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RESEARCH

A Study of Bioactivities and Composition of a Cocktail of Supernatants Derived from Lactic Acid Bacteria for Potential Food Applications

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Abstract

Growing interests in replacing conventional preservatives and antibiotics in food and pharmaceutical industries have driven the exploration of bacterial metabolites, especially those from strains with generally recognized as safe (GRAS) status, such as lactic acid bacteria (LAB). In this study, a supernatant cocktail derived from multiple LAB strains was prepared and its bioactivities—antimicrobial, antibiofilm, antioxidant, cytotoxicity, and stability—were thoroughly investigated. The cocktail's main components were identified using thermal and protease treatments, gas chromatography coupled to mass spectrometry (GC–MS), and flame ionization detection (GC-FID). The results demonstrated that the supernatant cocktail had a broad inhibition spectrum and was effective against food-related bacterial indicators with the highest activity observed on *Bacillus cereus* ATCC9634 (inhibition zone sizes 12.33 mm) and the lowest on *Enterococcus faecium* DSM 13590 (3.31 mm). It showed dose- and time-dependent delaying effects on the growth of tested fungi. Regarding the antibiofilm activity, it was more effective in preventing biofilm formation (40% biofilm mass reduction) than in degrading preformed biofilm (20% reduction). Additionally, the cocktail showed antioxidant capacity of 10.1 ± 0.3 g Trolox equivalent (TE)/kg and dose-dependent cytotoxicity on HEK-293 and HT-29 cell lines. The main bioactive compounds in this cocktail are organic acids (particularly acetic acid), volatiles, and bacteriocin-like compounds. The antimicrobial capacity of this supernatant cocktail was highly reproducible across different fermentation batches, and it remained highly stable at 4 °C. Overall, these findings provided novel insights into the functional potentials of LAB metabolites, broadening their application as customizable biopreservatives for food and pharmaceutical industry.

Keywords Lactic acid bacteria · Cell-free supernatant · Postbiotics · Bioactivities · Short-chain fatty acids · Food applications

Introduction

Lactic acid bacteria (LAB) represent a diverse group of gram-positive and coccus- or rod-shaped bacteria, which ferment carbohydrates, producing lactic acid as their primary byproduct [1]. LAB can be identified from various sources, particularly non-pasteurized fermented foods, such as dairy products and vegetables (kimchi), in which they play roles in transforming compounds, developing flavours, and competing with other microorganisms [2, 3]. In addition, food-associated LAB, particularly the genera *Lactobacillus* and *Bifidobacterium*, are considered probiotics and have demonstrated health benefits by upregulating the growth of beneficial bacteria, downregulating inflammation, and maintaining the proper function of microbiota-gut-brain axis [4]. Evidence showed that probiotics

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can be applied as psychobiotics to enhance gut-brain communication and neurotransmitter production and reduce stress hormones and depression symptoms, contributing to host physical and mental health [4, 5].

Among different health benefits, the most well-known characteristic of probiotics is their interactions with the gut microbiome. Gastrointestinal (GI) tract harbours around 100 trillion microbes, which directly and indirectly interact with host metabolisms through energy extraction, bioactive molecule synthesis, and interaction with the immune system [6]. Studies have found healthy individuals exhibit a well-maintained balance between beneficial and pathogenic microorganisms. However, loss of this balance—dysbiosis of gut microbes—leads to increased risks of obesity, cardiovascular diseases, and age-related inflammation [4]. Consuming probiotic-rich foods, in the case of fermented foods, represents a good strategy to restore gut microbiota homeostasis. Studies have shown that *Lactiplantibacillus plantarum* strains isolated from Tibetan kefir possess probiotic traits, including potent antibacterial activities against food-relevant pathogens, high aerotolerance, and antioxidant properties [7]. Genome analysis revealed that *L. plantarum* K25 and YW11 strains encode gene clusters for colonization, carbohydrate metabolisms, and bacteriocin synthesis [8, 9].

Food matrix acts as a vehicle for delivering probiotic strains and their associated health benefits, while probiotics also contribute to preserving food quality and safety over time through their protective effects against spoilage and pathogenic strains. *Listeria* species, particularly *Listeria monocytogenes*, are notorious foodborne pathogens that cause listeriosis and pose serious risks to pregnant women and immunocompromised individuals [10]. *L. monocytogenes* contaminate foods and are able to survive under refrigerated temperature, leading to several foodborne outbreaks [11]. To address this challenge, *Pediococcus acidilactici* UL5 exhibited compelling anti-listerial activity by producing pediocin PA-1 [12]. In addition, the feasibility of probiotics as biopreservatives to replace conventional chemicals and salts has been extensively investigated using various food models, including dairy, non-dairy plant-based, fish, and fishery products [13, 14]. However, the direct application of LAB strains in foods is still facing some challenges. The inherent qualities of the food matrix, along with storage conditions like temperature, greatly affect the protective effectiveness of LAB and can sometimes lead to undesirable fermentation, particularly in fresh food products [15–18]. Additionally, low temperatures can inhibit LAB's metabolic activities, like bacteriocin production, reducing their protective function during storage [19, 20]. To overcome these obstacles, utilizing LAB metabolites offers an attractive solution [21].

Cell-free supernatants (CFS) from LAB are gaining as natural biopreservatives due to their attractive antimicrobial, antioxidant, and anti-inflammatory properties [22]. A CFS generally indicates the fermentate that contains residual nutrients and bioactive metabolites like organic acids, bacteriocins, vitamins, extracellular polysaccharides, bacteriocins, hydrogen peroxide, enzymes, and ethanol [22, 23]. While organic acids (acetic and lactic acid) and bacteriocins have been effective as microbial decontaminants, prolonged exposure may lead to resistance [16–19]. Combining active metabolites, such as acid-bacteriocin mixtures, offers enhanced protection and reduces this risk [24–27]. Additionally, CFS also demonstrate high stability, long shelf life, and minimal impact on food quality, effective both as additives in foods and packaging systems [22, 28].

Although the antimicrobial properties of LAB-derived CFSs have been extensively studied both *in vitro* and in food models, optimizing their efficiency and ensuring reproducibility remain areas that need further exploration. Additionally, examining key characteristics like composition, bioactivity, and safety is crucial for selecting the appropriate food matrix to maximize protective efficiency without compromising sensory qualities. Thus, this study aims to (1) assess the antibacterial activity of LAB-CFSs after 24- and 48-h incubation; (2) create a broad-spectrum antibacterial CFS mixture (CFSM) by coculturing strains or combining supernatants; (3) evaluate its antibacterial, antifungal, antibiofilm, antioxidant, and cytotoxicity properties; (4) analyse its volatile and chemical composition; and (5) assess its stability and reproducibility under various conditions (Fig. 1).

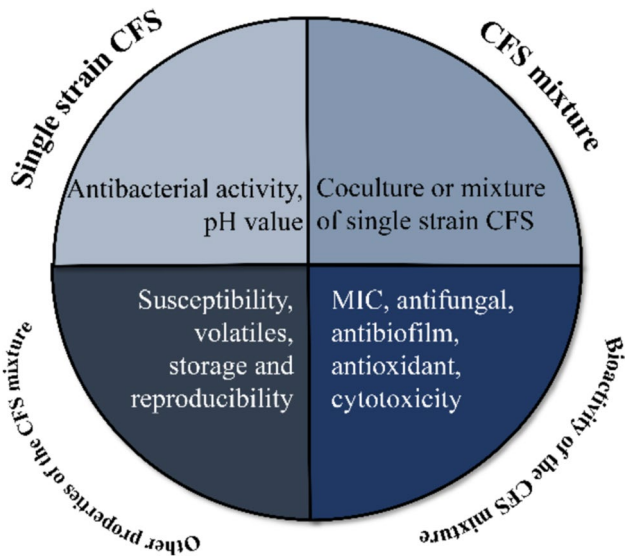

Fig. 1 Scheme of the study

Materials and Methods

Microorganisms and Culture Conditions

LAB strains were isolated either from human stool samples or bee guts, stored in the microbial collection at the University of Camerino (Camerino, Italy), and further identified by 16S RNA gene sequence and MALDI-TOF MS analysis (Bruker Daltonics, Germany) (Table 1). *Lactiplantibacillus plantarum* IMC 509 was kindly provided by SYNBIOTEC Laboratori Srl (Camerino, Italy). *Lactiplantibacillus plantarum* API6 and *L. plantarum* API1 were isolated from worker bees. In addition, LAB strains—*Limosilactobacillus fermentum* 22A, *Ligilactobacillus salivarius* 26C, *Pediococcus acidilactici* 46A, and *Latilactobacillus curvatus* L-A1—were isolated from human faecal samples. All strains were reactivated by growing in de Man, Rogosa, and Sharpe (MRS) broth (VWR, Leuven, Belgium), at 37 °C for 24–48 h, aerobically.

Indicator strains were obtained either from culture collections or food isolation (Table 1). The strains were grown in tryptone soya broth (TSB, OXOID, Basingstoke, UK) for 24–48 h, at 37 °C under aerobic conditions.

Species of *Aspergillus* and *Penicillium* are commonly associated with foods, to investigate the protective effect of LAB metabolites on fungal contamination. The following strains, *Penicillium chrysogenum*, *Aspergillus fumigatus*, and *Penicillium brevicompactum*, were obtained from the microbial collection at the University of Camerino (Camerino, Italy). They were streaked and incubated on Sabouraud dextrose agar (SDA, VWR) at 25–30 °C aerobically for 5 days.

Preparation of Cell-Free Supernatants (CFSs) and CFSs Mixture

LAB strains were inoculated individually in sterile MRS broth with an initial bacterial concentration of 10^7 CFU/ml. After incubation at 37 ± 1 °C for 24 h and 48 h, prior to the extraction, the pH value of each sample was measured by Jenway 3510 pH meter (Barloworld Scientific Ltd.,

Table 1 Sources and culture conditions of bacterial strains

	Source	Growth conditions (37 °C)
LAB strains		
Lactiplantibacillus plantarum IMC 509	Human	MRS broth
Lactiplantibacillus plantarum 24H	Human	MRS broth
Lactiplantibacillus plantarum 2.1B	Human	MRS broth
Lactiplantibacillus plantarum API6	Bee	MRS broth
Lactiplantibacillus plantarum API1	Bee	MRS broth
Limosilactobacillus fermentum 27D3F	Human	MRS broth
Limosilactobacillus fermentum 22A.2	Human	MRS broth
Ligilactobacillus salivarius 26C	Human	MRS broth
Pediococcus acidilactici 46A	Human	MRS broth
Latilactobacillus curvatus L-A1	Human	MRS broth
Indicator strains		
Gram-negative bacteria		
Escherichia coli ATCC 13706[a]	Culture collection	TSB broth
Pseudomonas aeruginosa DSM 1117[b]	Culture collection	TSB broth
Salmonella enterica subsp. *enterica sv.* Enteritidis DSM 14221	Culture collection	TSB broth
Proteus mirabilis prmi 27/77/IMV4[c]	Clinical isolated	TSB broth
Gram-positive bacteria		
Bacillus cereus ATCC 9634	Culture collection	TSB broth
Enterococcus faecium DSM 13590	Culture collection	TSB broth
Listeria monocytogenes 306	Food isolated	TSB broth
Staphylococcus aureus subsp. *aureus* ATCC 25923	Culture collection	TSB broth

[a]*ATCC*, American Type Culture Collection

[b]*DSM*, German Collection of Microorganisms and Cell Cultures

[c]*IMV*, Institute of Microbiology and Virology, Ukraine

MRS de Man, Rogosa and Sharpe, *TSB* tryptone soya broth

Staffordshire, UK) in triplicate. After specific incubation time (24 and 48 h), the CFSs of each strain were extracted by centrifugation (6000 rpm for 20 min at 4 °C) and further sterilized using 0.22-µm pore size filters (MF-Millipore®, Merck KGaA, Darmstadt, Germany). All the supernatants were stored at − 20 °C until use.

To broaden the inhibitory spectra of the CFS, CFSM with different formulations were prepared either by mixing an equal volume of selected CFSs that exhibited the largest inhibition zone against each target bacteria or by coculturing selected LAB strains whose CFSs exhibited the highest inhibition activity (Table 2). The antimicrobial efficiency and spectra of prepared CFSMs were determined using the above-described agar-well diffusion (AWD) method.

The pH values of individual CFSs or the prepared CFSMs were measured after 24- or 48-h incubation using a digital pH meter equipped with a food probe (Mettler Toledo, Columbus, UK). The measurement was conducted in triplicate.

Antibacterial Activity of CFSs

The antimicrobial activity of the individual CFS was evaluated through the agar-well diffusion method (AWD) as described by Koohestani et al. [29] with slight modifications. Briefly, bacterial suspension of target strains was prepared using a sterile physiological saline solution with a concentration of 10^7 CFU/ml. Eight-millimetre circular wells were cut on Mueller Hinton agar (MH, OXOID) with standardized thickness (20 ml/plate) using a sterile cork borer, and each pathogenic strain was spread onto MH agar surface using a sterile cotton swab. An aliquot of 100 µl of CFS was injected into the well, and 100 µl of sterile MRS broth was used as a negative control. All the plates were incubated at 37 °C for 24 h, aerobically. After incubation, the total halo diameter was measured using a calliper. The inhibition zone was considered by subtracting well diameter from zone diameter. The CFS that exhibited the largest inhibition zone for each test strain was chosen for preparing the cell-free supernatant mixture (CFSM).

Bioactivity of the CFSM

Antifungal Activity

The CFSM of individual CFSs was selected for the following tests. The antifungal capacity of the CFSM was evaluated according to a method described by Wang et al. [30] with slight modifications. Briefly, the spore's suspension was prepared by dislodging spores from the hyphae with sterile distilled water (10 ml) and sterile glass spreaders. The suspension was further filtered through sterile cotton filter to remove conidia and mycelia. The concentration of suspension was adjusted to 1×10^5 spores/ml using the spectrophotometer (UV-1601, Shimadzu, Japan) at OD_{600nm}. In the meanwhile, molten agar (20 ml) mixed with different concentrations (2.5%, 5%, 10%, 20%) of CFSM or sterile MRS broth (control) was poured into a 90-mm sterile Petri dish plate and solidified underflow. An aliquot (10 µl) of spore suspension was spotted on the centre of the agar and incubated at 30 °C for 7 days. Colony diameters were measured every 24 h until the control agar was completely covered by the fungus. Radial growth inhibition percentages were calculated using the formula:

$$\text{Radial inhibition}(\%) = \frac{Rc - Ri}{Rc} \times 100$$

Rc represents the radial of the control plate; Ri represents the radial of the plate containing CFSM suspension.

Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the CFSM against target bacteria was evaluated using the method described by the Clinical and Laboratory Standards Institute (CLSI) [31] with slight modifications. Firstly, the CFSM was concentrated by lyophilization. Two-fold serial dilution of the CFSM was made from 1000 to 1.85 mg/ml using sterile Muller-Hinton broth (MHB, OXOID). An aliquot of 100 µl of CFSM dilution and an

Table 2 Supernatant mixtures prepared by mixing selected single supernatant or coculturing selected strains

LAB strains	Preparation method			
	Cell-free supernatant mixture composition	Coculture formulation		
		1	2	3
Lactiplantibacillus plantarum IMC 509	24 h			
Lactiplantibacillus plantarum 2.1B	48 h		x	x
Lactiplantibacillus plantarum API1	24 h + 48 h	x		x
Pediococcus acidilactici 46A	24 h + 48 h	x	x	x
Ligilactobacillus salivarius 26C	24 h	x	x	x

24 h and 48 h indicate the supernatant extracted after 24- or 48-h incubation in broth; x indicates the strain is included in the coculture formulation (1, 2, 3)

equal volume of bacterial suspension (1×10^6 CFU/ml of MHB) were added into a 96-well microtiter plate. Positive control was composed of the same amount of bacterial suspension and sterile MHB, whereas sterility control contained only sterile MHB. The microtiter plate was incubated at 37 °C for 18–24 h. After that, the well with no visible growth of bacteria was considered the minimum inhibition concentration of CFSM against that strain. A quantity of 0.1 ml of broth MIC well was plated on TSA agar to determine the minimum bactericidal concentration (MBC).

Antibiofilm Activity—Biofilm Inhibition

The biofilm inhibition ability was assessed using a 24-well flat-bottomed polystyrene microtiter plate and crystal violet staining method as described by Sornsenee et al. [32] with minor modifications. Bacterial suspensions of the following strains—*P. aeruginosa* DSM 1117, *E. coli* ATCC 13706, *L. monocytogenes* 306, and *S. aureus* subspecies *aureus* ATCC 25923—were prepared using sterile MHB with a final concentration of 10^6 CFU/ml. The biofilm-forming ability of tested strains was pre-assessed by the tube method [33]. The CFSM solutions of 2MIC (31.25 mg/ml) and MIC (15.63 mg/ml) concentrations were prepared also with sterile MHB. An equal volume of bacterial suspension and the CFSM solutions were loaded into a 24-well plate and incubated at 37 °C for 24 h. Positive control was composed of bacterial suspension and MHB, while negative control contained MHB only. To investigate the impact of temperature fluctuation on the antibiofilm efficiency of the CFSM, another 24-well plate was firstly incubated at 4 °C for 12 h and then at 37 °C for further 12 h. After incubation, cell suspension was removed and washed with sterile phosphate-buffer solution (PBS, pH 7.4, Sigma-Aldrich, St. Louis, USA); then, the microtiter plate was left to dry under the flow of biosafety cabinet for 1 h. Two millilitres of crystal violet solution (0.1% w/v, bioMérieux SA, France) was added into the dried well and incubated for 30 min. Then, the staining solution was removed from each well and it was washed twice using 2-ml sterile distilled water to remove excess staining. The microtiter plate was dried again under the abovementioned conditions. The quantification of biofilm was obtained by adding 2 ml of acetic acid (99%, Sigma-Aldrich) per well, and the optical density of each well was measured using a microplate reader (FLUOstar Omega, BMG LABTECH, Germany) at OD_{540nm}. The biofilm inhibition activity was calculated using the following formula:

$$\text{Biofilm inhibition}(\%) = \frac{(ODC - ODT)}{ODC}$$

ODC indicates the OD value of the positive control well; ODT represents the OD value of treated wells.

Antibiofilm Activity—Biofilm Removal

To assess the biofilm removal ability of the CFSM, a 24-well plate was inoculated firstly with bacterial suspension (*P. aeruginosa* DSM 1117, *E. coli* ATCC 13706, *L. monocytogenes* 306, *S. aureus* ATCC 25923, 10^6 CFU/ml) and incubated at 37 ± 1 °C for 24 h. Wells inoculated with only MH broth were used as negative control (sterility). After incubation, the cell suspension was discarded, and the plate was washed three times using sterile phosphate-buffered saline (PBS, pH = 7.4, Sigma-Aldrich) and allowed to dry under the flow of biosafety cabinet. Then, 2 ml of CFSM prepared in MHB with the following concentrations—2MIC, MIC—was added to the wells and incubated at 37 °C for 24 h. Two millilitres of sterile MHB was added as a negative control. After incubation, the CFSM inside each well was decanted and plates were washed again using PBS and air-dried. The quantification method was described in the "Antibiofilm Activity—Biofilm Inhibition" section.

DPPH Reducing Activity Determination

The antioxidant activity of the CFSM was determined using the DPPH method according to Nzekoue et al. [34] with slight modifications. Briefly, CFSM-ethanol solutions at 100 mg/ml and 10 mg/ml were prepared by dissolving 100 mg or 10 mg of freeze-dried lyophilized powder of CFSM in 1 ml of ethanol solution (99%) (Merck, Darmstadt, Germany). Then, 0.5 ml of this CFSM solution was mixed with 4.5 ml of DPPH solution. The mixture was shaken and then kept in darkness for 30 min. After incubating, the absorbance of the sample was determined spectrophotometrically (Agilent Cary 8454 UV–Visible, Agilent Technologies, USA) at 517 nm. The results were expressed as µg TE/ml sample solution (Trolox equivalent antioxidant capacity, TEAC), obtained by using a standard curve with Trolox as a reference antioxidant [28]. The curve was obtained by comparing the absorbance difference between blank and reaction mixture containing standard Trolox (Aldrich Chemical Company, Steinheim, Germany) (1–100 ppm) at 517 nm.

$$\text{DPPH scavenging activity}(\%) = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \times 100$$

Cytotoxicity Assay

The cytotoxicity of the CFSM on HT-29 and HEK-293 cell lines was assessed using MTT assays with slight modifications [35]. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal bovine serum (10%), penicillin/streptomycin (1%),

and L-glutamine at 37 °C in an atmosphere of CO_2 (5%). For cytotoxicity assay, 1.5×10^4 cells/well (HEK-293 cells) and 1.0×10^4 cells/well (HT-29 cells) in 50-µl complete medium were seeded in a 96-well microtiter plate and grew at the abovementioned conditions. The day after, 50-µl fresh medium containing appropriate concentrations ranging from 3 to 21 mg/ml of CFSM or medium without CFSM as control was added. After 24-h treatment, the plates were washed with PBS and added fresh medium with 10 µl of 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT) solution (Sigma-Aldrich) (5 mg/ml in PBS) followed by an incubation in dark for 4 h.

The purple formazan crystals formed inside each well were dissolved in 100 µl of the dimethyl sulfoxide (DMSO), and the absorbance of each well was measured at 540 nm in Multiskan Ascent 96/384 Plate Reader. Each concentration was evaluated on eight replicates and the assay was repeated three times. The concentration of the compound was considered cytotoxic when the applied concentration resulted in ≥ 30% viability reduction [36].

Susceptibility of Antibacterial Activity to Heat, pH, Enzyme Treatments

The CFSM was treated with the conditions as reported in Table 3 [36], and the antimicrobial efficiency of the treated CFSM was evaluated using an agar-well diffusion test as described above. To assess organic acid functions, CFSM was neutralized to pH = 3, 7, and 9 with NaOH (1 N) or HCl (1 N). To verify the thermostability of the active compounds, heat treatments, including 100 °C and 121 °C (autoclave) for 15 min, were applied on the CFSM. For verifying the presence of bacteriocin-like substances and hydrogen peroxide (catalase), the neutralized CFSM (pH = 7, proper for the activity of enzyme) was treated separately using the following enzymes (all from Sigma-Aldrich): α-chymotrypsin, proteinase k (1 mg/ml), catalase, lysozyme. One milligram per millilitre of pepsin (Sigma-Aldrich) was added into acidified CFSM (pH = 3). After incubation, all enzyme-treated samples were heated for 5 min at 100 °C to inactivate the remaining enzymes. The CFSM without any treatment was used as a control. The residual antimicrobial activity against indicator strains was determined via a well diffusion test.

Table 3 Different treatments on the CFSM

Treatments	Conditions
pH	Value of 3 Value of 7 Value of 9
Heat	100 °C, 15 min 121 °C, 15 min
Enzyme	α-Chymotrypsin (pH 7, 37 °C, 2 h) Catalase (pH 7, 37 °C, 2 h) Proteinase K (pH 7, 37 °C, 2 h) Lysozyme (pH 7, 30 °C, 2 h) Pepsin (pH 3, 30 °C, 2 h)
Control	Untreated CFSM

Short-Chain Fatty Acids and Volatile Organic Compounds (VOC) of the CFSM

Short-chain fatty acids (SCFAs) were quantified in CFSM following procedures reported by Scortichini et al. [36] and Chang et al. [37]. Briefly, a 0.25-g aliquot of the sample was weighted in a 2-ml vial, acidified by 200 µl of sulphuric acid (50% w/v), supplemented with 10 µl of internal standard solution (valeric acid, 109.5 mM in ethyl ether), and stirred with the help of a vortex device for 1 min. Then, ethyl ether (0.8 ml) was added to extract SCFAs. The mixture was stirred for 2 min with the help of a vortex device and then centrifuged (5 min, 2800 rpm) to separate the upper organic phase that was transferred into another vial. The remaining aqueous phase was extracted two more times by ethyl ether as in the previous extraction. The collected organic phase was analysed by GC-FID (6850 Agilent Technologies, Santa Clara, CA, USA) injecting 0.5 µl of the ethyl ether solution and using a nitroterephthalic acid modified polyethyleneglycol (PEG) column (DB-FFAP, 25 m, 0.25 mm i.d., 0.25-µm film thickness, purchased from Agilent Technologies, Santa Clara, CA, USA). The GC injector was maintained at 280 °C. The injection was performed in splitless mode (splitless time 3 min). The oven temperature was initially set at 40 °C for 3 min and programmed at a rate of 20 °C/min to 160 °C and then at 40 °C/min to 245 °C which was held for 1.87 min, resulting in a total run time of 13 min. The carrier gas was hydrogen at a flow rate of 3.70 ml/min. The FID temperature was maintained at 250 °C.

Volatile organic compounds from CFSM were extracted by headspace solid-phase microextraction (HS-SPME) and analysed through a GC–MS with autosampler PAL3 (Agilent, Santa Clara, CA, USA, Agilent 7890B GC Hardware with Agilent 5977 Series MSD). Briefly, 2 ml of sample was incubated at 35 °C and agitated at 250 rpm for 20 min. Then, the SPME fibre (CAR/PDMS) was automatically inserted in the sample headspace and remained for 20 min for adsorption. Following HS-SPME, the fibre was automatically injected into the GC injector (260 °C). A desorption time of 5 min was sufficient to desorb analytes from the fibre. Cleaning was automatically performed with the PAL system by inserting the fibre in the conditioning port at 230 °C for 20 min after each process. The VOC separation was carried

out through a DB-WAX (0.25 mm × 60 m, 0.25 µm) (Agilent 122–7062, CA, USA) with helium as carrying gas at a flow rate (He) of 1.2 ml min^{-1} under spitless mode. The temperature for the column was programmed as follows: from 35 °C (4 min) to 120 °C (2.5 °C per min), from 120 to 250 °C (15 °C per min), then 250 °C for 3.33 min; total run time was 50 min. The VOC were ionized through electron ionization (EI) mode and data were acquired in full SCAN mode. Linear chain alkanes (C6–C26) were used to calculate retention indices. Thus, the detected VOCs were identified by comparing their retention indices and mass spectra with those of standards from the US National Institute of Standards and Technology database (NIST-USA, http://webbook.nist.gov).

Stability and Reproducibility of the CFSM

In addition, the antimicrobial efficiency of the CFSM from the same batch during refrigerated storage (4 °C) and the reproducibility of CFSM from different batches were checked using the agar-well diffusion (AWD) method as described above.

Statistical Analysis

All analyses were performed in triplicate. Values were expressed as means ± standard deviations. Statistical analysis was conducted by SPSS (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) with the Tukey's HSD test. Graphical works were done using Prism 9 (GraphPad, San Diego, CA, USA) and R (R Core Team (2020). Differences were considered statistically significant when $p < 0.05$.

Results

Inhibitory Spectrum and pH Value of CFSs

The antimicrobial activity of CFSs from ten LAB strains extracted after 24- and 48-h incubation was shown in

Table 4 Inhibition zone (mm) of LAB cell-free supernatants (CFSs) against target strains

LAB-CFSs		Inhibition zone (mm)							
		E. coli ATCC 13706	*P. aeruginosa* DSM 1117	*S. enteritidis* DSM 14221	*L. monocytogenes 306*	*B. cereus* ATCC 9634	*S. aureus* ATCC 25923	*P. mirabilis* IMV4	*E. faecium* DSM 13590
***L. plantarum* IMC 509**	24 h	5.69 ± 0.74^{bcB}	3.25 ± 1.47^{bA}	5.63 ± 1.72^{cdeB}	—	$\mathbf{17.91 \pm 1.18^{kC}}$	6.86 ± 0.63^{bB}	3.42 ± 0.28^{abA}	—
	48 h	6.34 ± 0.74^{bcB}	8.97 ± 0.72^{iC}	5.58 ± 0.66^{cdeB}	—	14.91 ± 0.41^{ijD}	—	2.94 ± 0.47^{abA}	—
***L. plantarum* 2.1 B**	24 h	5.16 ± 0.44^{bB}	3.18 ± 0.95^{bA}	6.71 ± 0.76^{eC}	—	16.78 ± 1.29^{jkD}	6.80 ± 0.79^{bC}	3.64 ± 0.63^{abA}	—
	48 h	$\mathbf{7.25 \pm 0.85^{cC}}$	9.07 ± 0.66^{iD}	5.85 ± 0.41^{cdeB}	—	14.21 ± 0.72^{ghiE}	—	4.28 ± 0.81^{bcA}	—
***L. plantarum* 24H**	24 h	5.27 ± 0.92^{bB}	3.08 ± 1.18^{bA}	6.41 ± 0.41^{deBC}	—	16.75 ± 1.82^{jkD}	7.48 ± 1.06^{bcC}	5.55 ± 0.88^{cBC}	—
	48 h	6.10 ± 1.41^{bcB}	8.72 ± 1.05^{hiC}	5.51 ± 0.28^{cdeB}	—	14.58 ± 0.83^{hiD}	—	3.36 ± 0.57^{abA}	—
***L. plantarum* API6**	24 h	4.93 ± 0.99^{bA}	6.87 ± 0.27^{fgB}	6.28 ± 0.39^{deAB}	—	17.68 ± 0.89^{kC}	5.81 ± 1.41^{abAB}	5.27 ± 1.06^{cAB}	—
	48 h	5.10 ± 1.03^{bB}	8.24 ± 0.71^{ghC}	5.16 ± 0.81^{bcdB}	—	13.20 ± 0.64^{fghiD}	—	2.79 ± 0.40^{aA}	—
***L. plantarum* API1**	24 h	4.71 ± 1.61^{abA}	6.58 ± 0.53^{efB}	$\mathbf{6.84 \pm 0.59^{eB}}$	—	17.14 ± 0.65^{kD}	$\mathbf{9.16 \pm 0.74^{cC}}$	7.78 ± 0.62^{dBC}	—
	48 h	4.52 ± 1.27^{abB}	$\mathbf{9.10 \pm 0.63^{iC}}$	3.66 ± 1.13^{abAB}	—	14.36 ± 1.53^{ghiD}	—	2.58 ± 0.51^{aA}	—
***L. fermentum* 27D3F**	24 h	—	2.77 ± 0.45^{abA}	—	—	12.89 ± 0.70^{fghC}	4.12 ± 1.23^{aB}	—	—
	48 h	—	4.82 ± 0.55^{cA}	—	—	8.09 ± 0.84^{cdB}	—	—	—
***L. fermentum* 22A.2**	24 h	—	1.51 ± 0.24^{aA}	—	1.74 ± 0.17^{aA}	6.71 ± 0.33^{bcB}	—	—	—
	48 h	—	2.33 ± 0.93^{abA}	—	5.20 ± 1.22^{bC}	3.75 ± 0.45^{aB}	—	—	—
***P. acidilactici* 46A**	24 h	2.88 ± 0.34^{aA}	5.12 ± 0.55^{cdB}	3.39 ± 0.64^{aA}	5.28 ± 0.39^{bB}	11.29 ± 0.56^{efB}	—	—	—
	48 h	4.52 ± 0.86^{abAB}	6.25 ± 0.67^{cdeB}	—	$\mathbf{8.78 \pm 0.94^{dC}}$	9.54 ± 1.57^{deC}	—	2.62 ± 1.36^{aA}	—
***L. curvatus* L-A1**	24 h	—	—	—	—	4.72 ± 0.61^{a}	—	—	—
	48 h	—	—	—	—	4.88 ± 0.69^{ab}	—	—	—
***L. salivarius* 26C**	24 h	2.84 ± 0.56^{aA}	6.13 ± 0.46^{cdeC}	3.48 ± 0.55^{aAB}	5.47 ± 0.27^{bC}	12.62 ± 0.45^{fgD}	—	3.38 ± 0.34^{abAB}	$\mathbf{3.98 \pm 0.48^{B}}$
	48 h	4.99 ± 0.54^{bAB}	5.10 ± 0.08^{cdB}	4.43 ± 0.12^{abcA}	7.55 ± 0.39^{cC}	10.07 ± 0.42^{eE}	—	$\mathbf{8.56 \pm 0.22^{dD}}$	—

a–k represent statistical difference of each column ($p < 0.05$); A–K represent significant difference of each row. Values in bold represent the supernatant that showed the highest inhibition zone

Table 4. Generally, the inhibitory efficiency was highly influenced by the species of producer strain and the CFS incubation hours. CFSs of *L. plantarum* strains (IMC 509, 24H, 2.1B, API6, API1) exhibited higher activity against *E. coli*, *P. aeruginosa*, *S. Enteritidis*, *S. aureus*, and *B. cereus* compared to other CFSs, but no inhibitory activity was observed on *E. faecium* and *L. monocytogenes*. Supernatants of *L. salivarius* 26C and *P. acidilactici* 46A exhibited a broad inhibitory spectrum. *L. salivarius* 26C was active in both gram-positive and negative indicators, especially *P. mirabilis* and *E. faecium*. *P. acidilactici* 46A-CFS of 48 h exhibited strong anti-listerial activity (inhibition zone = 8.78 mm), suggesting the production of pediocin by this strain. By contrast, CFSs of *L. fermentum* strains (27D3F and 22A.2) and *L. curvatus* L-A1 showed a relatively narrow inhibitory spectrum.

In this study, a wider inhibition zone was found in CFSs of 48-h incubation against *E. coli*, *P. aeruginosa*, and *L. monocytogenes*, whereas other indicator bacteria were more sensitive to CFSs of 24 h. It is to be noticed that the anti-*S. aureus* and anti-*E. faecium* effects were only observed in 24-h CFSs, and the inhibition activities vanished after prolonged incubation (Fig. 2).

Inhibitory Spectrum and pH Value of CFS Mixture

To obtain a supernatant cocktail with a broad inhibition spectrum, the antibacterial activities of supernatant mixtures prepared either by coculturing multiple strains or mixing selected supernatants were investigated. Figure 3 shows the inhibition efficiency (mm) of the CFS

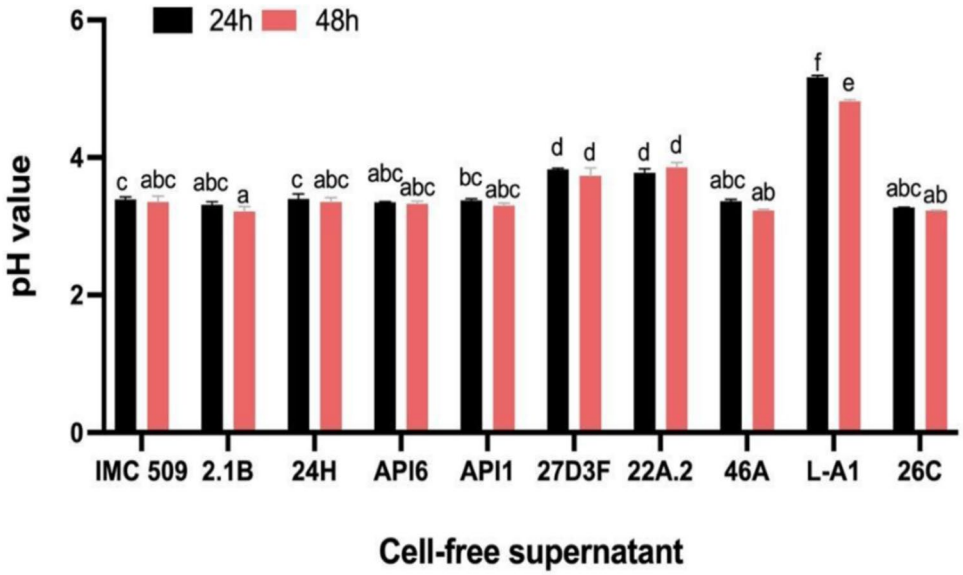

Fig. 2 pH value of LAB supernatants. Bars denoted by different letters indicate significant differences ($p < 0.05$, ANOVA, Tukey HSD)

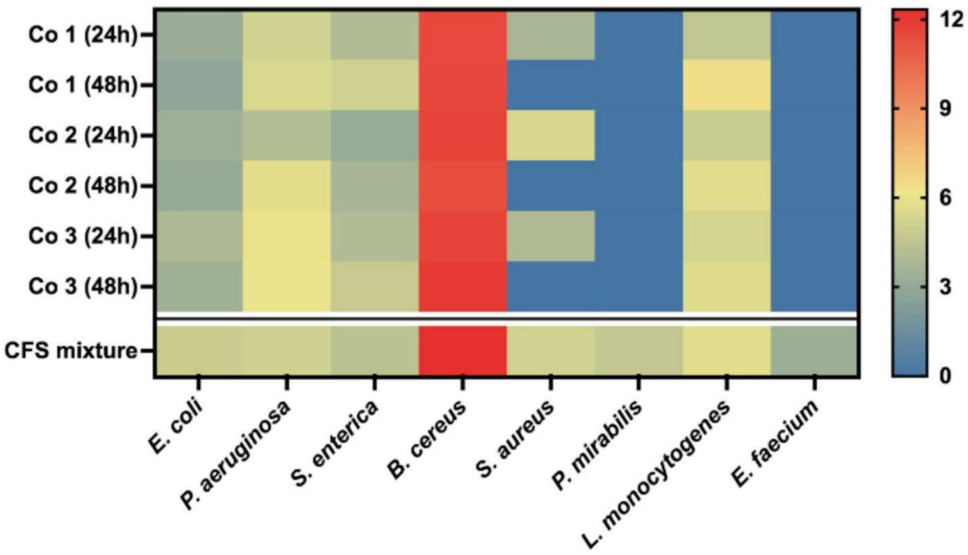

Fig. 3 Inhibition zone (mm) of the CFSM. Co 1: coculture supernatant of *L. plantarum* API1, *P. acidilactici* 46A, *L. salivarius* 26. Co 2: coculture supernatant of *L. plantarum* 2.1B, *P. acidilactici* 46A, *L. salivarius* 26C. Co 3: coculture supernatant of *L. plantarum* API1, *L. plantarum* 2.1B, *P. acidilactici* 46A, *L. salivarius* 26C. CFS mixture composed of an equal volume of the following single supernatants: IMC 509 (24 h), 2.1B (48 h), API1 (24 h), API1 (48 h), 46A (24 h), 46A (48 h), 26C (24 h)

mixture extracted from 24 and 48 h of cocultures (CFS-Co) or prepared by mixing individual CFS (CFSM). Like single CFS, CFS-Co (Co 1, Co 2, and Co 3) showed no inhibitory activity against *E. faecium* and *P. mirabilis*. The anti-*S. aureus* activity was not found in supernatants of 48-h cocultures. Compared to supernatants of cocultures composed of three strains (Co 1 and Co 2), the supernatant of Co 3 (coculture composed of *L. salivarius* 26C, *P. acidilactici* 46A, *L. plantarum* API1, *L. plantarum* 2.1B) exhibited slightly wider inhibition zone (supplementary Table 1S). The CFS-Co showed similar pH values, ranging from 3.65 to 3.71 (Fig. 4). By contrast, although the CFSM composed by single CFSs exhibited the highest pH value (3.83, Fig. 4) ($p < 0.05$), CFSM inhibited the growth of all indicators. Larger inhibition zone was observed in the CFSM against *E. coli* ($p < 0.05$), *B. cereus* ($p < 0.05$), *P. mirabilis* ($p > 0.05$), and *E. faecium* ($p > 0.05$) than other supernatant mixtures (supplementary Table 2S).

According to the aim of the study—to develop a CFS with a broad inhibitory spectrum—the CFS mixture composed of selected supernatants was chosen for further analysis.

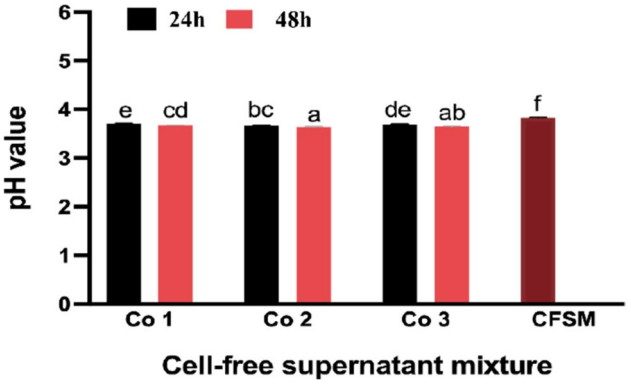

Fig. 4 pH value of the CFS mixtures. Co 1: coculture supernatant of *L. plantarum* API1, *P. acidilactici* 46A, *L. salivarius* 26. Co 2: coculture supernatant of *L. plantarum* 2.1B, *P. acidilactici* 46A, *L. salivarius* 26C. Co 3: coculture supernatant of *L. plantarum* API1, *L. plantarum* 2.1B, *P. acidilactici* 46A, *L. salivarius* 26C. CFS mixture composed of an equal volume of the following single supernatants: IMC 509 (24 h), 2.1B (48 h), API1 (24 h), API1 (48 h), 46A (24 h), 46A (48 h), 26C (24 h)

Antifungal Activity of the CFSM

The CFSM exhibited a concentration- and time-dependent inhibition effect on *Aspergillus fumigatus*, *Penicillium brevicompactum*, and *Penicillium chrysogenum* as shown in Fig. 5. The inhibition percentage increased as the concentration of the CFSM increased. At lower concentrations (2.5–10%), the CFSM inhibited similarly on the growth of fungal strains. Twenty percent CFSM showed the highest inhibition activity on *P. chrysogenum* by completely suppressing its growth up to 48 h. Moreover, the highest inhibition percentage was observed on *P. chrysogenum* even after 96 h of incubation.

However, the antifungal activity of the CFSM gradually decreased as the agar plate incubation time increased. At 96-h incubation of fungi with supernatant, the efficacy of CFSM against all tested fungi was still detectable with inhibition concentrations of 5%, 2.5%, and 2.5% for *A. fumigatus*, *P. brevicompactum*, and *P. chrysogenum*, respectively.

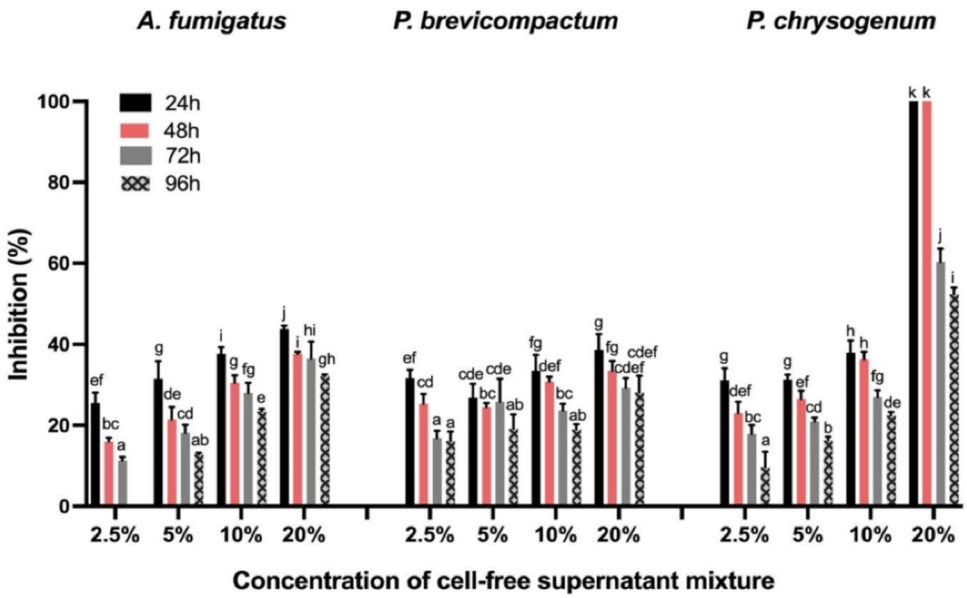

Fig. 5 Antifungal activity of the CFS with concentrations, ranging from 2.5 to 20% (v/v). Bars denoted by different letters indicate significant differences ($p < 0.05$, ANOVA, Tukey HSD) among concentrations within the same strain

Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) of the CFSM

To evaluate the minimum concentrations of the CFSM to exert inhibitory or bactericidal effects, the MIC and MBC values were determined against the target indicator. As shown in Table 5, the MIC value for *L. monocytogenes*, *B. cereus*, and *E. coli* was 15.63 mg/ml, which was half of the MIC of the CFSM against other bacterial strains (31.25 mg/ml). However, the MBC values for each strain were higher than the MIC values.

Antibiofilm Activity

The CFSM was more effective in preventing biofilm formation (Fig. 6A) than degrading preformed biofilm (Fig. 6B). Higher concentration of the CFSM (2MIC) showed enhanced biofilm-prevention efficiency ($p < 0.05$), especially on *P. aeruginosa* (20% increment). On the contrary, *L. monocytogenes* generally required a higher amount of the CFSM (2MIC) to prevent its biofilm formation. Regarding the temperature fluctuation (4 °C for the first 12 h followed by 37 °C for another 12 h) on CFSM antibiofilm efficacy, results showed that temperature fluctuation enhanced the CFSM-anti-biofilm efficiency against *P. aeruginosa*, but lower inhibition percentages were observed on preventing biofilm formation of *E. coli* and *L. monocytogenes*.

As for its biofilm degradation capacity, the assay was conducted only at 37 °C due to the low degradation capacity observed previously. Although the concentration-dependent efficiency of the CFSM was not observed on the preformed biofilm of *P. aeruginosa* as observed above, a higher degradation percentage (20%) was observed on *P. aeruginosa* biofilm than other strains. At 37 °C, the CFSM treatment (2MIC) removed 20% of the preformed biofilm of *P. aeruginosa* and *E. coli*, while only 6% and 9% biofilm degradation on *L. monocytogenes* and *S. aureus*. At 1MIC, the CFSM was more effective in inhibiting (20%) than degrading the biofilm of *E. coli* (4.9%) and *S. aureus* (2.8%) at 37 °C.

Table 5 Minimum inhibition concentration and minimum bactericidal concentration of the CFSM (mg/ml)

Bacteria	MIC	MBC
E. coli ATCC 13706	15.63	> 15.63
S. Enteritidis DSM 14221	31.25	> 31.25
P. mirabilis 27/77/IMV4	31.25	> 31.25
P. aeruginosa DSM 1117	31.25	> 31.25
B. cereus ATCC 9634	15.63	> 15.63
E. faecium DSM 13590	31.25	> 31.25
S. aureus ATCC 25923	31.25	> 31.25
L. monocytogenes 306	15.63	> 15.63

Antioxidant Activity

To evaluate the antioxidant capacity of the CFSM, the DPPH radical scavenging method was applied, yielding a result of 10.1 ± 0.3 g Trolox equivalent/kg of CFSM.

Cytotoxicity Assay

The toxicity of CFSM was evaluated on two cell lines. Figure 7 shows that the CFSM exerted a concentration-dependent cytotoxicity on both cell lines—HEK-293 and HT-29 cells—after 24-h treatment. The CFSM treatment exhibited no toxicity to both cell lines at lower concentrations (< 9 mg/ml). At a concentration of 9 mg/ml, there was no significant cytotoxicity on HEK-293 cells, but the viability of HT-29 cells started to drop. Noticeable cytotoxicity was observed on both cell lines when the concentration of CFSM was above 12 mg/ml. In addition, the half maximal inhibitory concentration (IC_{50}) of HT-29 (12.43 mg) was lower than the IC_{50} of HEK-293 (13.15 mg) (Fig. 7B and D).

Antibacterial Susceptibility to Heat, pH, Enzyme Treatments

The preliminary identification of CFSM antibacterial compounds using different treatments is shown in Fig. 8. Its antibacterial activity was enhanced by acidification and resisted thermal conditions, but it was sensitive to neutralization, catalase, and enzyme treatments. High temperatures (100 °C and 121 °C) only abolished the CFSM activity against *E. faecium*. Under neutral and alkaline conditions, the antibacterial capacity of the CFSM was gone. In addition, the antimicrobials present in CFSM were sensitive to chymotrypsin, proteinase K, lysozyme, and catalase.

Short-Chain Fatty Acids (SCFAs) and Volatile Organic Compounds (VOC) of the CFSM

Four major SCFAs (Table 6) were detected and quantified. The dominant SCFA of the CFSM was acetic acid. Although the concentrations were low, propionic acid and butyric acid were also detected in the supernatant. The level of isovaleric acid, isocaproic acid, and caproic acid was below the detection limit. As for the repeatability of the assay, the RSD values range from 1.29 to 8.72% for acetic acid and propionic acid respectively.

Overall, 32 volatile organic compounds were identified from CFSM analysis including carbonyl compounds, organic acids, and alcohols as main chemical classes (Table 7). Moreover, sulphur-containing compounds such as dimethyl

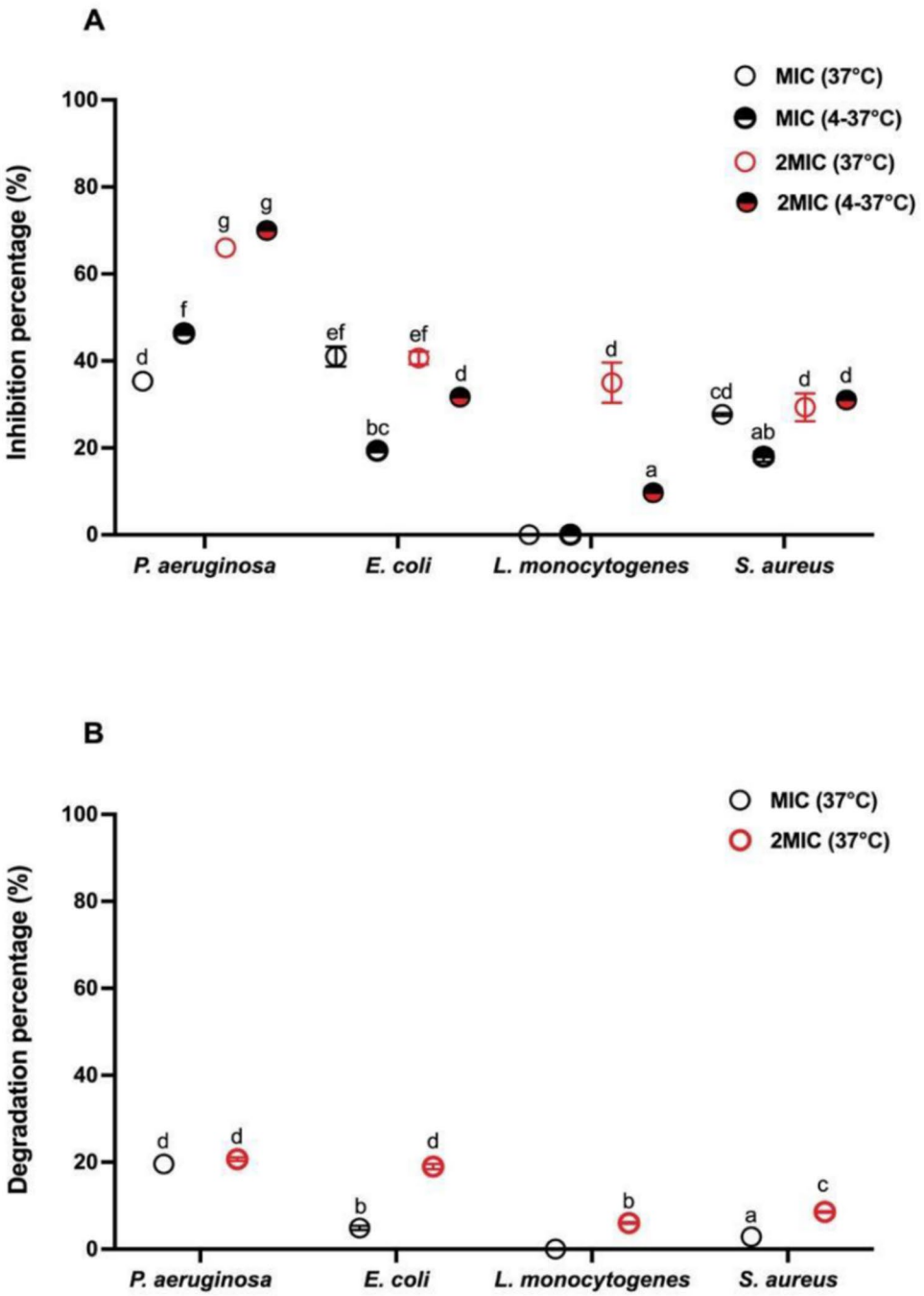

Fig. 6 Antibiofilm activity of the cell-free supernatant mixture. **A** Biofilm inhibition of the CFSM (MIC and 2MIC) at 37 °C and 4–37 °C; **B** biofilm degradation ability of the CFSM (MIC and 2MIC) at 37 °C. Different letters above each value indicate significant differences ($p < 0.05$, ANOVA, Tukey HSD)

disulfide and dimethyl trisulfide, which are produced from L-methionine, were also observed.

Storage Stability and Reproducibility of the CFSM

The antibacterial activity of CFSM produced from three different batches under different storage conditions (− 20 °C and 4 °C for 5 months, 4 months, and fresh) was assessed. The results showed that although there were slight variations in the inhibition zone diameters, the antimicrobial spectrum and efficiency were similar among different batches (Fig. 9). In addition, the CFSM stored at lower storage temperature (− 20 °C) showed slightly reduced activity against *S. aureus* and *S. enteritidis* after 4-month storage compared to that stored at 4 °C. Longer refrigerated storage (5 months, 4 °C) did not affect the overall antibacterial activity of the CFSM, which was like fresh CFSM.

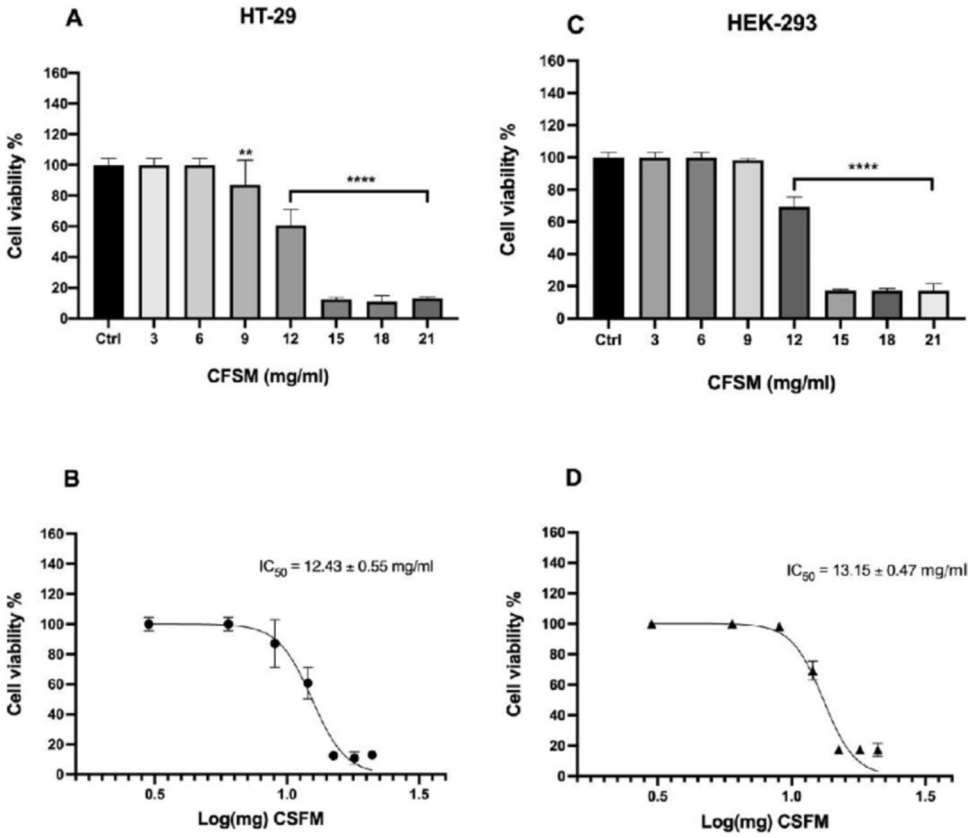

Fig. 7 Cytotoxicity of different concentrations of the supernatant mixture (CFSM, 3–21 mg/ml) was assessed on human colon cancer (HT-29, **A**, **B**) and human embryonic kidney 293 (HEK-293, **C**, **D**) cell lines after 24-h treatment using the MTT assay. Ctrl, positive control. The data are shown as means ± standard deviations. The symbols * and **** represent values that are different from control, $p < 0.05$ and $p < 0.0001$ respectively (ANOVA, Dunnett's multiple comparison test). IC_{50} value of each cell line was determined by fitting the results into the sigmoid-Emax model using nonlinear regression

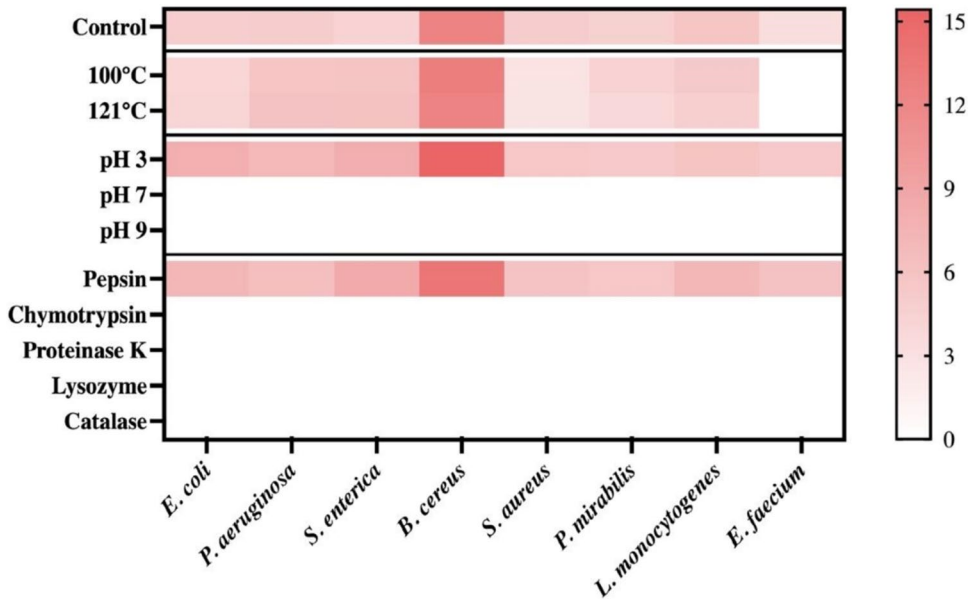

Fig. 8 Antibacterial efficiency of the CFSM after treatments

Discussion

Investigating the beneficial bioactivities of LAB metabolites offers new perspectives for their application across various industries. Specifically, combining select metabolites, in the form of postbiotics, allows for customized preservation strategies to tackle specific challenges faced by food products [38]. Although LAB metabolites have been extensively studied, the diverse bioactivity and feasibility of food applications remained underexplored. This work presents a broad-spectrum, antioxidant,

Table 6 Short-chain fatty acids (SCFA) of the cell-free supernatant mixture (CFSM)

SCFA	Molecular formula	Mean ± SD mmol/g	Repeatability RSD, % ($n = 3$)
Acetic acid	$C_2H_4O_2$	61.24 ± 0.79	1.29
Propionic acid	$C_3H_6O_2$	0.30 ± 0.03	8.72
Isobutyric acid	$C_4H_8O_2$	0.09 ± 0.01	6.63
Butyric acid	$C_4H_8O_2$	0.13 ± 0.01	6.29
Isovaleric acid	$C_5H_{10}O_2$	nd	nd
Isocaproic acid	$C_6H_{12}O_2$	nd	nd
Caproic acid	$C_6H_{12}O_2$	nd	nd

nd not detected

low-toxicity supernatant cocktail derived from LAB strains with great potential in food and pharmaceutical applications.

Microbial contamination is a primary cause of foodborne illness and food waste, leading to substantial economic loss [39]. Key pathogens of concern include *Salmonella enterica* and *Listeria monocytogenes* [40]. Due to the strong tolerance to processing and cold conditions, *L. monocytogenes* can survive and cause listeriosis, posing serious risks to susceptible individuals [41]. In this study, supernatants of *Pediococcus acidilactici* 46A, *L. salivarius* 26C, and *Limosilactobacillus fermentum* 22A.2 exhibited potent anti-listerial activity. *Pediococcus* spp. and *L. salivarius* are well-known for their potent anti-listerial metabolites—pediocins and salivaricins (class II bacteriocins)—which are particularly active against *L. monocytogenes*, respectively [42–44]. Additionally, digestive enzyme abolished the anti-listerial activity of supernatants, suggesting the possible anti-listerial metabolites are bacteriocins. Supernatants of *L. plantarum* ACA-DC287 inhibited the growth of *S. enterica* serovar Typhimurium [45]. Other strains—*E. coli*, *P. aeruginosa*, *S. aureus*, *B. cereus*, and *P. mirabilis*—were also sensitive to metabolites produced by *L. plantarum* strains, particularly *B. cereus*, likely due to higher acid production [46].

For food applications, preservative with a broad inhibition spectrum is considered a desirable quality as it offers complete protection from microbial contamination [47]. Studies showed that metabolite (bacteriocin) production of the producer is enhanced by coculturing with other bacteria, which act as stressors or competitors [48, 49]. Therefore, this study tested and compared the antibacterial spectrum of supernatant mixtures obtained by coculturing strains or mixing supernatants with powerful antibacterial activities (Table 4). Contrary to what has been reported in the literature, the supernatant mixture exhibited a broader spectrum than the coculture supernatant, suggesting that metabolites from individual strains may exert a synergistic antibacterial effect when combined. Similar results were reported in another study, where the combination of CFS from *Lactobacillus* species showed enhanced growth inhibitory effects on multidrug-resistant pathogens, such as *E. coli*, *S. aureus*, vancomycin-resistance *E. faecium* (VRE), *P. aeruginosa*, and *P. mirabilis* [50]. In addition, the minimum concentrations of the supernatant mixture (CFSM) required for inhibiting *P. mirabilis* and *E. faecalis* were lower than what was reported for CFS of *L. plantarum* (50 mg/ml) [51].

Fungal contamination often occurs during food processing and storage and contributes to mouldy odours (volatiles), deterioration, and spoilage [52]. Certain filamentous fungi, particularly *Aspergillus*, *Penicillium*, and *Fusarium* species, produce mycotoxins that are carcinogenic and harmful to human and animal health [53]. This study showed that CFSM effectively inhibited the growth of *P. chrysogenum*, likely through damaging fungal structures and suppressing gene expression by organic acids and antimicrobial peptides reported by other studies [22, 54–58]. In addition, the CFSM also showed strong preventive effects on biofilm formation, possibly by reducing planktonic cell growth, although it was less effective in degrading established biofilms, probably due to the protective matrix of extracellular polymers [59, 60]. Other studies also reported that higher levels of CFS were required to control biofilms, and that it was generally more effective in preventing than degrading biofilms [61].

Antioxidants are commonly used in foods to retard fat rancidity and undesirable off-flavours. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have raised public concern due to their adverse health effects [62]. In this study, CFSM exhibited potent radical scavenging properties. Similar results have been observed in CFS of different LAB strains [63, 64]. In addition, CFSM exhibited dose-dependent toxicity on the cell lines tested. Studies showed that supernatants with lower pH exhibited greater toxicity to cancer cells and that this toxicity was probably due to acids [65, 66].

Understanding the bioactive compounds present in the supernatant cocktail is essential for its applications. Its antimicrobial activity was sensitive to neutralization and enzymatic digestion, suggesting that the main active metabolites are acidic and proteinaceous compounds. Similarly, the bioactivity of CFSs of *Weissella cibaria* and *Lactobacillus* spp. was sensitive to neutralization [67, 68]. As our results suggested that the main antimicrobial compounds were acids, the SCFA and volatile profiles of the CFSM were further elucidated. As expected, acetic acid was the most abundant SCFA as *L. plantarum* strains are known acetic acid producers [69]. Although less abundant, propionic acid and butyric acid were likely involved in the antagonistic activity of the CFSM as observed in another study [70]. In contrast, the CFSM had a rich volatile profile (32 volatiles), including carbonyl compounds, organic acids, and alcohols. These volatiles are commonly found in dairy and meat products and impart characteristic flavours.

Table 7 Volatile compounds of the CFSM

No. of peak	R.T. (min)	Area	Probability (%)	Compound	Formula	MW	Flavour and taste
1	6.55	247,926.86	55.8	Acetone (methyl ketone)	C_3H_6O	58.08	Fruity, pungent, sweetish
2	9.021	N.Q	12.9	3-Methylbutanal	$C_5H_{10}O$	86.13	apple-like, powerful penetrating, acrid, warm, herbaceous, slightly fruit, nut-like
3	14.938	45,338.57	83.2	Disulfide, dimethyl	$C_2H_6S_2$	94	garlic-like, onion, sulphurous
4	18.06	N.Q	62.3	Orthoformic acid, triisobutyl ester	$C_{13}H_{28}O_3$	232	-
5	18.535	133,733.44	72.1	1-Butanol	$C_4H_{10}O$	74	Fruit
6	20.16	70,933.95	68	2-Heptanone (N-amyl methyl ketone)	$C_7H_{14}O$	114	Blue cheese, fruit, green, nut, spice
7	20.967	57,210.55	37.1	Dodecane	$C_{12}H_{26}$	170	-
8	21.468	96,508.88	30.3	1-Butanol, 3-methyl-/isoamyl alcohol	$C_5H_{12}O$	88	Burnt, cocoa, floral, malt
9	24.12	77,881.13	78	2-Methylpyrazine	$C_5H_6N_2$	94	Cocoa, green, hazelnut, popcorn, roasted
10	24.95	N.Q	92	Acetoin	$C_4H_8O_2$	88	Butter, creamy, green pepper
11	25.562	45,494.73	38.2	2-Propanone, 1-hydroxy-/ hydroxyacetone	$C_3H_6O_2$	74	Butter, herb, malt, pungent
12	26.817	132,999.4	69	Pyrazine, 2,5-dimethyl-/2,5-dimethylpyrazine	$C_6H_8N_2$	108	Cocoa, roast beef, roasted nut
13	27.116	49,643.26	78.8	Pyrazine, 2,6-dimethyl-, 1-oxide	$C_6H_8N_2$	108	-
14	29.356	67,053.42	98.3	Dimethyl trisulfide	$C_2H_6S_3$	126	Cabbage, fish, onion, sulphur
15	29.818	63,371.42	85.4	2-Nonanone	$C_9H_{18}O$	142	Fragrant, fruit, green, hot milk
16	30.268	53,737.46	34	Tetradecane	$C_{14}H_{30}$	198	-
17	31.527	760,057.98	74.2	Benzene, 1,3-bis(1,1-dimethylethyl)-/1,3-Di-tert-butylbenzene	$C_{14}H_{22}$	190	-
18	32.135	4,098,550.2	81.4	Acetic acid	$C_2H_4O_2$	60	Acid, fruit, pungent, sour, vinegar
19	35.441	95,807.24	65.7	Benzaldehyde	C_7H_60	106	Bitter almond, burnt sugar, cherry, malt, roasted pepper
20	35.955	N.Q	51.3	Propanoic acid/propionic acid	$C_3H_60_2$	74	Fat, fruit, pungent, silage, soy
21	37.231	81,282.61	93.3	Propanoic acid, 2-methyl- / isobutyric acid	$C_4H_8O_2$	88	Burnt, butter, cheese, sweat
22	38.712	N.Q	68.7	2-Undecanone	$C_{11}H_{22}O$	170	Fresh, green, orange, rose
23	39.616	148,678.77	90	Butanoic acid/butyric acid	$C_4H_8O_2$	88	Butter, cheese, sour
24	40.545	58,092.29	32.8	2-Methylbenzaldehyde	C_8H_8O	120	-
25	41.29	77,275.66	84.7	Butanoic acid, 3-methyl-/ isovaleric acid	$C_5H_{10}O_2$	102	Cheese, pungent
26	44.561	61,900.2	90.3	Oxime-, methoxy-phenyl-	$C_8H_9NO_2$	151	-
27	46.775	122,649	35.9	Benzaldehyde, 3,4-dimethyl-/3,4-dimethylbenzaldehyde	$C_9H_{10}O$	134	-
28	47.807	N.Q	63.7	Hexanoic acid/caproic acid	$C_6H_{12}O_2$	116	Cheese, oil, pungent, sour
29	47.978	44,207.73	35.3	2,6-Octadien-1-ol, 3,7-dimethyl-(Z)-/Nerol	$C_{10}H_{18}O$	154	Floral, fruit
30	50.227	41,252.67	74.4	Phenylethyl alcohol	$C_8H_{10}O$	122	Fruit, honey, lilac, rose, wine
31	54.83	47,979.37	36.7	Octanoic acid (heptane)	$C_8H_{16}O_2$	144	Cheese, fat, grass, oil
32	57.634	50,936.21	12.2	Nonanoic acid	$C_9H_{18}O_2$	158	Fat, green, sour

Short- and medium-chain fatty acids such as isovaleric, butanoic, and octanoic acids are VOCs that contribute to the cheesy and fatty odour of cheese [15, 71]. 3-Methylbutanal and acetoin are carbonyl compounds that contribute to barley malt and yoghurt notes, respectively [72, 73]. Our preliminary results suggest that CFSM could also be applied to foods with

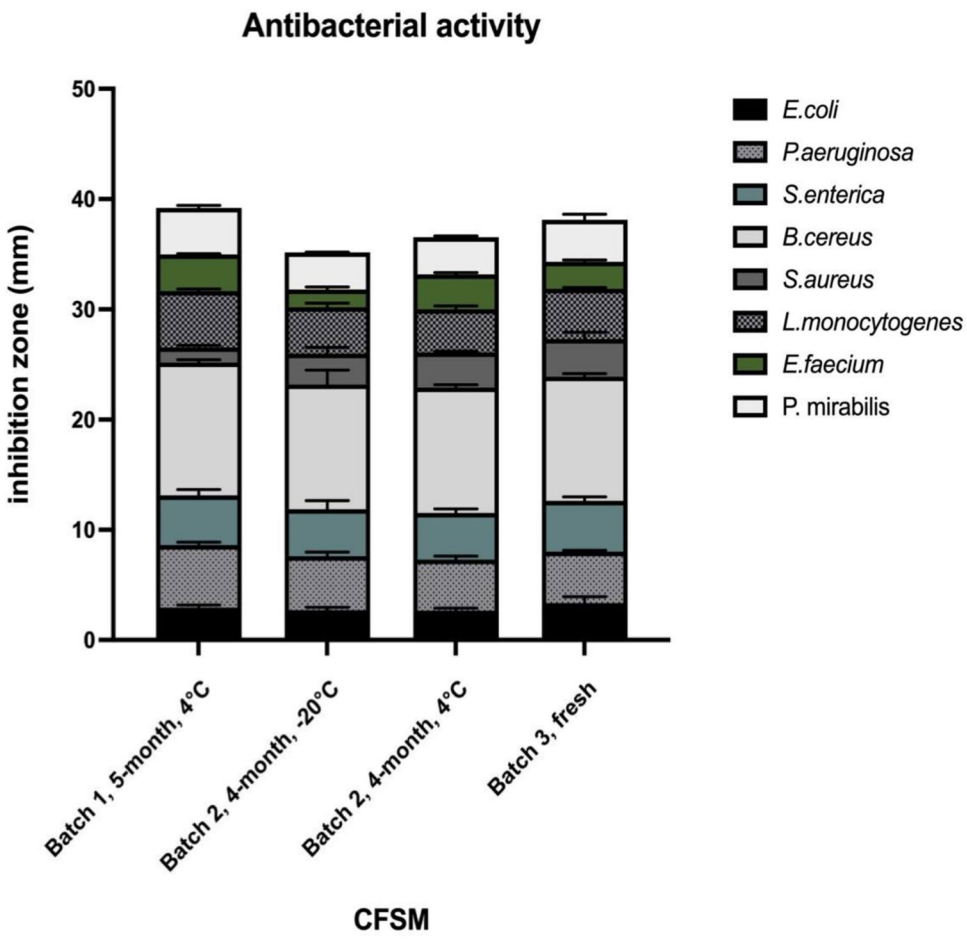

Fig. 9 Antibacterial efficiency of the CFSM produced from different batches under storage conditions

a similar volatile profile, although the concentrations of the main aroma compounds need to be determined. Further studies are needed to quantify the major volatiles present in CFSM and their interactions with the food system.

The stability and reproducibility of natural biopreservatives are of great interest for industrial applications. This study showed that CFSM maintained most of its antimicrobial efficacy under refrigerated conditions for 5 months, although anti-*S. aureus* activity was slightly reduced. This reduction may have been due to reduced levels of acetic acid, as suggested by other studies [74, 75]. In addition to high stability, the antimicrobial capacity of CFSM was highly reproducible even from different fermentation batches. Overall, our results indicated that the CFSM exhibited diverse bioactivity, low toxicity, and high stability.

Conclusion

In this study, a broad-spectrum LAB supernatant cocktail was prepared by combining metabolites from LAB strains including *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Pediococcus acidilactici*, and *Ligilactobacillus salivarius*. Various aspects of this supernatant cocktail, including chemical composition, bioactivity, cytotoxicity, and stability, have been thoroughly investigated. This cocktail is mainly composed of organic acids, volatiles, and bacteriocin-like substances. It shows potent inhibitory activity against food-related pathogenic and spoilage microorganisms and is effective in preventing biofilm formation. In addition, this cocktail showed antioxidant activity and low toxicity to human cells. Under refrigerated conditions, the antimicrobial capacity of this supernatant cocktail remained stable for 5 months and the efficacy is highly reproducible. This study highlights the potential of LAB supernatant as a novel and natural alternative to conventional chemicals and antibiotics, opening up new opportunities for their food and pharmaceutical applications.

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Data Availability All the data supporting the conclusions of this study are available from the corresponding authors upon reasonable request.

Declarations

Conflict of Interest The authors declare no competing interests.

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