0.359 ± 0.008^{ghiE}	0.657 ± 0.005^{fA}	0.614 ± 0.005^{fghB}	0.415 ± 0.003^{ghiD}	0.523 ± 0.008^{efC}
Myristoleic acid	C14:1	0.672 ± 0.006^{fgD}	1.459 ± 0.016^{efA}	0.811 ± 0.008^{fC}	0.416 ± 0.004^{ghiE}	0.885 ± 0.005^{dB}
Pentadecanoic acid	C15:0	Nd	Nd	0.643 ± 0.008^{fghA}	0.387 ± 0.009^{ghiB}	0.199 ± 0.007^{fghC}
Cis-10-pentadecenoic acid	C15:1	Nd	Nd	Nd	4.005 ± 0.004^{bA}	3.810 ± 0.0078^{bA}
Palmitic acid	C16:0	5.465 ± 0.103^{bA}	0.225 ± 0.005^{fD}	4.387 ± 0.049^{bB}	0.472 ± 0.009^{ghiC}	0.654 ± 0.069^{deC}
Palmitoleic acid	C16:1	Nd	0.424 ± 0.021^{fB}	0.759 ± 0.006^{fgA}	Nd	Nd
Heptadecanoic acid	C17:0	Nd	Nd	Nd	Nd	Nd
Cis-10-heptadecenoic acid	C17:1	Nd	Nd	Nd	Nd	Nd
Stearic acid	C18:0	1.834 ± 0.003^{dB}	22.888 ± 0.035^{abA}	1.775 ± 0.019^{dBC}	1.792 ± 0.013^{dBC}	1.72 ± 0.059^{cC}
Elaidic acid	C18:1N9T	Nd	Nd	Nd	Nd	Nd
Oleic acid	C18:1N9C	86.203 ± 0.024^{aA}	$5.196 \pm 0.008d^{efE}$	80.232 ± 0.039^{aD}	84.439 ± 0.029^{aB}	84.182 ± 0.012^{aC}
Linolelaidic acid	C18:2N6T	0.217 ± 0.004^{ghiC}	0.405 ± 0.004^{fA}	0.129 ± 0.001^{iE}	0.269 ± 0.003^{hiB}	0.162 ± 0.004^{ghD}
Linoleic acid	C18:2N6C	0.121 ± 0.002^{iD}	0.267 ± 0.007^{fA}	0.110 ± 0.002^{iD}	0.232 ± 0.050^{hiB}	0.156 ± 0.005^{ghC}
γ-linolenic acid	C18:3N6	Nd	Nd	Nd	Nd	Nd
Linolenic acid	C18:3N3	3.782 ± 0.020^{cB}	$5.635 \pm 0.025d^{efA}$	3.569 ± 0.016^{cC}	3.266 ± 0.014^{cD}	3.572 ± 0.060^{bC}
Arachidic acid	C20:0	1.109 ± 0.021^{efC}	32.006 ± 0.032^{aA}	1.381 ± 0.012^{eB}	1.044 ± 0.011^{eC}	0.942 ± 0.013^{dD}
Cis-11-eicosenoic acid	C20:1	0.447 ± 0.007^{ghiB}	18.824 ± 0.023^{bcA}	0.442 ± 0.006^{ghiB}	0.354 ± 0.006^{ghiC}	0.494 ± 0.013^{efgB}
Cis-11,14-eicosadienoic acid	C20:2	Nd	Nd	Nd	Nd	Nd
Cis-8,11,14-eicosatrienoic acid	C20:3N6	Nd	Nd	Nd	Nd	Nd
Arachidonic acid	C20:4N6	Nd	Nd	1.21 ± 0.017^{eA}	0.734 ± 0.010^{fgB}	0.360 ± 0.005^{efghC}
Cis-11,14,17-eicosatrienoic acid	C20:3N3	Nd	Nd	Nd	Nd	Nd
Cis-5,8,11,14,17-eicosapentaenoic acid	C20:5N3	Nd	Nd	Nd	Nd	Nd
Heneicosanoic acid	C21:0	Nd	Nd	Nd	Nd	Nd
Behenic acid	C22:0	Nd	Nd	Nd	Nd	Nd
Erucic acid	C22:1N9	Nd	Nd	0.168 ± 0.003^{iA}	Nd	0.155 ± 0.007^{ghA}
Cis-13, 16 docosadienoic acid	C22:2	Nd	Nd	1.317 ± 0.023^{eA}	0.729 ± 0.009^{fgB}	0.363 ± 0.014^{efghC}
Docosahexaenoic acid	C22:6N3	Nd	Nd	Nd	Nd	Nd
Tricosanoic acid	C23:0	Nd	Nd	Nd	Nd	Nd
Tetracosanoic acid	C24:0	Nd	Nd	Nd	Nd	Nd
Cis-tetracosenoate acid	C24:1	Nd	Nd	Nd	Nd	Nd
SFA		8.57 ± 0.056^{bBC}	56.61 ± 0.098^{aA}	11.34 ± 0.054^{bB}	5.63 ± 0.027^{bC}	5.75 ± 0.029^{bC}
MUFA		87.32 ± 0.064^{aA}	25.75 ± 0.034^{bB}	82.40 ± 0.064^{aA}	89.20 ± 0.074^{aA}	89.65 ± 0.088^{aA}
PUFA		4.12 ± 0.015^{bB}	6.26 ± 0.024^{cB}	6.26 ± 0.023^{bB}	5.17 ± 0.034^{bB}	4.60 ± 0.026^{bB}
ω−3		3.782 ± 0.029^{bB}	5.584 ± 0.016^{aA}	3.539 ± 0.018^{bBC}	3.266 ± 0.023^{bC}	3.572 ± 0.035^{bBC}
ω−6		0.337 ± 0.013^{cC}	0.678 ± 0.015^{bB}	1.441 ± 0.012^{bA}	1.171 ± 0.019^{bA}	0.67 ± 0.011^{cB}
ω−9		86.203 ± 0.038^{aA}	5.166 ± 0.037^{aB}	80.394 ± 0.065^{aA}	84.439 ± 0.056^{aA}	84.33 ± 0.045^{aA}

[a–i]Represent statistically significant differences among different fatty acids within each LAB strain; [A–D]Represent statistically significant differences among different LAB strains within each fatty acid; LP, *L. plantarum* NBRC 3070; LA, *L. acidophilus* ATCC 4356; LC, *L. casei* ATCC 393; LGG, *L. rhamnosus* GG ATCC 53103; and BAL, *B. animalis* subsp. *lactis* ATCC 27673, *Nd* not detected, *SAF* Saturated fatty acid, *MUFA* Monounsaturated fatty acid, *PUFA* Polyunsaturated fatty acid, *ω−3* Omega-3 fatty acid, *ω−6* Omega-3 fatty acid, *ω−9* Omega-9 fatty acid

higher MUFA levels. LA 4356 had a dominant SFA profile, whereas LGG 53103 and BAL 27673 displayed a more balanced FA distribution. These results support the previous findings [134], that long and medium-chain FAs, especially oleic and linoleic acid, have significant antimicrobial effects against Gram-positive foodborne pathogens. In summary, the FA composition of the LAB CFSs not only differed among strains but also reflected their potential contribution to antimicrobial properties. The presence of key MUFAs and PUFAs suggests a role

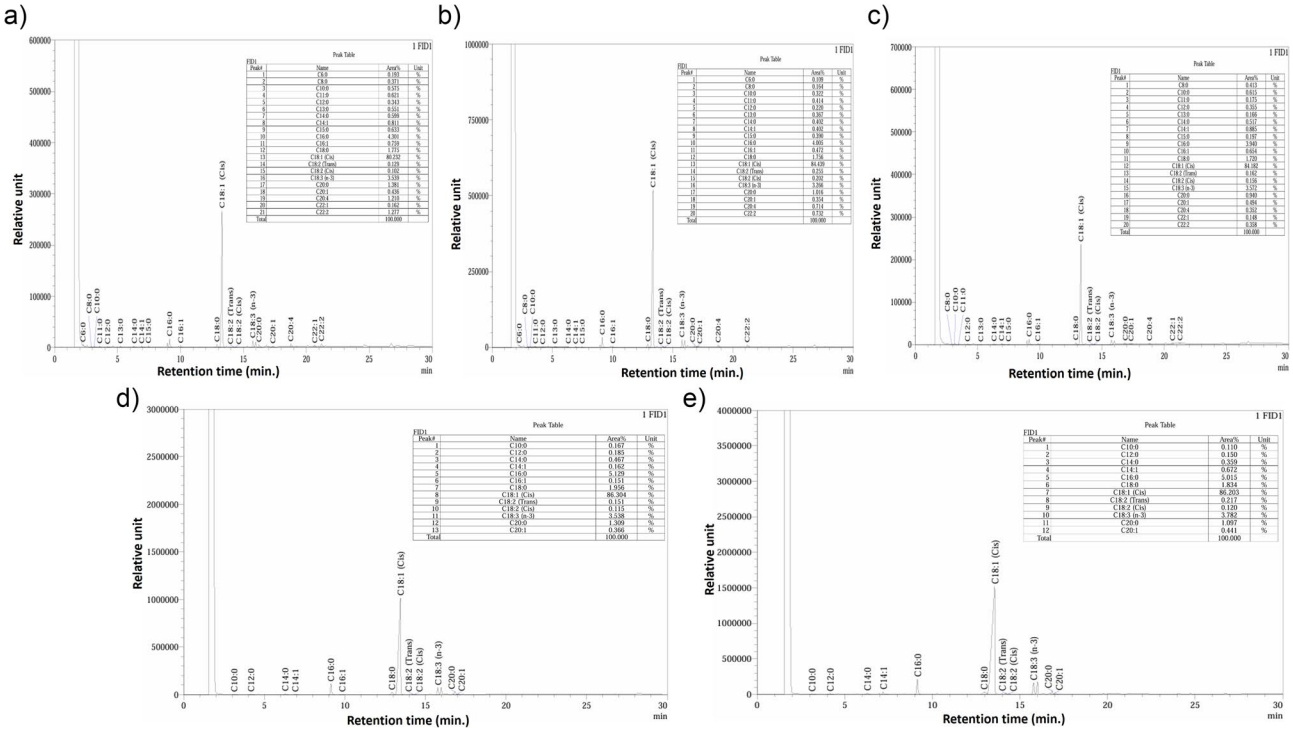

Fig. 7 The chromatogram in the gas chromatography-mass spectrometry system (GC–MS). The GC–MS of saturated and unsaturated fatty acid content in lyophilized cell-free supernatant (cCFS) of *L. casei* ATCC 393 (**a**), *L. rhamnosus* GG ATCC 53103 (**b**), *B. animalis* subsp. *lactis* ATCC 27673 (**c**), *L. acidophilus* ATCC 4356 (**d**), and *L. plantarum* NBRC 3070 (**e**)

in disrupting pathogen cell membranes or modulating microbial interactions, highlighting the multifaced antimicrobial mechanisms of LAB-derived postbiotics.

Storage stability of postbiotics in CFS

The long-term stability of antimicrobial substances in LAB-derived CFS is crucial for their potential in food preservation, especially under conditions that simulate commercial and domestic storage. Ideally, these bioactive compounds should retain their efficacy at refrigeration or ambient temperatures, minimizing the need for cold-chain logistics. In this study, CFS from five LAB strains were stored for 30 days at both 4 °C and −20 °C. Antimicrobial activity was measured via agar well assays against *E. coli* ATCC 25922, *Salm. Typhimurium* ATCC 14028, *Staph. aureus* ATCC 43300, and *P. aeruginosa* ATCC 10145 at 15- and 30-day intervals. Corresponding pH changes were also monitored. Overall, the antimicrobial efficacy of the CFS remained stable against pathogens during the storage period, particularly at −20 °C (Supplementary Tables S6-S10). However, a notable decline ($p < 0.05$) was observed in LGG 53103-derived CFS after 30 days, with final pH values reaching 4.01–4.19. This suggests that acidic degradation of active compounds may contribute to reduced efficacy, underscoring the importance of pH for long-term storage [135, 136]. Our findings are consistent with previous reports of Koohestani et al. [16], who observed comparable stability in CFS from *L. acidophilus* LA-5 and *L. casei* for four weeks at 4 or 25 °C. Similarly, [137] found that CFS from *Enterococcus faecalis* maintained activity for only one month at varying temperatures but showed a 61% reduction in antibacterial activity at 37 °C, attributed to the degradation of low molecular weight peptides. Arrioja-Bretón et al. [52] further demonstrated that temperature significantly impacts the antibacterial persistence of CFS from *L. plantarum* NRRL B4496, *L. saki* NRRL B1917, and *L. rhmnosus* NRRL B442 over a 20-weeks period. These results affirm that LAB-derived postbiotics, particularly when stored at sub-zero temperatures, are relatively stable and retain bioactivity. Their ability to inhibit both Gram-positive and Gram-negative bacteria supports their potential application as natural preservatives to extend product the shelf-life of perishable food products.

Conclusion

Given the growing threat of antimicrobial resistance (AMR) in foodborne pathogens, LAB-derived postbiotics offer a promising natural alternative to chemical preservatives. In the present study, we evaluated the antimicrobial spectrum, production kinetics, and physicochemical properties of postbiotics from five LAB strains: LP 3070, LA 4356, LC 393, LGG 53103, and BAL 27673. All strains demonstrated broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative pathogens, with LP 3070 and LC 393 exhibiting the strongest

effects, particularly after 16 h of incubation. Peak activity was sustained up to 36 h, indicating a viable window for postbiotic preparation. The postbiotics remained stable under various environmental conditions, while BLIS was sensitive to proteolytic enzymes, confirming its proteinaceous nature. SDS-PAGE analysis revealed molecular weights between < 3.3 and 6.5 kDa for BLIS from LP 3070, LA 4356, and LGG 53103. In addition to BLIS, the presence of antimicrobial compounds such as lactic, acetic, and oleic acids further contributed to the inhibitory effects. Despite these promising findings, several limitations must be acknowledged. First, the antimicrobial activity was not validated in a meat model or complex food matrix. Second, only reference strains were tested, which may not reflect the full spectrum of real-world pathogens. Third, while the molecular weight of BLIS was determined primarily, detailed purification and amino acid sequence were not performed. Fourthly, although physicochemical stability under laboratory conditions was confirmed, real-life food system applications remain to be validated. Fifth, there existed limitations regarding the absence of stability testing at extremely low pH and bile salt tolerance. Lastly, the scalability of postbiotics production under industrial settings was not addressed, which is critical for commercial viability. To fully establish the applicability of LAB-derived postbiotics in food systems, future investigations should include molecular characterization of BLIS using advanced proteomic techniques (e.g., MALDI-TOF), in vivo validation using real food models, stability trials under commercial storage conditions (especially lower pH and bile salt matrix), and broad-spectrum screening against emerging AMR pathogens. Moreover, strategies to overcome production-scale challenges (process optimization, cost-effective substrates, and formulation technologies) should be explored to facilitate industrial application. Such studies would strengthen the evidence base for incorporating LAB-derived postbiotics into functional foods and bio-preservation strategies.

Abbreviations

ATCC	American Type Culture Collection
AMR	Antimicrobial resistance
BLIS	Bacteriocin-Like Inhibitory Substances
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
CFS	Cell-Free Supernatant
CFU	Colony forming unite
rCFS	Raw cell-Free Supernatant
nCFS	Neutralized cell-Free Supernatant
cCFS	Lyophilized cell-Free Supernatant
GC-MS	Gas chromatography-mass spectrometry
H_2O_2	Hydrogen peroxide
HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
kDa	Kilodalton
L	Liter
LAB	Lactic acid bacteria
LB	Luria Bertani
M	Molarity
MDR	Multi-drug resistance
MRS	De Man Rogosa Sharpe agar
MIC	Minimum inhibitory concentration
mg	Milligram
mL	Milliliter
µL	Microliter
µm	Micrometer
MUFA	Monounsaturated fatty acid
NBRC	National Institute of Technology and Evaluation, Biological Resource Center
NITE	National Institute of Technology and Evaluation
nm	Nanometer
NRRL	USDA-ARS Culture Collection
OD	Optical density
PBS	Phosphate Buffered Saline
PUFA	Polyunsaturated fatty acid
rpm	Revolutions Per Minute
SFA	Saturated fatty acid
SEM	Standard Error of Mean
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis
UiTM	Universiti Teknologi MARA
USA	United States of America
UK	The United Kingdom
v/v	Volume per volume

Supplementary Information

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Supplementary Material 1

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Authors' contributions

Conceptualization: AQS, SAA, KAK, MMR; Data curation: AQS, MMR; Formal analysis: MMR; Funding acquisition: MSKS; Project administration: MSKS; Supervision and Visualization: AQS, SAA, KAK, MRIF, MSKS; Writing—original draft: MMR; Writing—review & editing: AQS, SAA, KAK; Final manuscript: All authors reviewed the manuscript and approved it.

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Data availability

This article and its supplementary materials include all the essential data generated and analyzed during this study, including experimental data. The commercial lactic acid bacterial strain of ***L. plantarum*** is from NBRC, and other ***Lactobacillus*** strains, ***B. animalis***, and all pathogens tested are from ATCC strains.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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