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Research article

Analysis and identification of the main antimicrobial metabolites of *Lactobacillus plantarum* LPZN19 ☆

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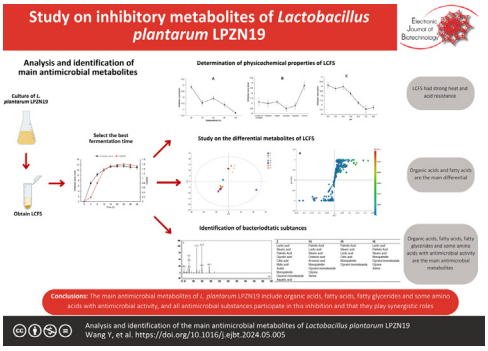
GRAPHICAL ABSTRACT



Study on inhibitory metabolites of *Lactobacillus plantarum* LPZN19

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ABSTRACT

Background: *Lactobacillus plantarum* can produce many secondary metabolites, some of which have antibacterial effects. This study aimed to explore the main antimicrobial metabolites of *Lactobacillus plantarum* LPZN19.

Results: The results of antibacterial activity after fermentation for different durations showed that the metabolites from the LPZN19 cell-free supernatant (LCFS) after 24 h had the strongest antibacterial activity, which was confirmed by the highest contents of organic acids and fatty acids in the LCFS after 24 h. Lactic acid, phenyllactic acid, malic acid, aspartic acid, dodecanoic acid and propionic acid were the main differentially abundant metabolites. LCFS was separated by semi-preparative liquid chromatography to obtain 4 antibacterial parts, mainly organic acids such as lactic acid, glycolic acid, and citric acid, and fatty acids such as stearic acid, palmitic acid, and octanoic acid. In addition, fatty glycerides and amino acids with antimicrobial activity were included.

Conclusions: Our findings indicate that the main antimicrobial metabolites of *L. plantarum* LPZN19 include organic acids, fatty acids, fatty glycerides and some amino acids with antimicrobial activity, which not only clarifies the main antimicrobial metabolites of *L. plantarum* LPZN19 but also provides an effective method for rapid screening of antimicrobial substances.

☆ Audio abstract available in Supplementary material.

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1. Introduction

Lactic acid bacteria are widely used in the processing and production of fermented food. They have many beneficial health effects, among which antibacterial action is one of the most important [1,2]. There are two reasons why lactic acid bacteria exert antibacterial effects. On the one hand, lactic acid bacteria can antagonistically affect pathogenic microorganisms in microecological environments. On the other hand, lactic acid bacteria produce organic acids (lactic acid, acetic acid, formic acid, propionic acid, etc.), fatty acids (caprylic acid, palmitic acid), and other metabolites during fermentation, which can significantly reduce the pH of the environment, thus leading to antibacterial effects [3,4,5].

In recent years, breakthroughs in metabolomics technology have made progress in studying metabolic pathways and monitoring and optimizing the fermentation process of lactic acid bacteria. Zha et al. [6] used ultrahigh-performance liquid chromatography combined with time-of-flight mass spectrometry to study the changes in metabolites of Lactobacillus plantarum-fermented milk, which provided a valuable reference for the development and improvement of fermented dairy products made with this strain in the future. Park et al. [7] investigated the changes in flavor substances during the fermentation of kimchi through GC-MS, providing a theoretical basis for controlling the quality of kimchi.

During the early stages of this study, we screened one probiotic, *Lactobacillus plantarum* LPZN19, and one putrid, *Proteus mirabilis* PM18, from fermented sausages. The results of antibacterial experiments of *L. plantarum* LPZN19 on *P. mirabilis* PM18 showed that LPZN19 disrupted the normal cell wall and membrane of PM18, damaging its morphology and structure and inhibiting its growth [8]. However, it is unclear what *L. plantarum* LPZN19 produces antibacterial substances. Moreover, there are few studies on the antimicrobial metabolites of *L. plantarum*, except for those of Mao et al. [9]. In this research, GC–MS and metabonomics were used to analyze the metabolites of LCFS, determine the markers of antibacterial differences, analyze the main antibacterial substances, and preliminarily separate the LCFS by semi-preparative liquid chromatography. The antibacterial component was obtained and analyzed, hoping to provide a further reference for the antibacterial mechanism of *L. plantarum* LPZN19 against spoilage microorganisms.

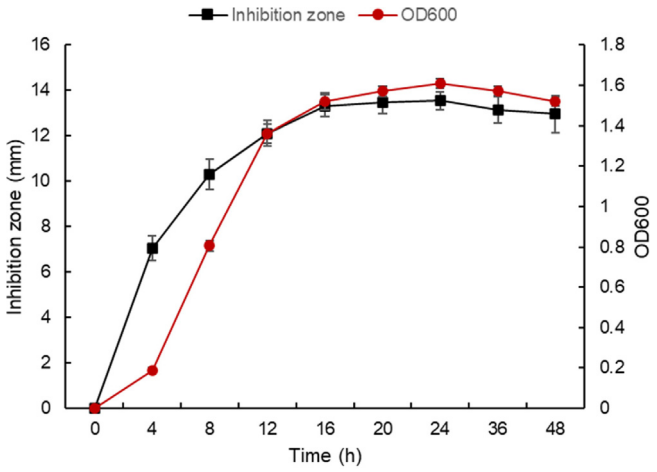


Fig. 1. Antibacterial activity at different fermentation times.

2. Materials and methods

2.1. Reagents and strains

Compound protease (1.36×10^4 U/g), pepsin (3.5×10^3 U/g), trypsin (2.5×10^3 U/g), papain (9.32×10^3 U/g) and catalase (5×10^3 U/g) were obtained from Beijing Boao Tuoda Technology Co., Ltd. (Beijing, China). Bis-(trimethylsilyl)-trifluoroaceta mide (BSTFA) was purchased from Regis Technologies, Inc. (IL, USA). Methanol, chloroform, L-2-chlorophenylalanine, and pyridine were obtained from Shanghai Heng Bo Biological Technology Co. (Shanghai, China). Unless otherwise indicated, all other reagents were obtained from Alfa Aesar Chemical Co., Ltd. (Tianjin, China).

L. plantarum LPZN19 (accession number: MW282954) *and P. mirabilis* PM18 (accession number: MT730122) were isolated from spontaneously fermented Sichuan sausages and identified by a previous study. *L. plantarum* was grown on De Man Rogosa Sharpe (MRS) broth, and *P. mirabilis* was incubated in Luria–Bertani (LB) broth and cultured in an incubator (Yiheng Technology Co., Ltd., Shanghai, China) at 37°C for 24 h.

Table 1
Content of organic acids and fatty acids in different fermentation times (mg/mL).

Time (h)	Lactic acid	Stearic acid	Palmitic Acid	Glycolic acid	Citric acid	Malic acid
0	0	0	0	0	0	0
4	7.32 ± 0.27^{d}	2.9 ± 0.45^{a}	2.17 ± 0.62^{b}	0.34 ± 0.55^{de}	4.11 ± 0.05^{e}	0.3 ± 0.42^{b}
8	13.86 ± 0.08^{b}	2.95 ± 0.5^{a}	2.8 ± 0.36^{ab}	0.7 ± 0.62^{bcd}	6.87 ± 0.17^{d}	0.77 ± 0.15^{b}
12	15.97 ± 0.96^{a}	2.41 ± 0.77^{a}	3.09 ± 0.45^{a}	0.61 ± 0.31^{cde}	13.84 ± 0.05^{c}	0.91 ± 0.98^{b}
24	16.71 ± 0.97^{a}	2.57 ± 0.43^{a}	2.92 ± 0.38^{a}	1.07 ± 0.10^{bc}	17.62 ± 0.24^{a}	2.9 ± 0.44^{a}
36	12.16 ± 0.35^{c}	2.11 ± 0.28^{a}	2.97 ± 0.15^{a}	1.29 ± 0.12^{b}	16.35 ± 0.27^{b}	3.22 ± 0.65^{a}
48	13.83 ± 0.28^{b}	2.3 ± 0.9^{a}	3.23 ± 0.20^{a}	2.12 ± 0.19^{a}	13.78 ± 0.36^{c}	3.67 ± 0.74^{a}

Different letters (a-e) within a column indicate significant differences in organic acid content among the different durations ($p < 0.05$).

2.2. Activation of strains

LPZN19 and PM18 stored in glycerol were thawed on MRS and LB agar media. Single colonies were selected and inoculated in 50 mL of MRS or LB broth with a 2% inoculation amount and activated for three generations.

2.3. Preparation of the LCFS

These materials were prepared as described previously [3] with some modifications. The preserved LPZN19 was inoculated into an MRS broth medium and cultured to normal metabolic levels for two generations. The bacterial culture broth was then centrifuged at 8000 rpm for 10 min and filtered through a 0.45 μm Millipore membrane filter membrane to obtain LCFS.

2.4. Determination of the antibacterial activity and content of organic acids and fatty acids at different fermentation times

The activated LPZN19 was subsequently transferred to 200 mL of MRS broth supplemented with 2% inoculum, after which the mixture was cultured at 37°C for 48 h. The fermentation broth was removed after 0, 4, 8, 12, 24, 36, and 48 h, after which the OD_{600} was determined by a spectrophotometer (Thermo Scientific, Wilmington, USA). Preparing the LCFS from the above time period, the Oxford cup method determined the bacteriostatic activity of the LCFS against PM18 [10]. Furthermore, the contents of organic acids and fatty acids in the LCFs during the corresponding period were determined by HPLC (Thermo Scientific, San Jose, California, USA) according to previous methods using an Agilent ZORBAX SB-Aq column (5 μm, 250 × 4.6 mm) [11]. Chromatography was conducted with a gradient of 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.6 mL/min and a column temperature of 50°C. The contents of various organic acids and fatty acids were calculated according to the peak time and peak area.

2.5. Exploring the physicochemical properties of antibacterial metabolites

The influence of temperature, pH, and enzyme on the antibacterial activity of the LCFS was determined by the Oxford cup method according to previous methods [12] with some modifications. The specific procedure was as follows: LCFS was treated for 30 min at −20, 4, 30, 60, 80, 100, or 121°C, after which the change in antibacterial activity of the LCFS was tested. The pH of the LCFS was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, or 8.0 with 1 mol/L HCl and 1 mol/L NaOH, after which the pH was adjusted back to the initial pH by a pH meter (Yiheng Technology Co., Ltd., Shanghai, China) after the pH was maintained for 1 h. Afterward, the antibacterial activity of the LCFS was detected. LCFS were treated with trypsin, pepsin, or proteinase K. The enzyme solution was adjusted to a concentration of 1 mg/mL. The temperature and pH were adjusted to the optimum values for the following enzymes: compound protease (45°C, 6.5), pepsin (37°C, 2.0), trypsin (37°C, 7.0), papain (60°C, 6.5) and catalase (37°C, 7.2). The enzyme solution and the LCFS were mixed in equal volumes for 3 h, and before evaluating the antibacterial activity, the pH of the LCFS was readjusted to the initial pH. LCFS without enzyme treatment served as a control.

2.6. GC-MS analysis of the metabolites

The metabolites of LPZN19 were analyzed as described previously [13] with some modifications. The LCFS were extracted for different durations with ethyl acetate, and the extraction mixture was dried by a nitrogen blower and derivatized at 85°C in 35 μl of pyridine and 35 μl of N,O-bistrimethylsilyl trifluoroacetamide for 60 min at 70°C. After standing at room temperature for 30 min, the sample (1 μL) was injected with the Agilent 7683 autosampler in splitless mode onto a DB-5MS GC capillary column

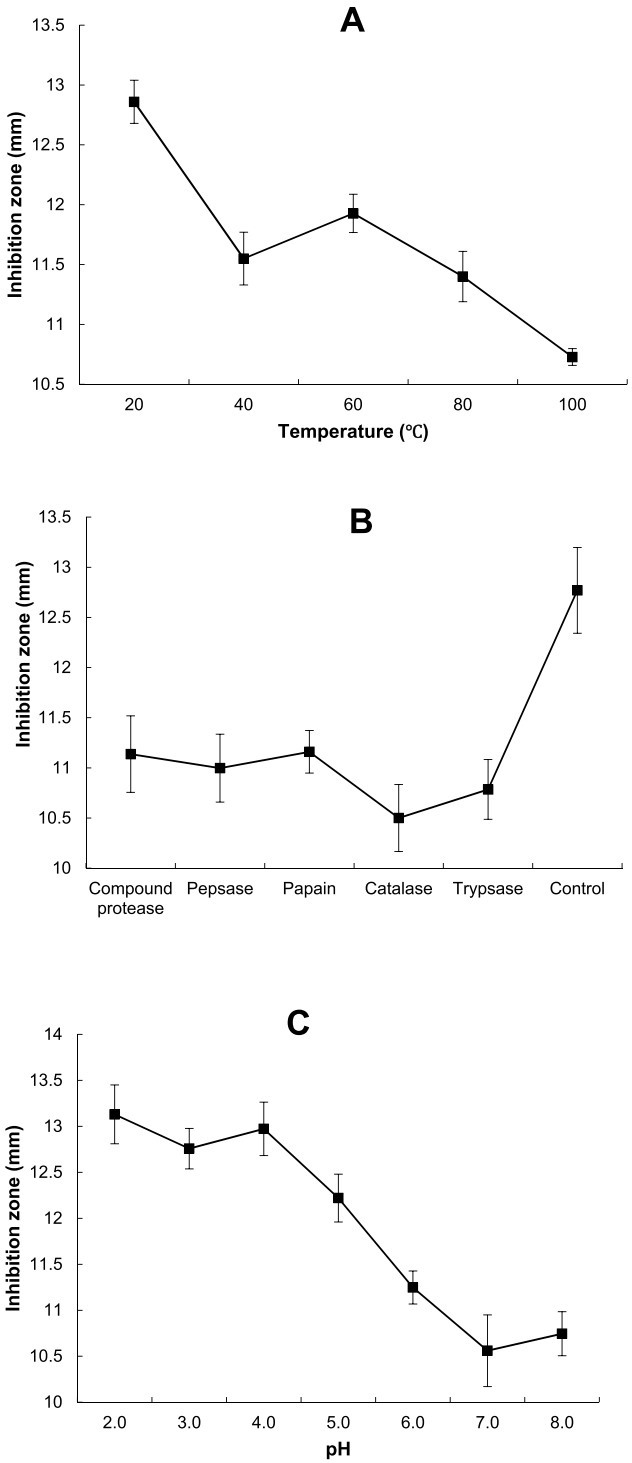


Fig. 2. Effects of different temperatures, enzyme treatments, and pH on antibacterial activity. A: temperature; B: enzyme treatment; C: pH.

(Agilent Technologies, 60 m × 0.250 mm × 0.25 μm; Agilent 7890 GC) coupled to a 5975 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) with an EI ionization source, which was set to 230°C. Each sample was run in duplicate, beginning with a blank sample consisting of 200 μL of methanol. The temperature of the inlet was set at 280°C. Ultrapure helium gas was used as the mobile phase. The oven temperature was set at 70°C for 2 min, with a subsequent temperature gradient of 10°C per minute until a final temperature of 200°C was reached, after which the temperature was held for 10 min. Mass spectra ranging from 30 to 550 *m*/*z* were recorded.

2.7. Multivariate statistical analysis

All GC-selected peaks were searched and identified using the NIST Mass Spectral Library, and the peak area normalization method was used to calculate the relative percentage of each component [9,14]. The collected data were processed by Simca 14.1 software for principal component analysis, and total metabolic differences were observed. The samples were analyzed using the OPLS-DA method to identify the metabolites that could significantly distinguish the two samples [15].

2.8. Isolation and identification of antibacterial substances

The isolation and identification of antibacterial substances and metabolites were performed as described previously [16] with some modifications. The procedure was as follows: 200 mL of fermentation supernatant was freeze-dried, after which the product was redissolved in 10 mL of sterile water. The redissolved product was extracted with ethyl acetate at a volume ratio of 1:1.5 and then redissolved in 10 mL of sterile distilled water after nitrogen flow. The above-treated samples were separated by semi-preparative liquid chromatography (Waters 1525, USA) using a preparative Waters XBridge Prep C_{18} (5 μm, 10 mm × 150 mm) at a flow rate of 3 mL/min, and the effluence was monitored at 210 nm. The peak-partition multi-tube collection method was adopted, and one tube was collected every 1 min (flow rate: 3 mL/min, collection tube: 10 mL). Chromatography was conducted with a gradient of methanol (solvent A) and 0.05% trifluoroacetic acid (solvent B) as follows: 0 min, A/B (10:90); 30 min, A/B (100:0); 35 min, A/B (100:0); 40 min, A/B (10:90); and 45 min, A/B (10:90). The isolated antimicrobial agents were identified by GC-MS (**Section 2.6**).

2.9. Determination of the antibacterial activity of organic acids and fatty acids

A pure acid solution and six mixed acid solutions with the same concentration of each acid (lactic acid, acetic acid, propionic acid, citric acid, and phenyllactic acid) were added to the LCFS for the strongest antibacterial ability period, according to the results of the determination of organic acids and fatty acids in the LCFS. The antibacterial effect on PM18 was determined by the Oxford cup method.

2.10. Statistical analysis

All analyses were performed in triplicate. The collected data were subjected to a one-way analysis of variance (ANOVA). The statistical significance of differences in group means was evaluated at $P < 0.05$ using Duncan's multiple range tests. Statistical data analysis was carried out in SPSS 22.0 (SPSS, Inc., Chicago, IL, USA).

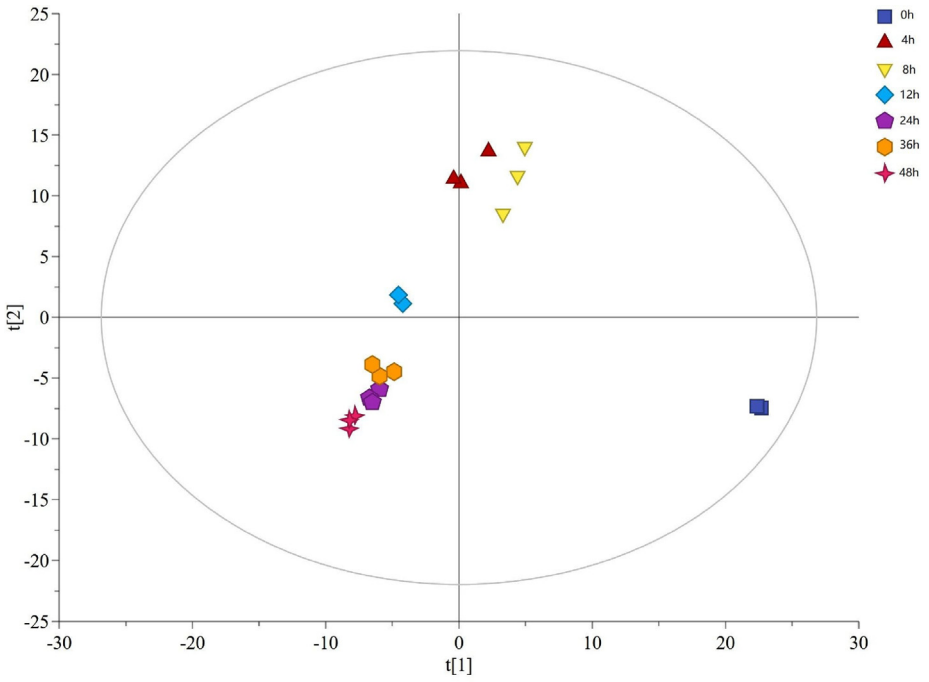


Fig. 3. GC-MS spectrum PCA score plots of the cell-free supernatant of *L. plantarum* LPZN19 (LCFS) at different fermentation times.

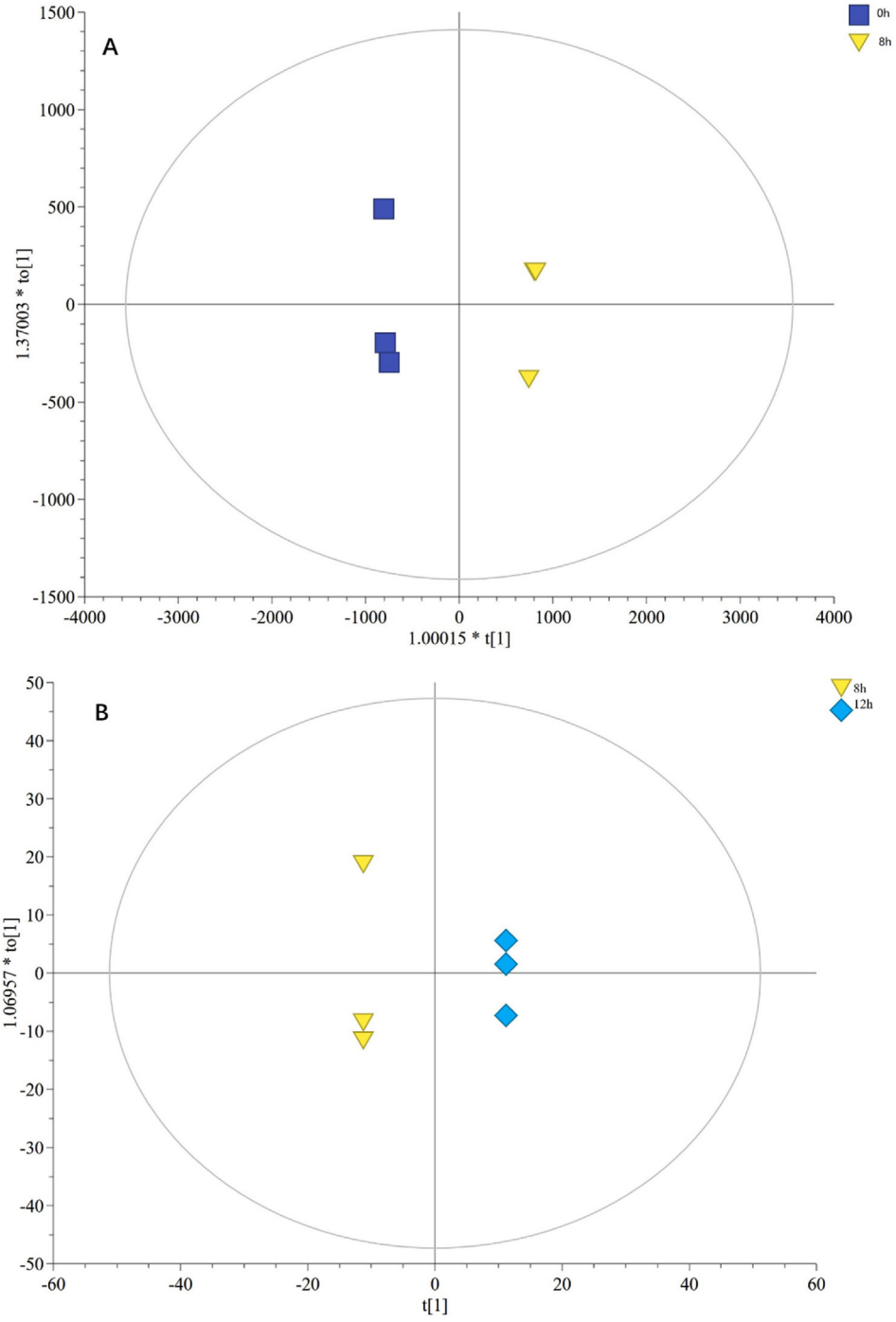


Fig. 4. OPLS-DA diagram of LCFS at different fermentation times. A: 0 and 8 h; B: 8 and 12 h; C: 12 and 24 h. The greater the distance between the two time points was, the greater the difference between the metabolites in the LCFS.

3. Results

3.1. Determination of the antibacterial activity of LCFS fermented for different durations

The inhibitory effect of the fermentation supernatant was positively correlated with the growth of the bacteria, as shown in Fig. 1. Specifically, LPZN19 entered the logarithmic phase after 4 h and reached a plateau phase after 16 h. LCFS had no bacteriostatic ability after 0 h, and the bacteriostatic ability significantly improved after 0–4 h. Similarly, the bacteriostatic diameter was close to 8.65 mm after 4 h. The antibacterial ability gradually increased from 4 h to 24 h, and the antibacterial diameter reached a maximum of 13.54 mm after 24 h. After 24 h of fermentation, there was no significant difference in antibacterial ability. As shown in Table 1, the contents of organic acids and fatty acids in the LCFS increased sharply at 0–4 h, and the bacteriostatic ability was related to the continuous increase in the amount of organic acids and fatty acids during the growth of the bacteria.

3.2. Study on the physicochemical properties of LCFS

The physicochemical properties of the LCFS are shown in Fig. 2A. Fig. 2A shows that the antibacterial activity decreased with increasing treatment temperature, and there was still antibacterial activity after treatment at 100°C for 30 min, indicating that the LCFS had good heat resistance. From Fig. 2B, it can be seen that the inhibitory effect of LCFS after complex protease, pepsin, trypsin, papain, and catalase treatment was significantly reduced, indicating that LCFS was more sensitive to these enzymes. Moreover, the antibacterial activity of the catalase treatment was lower than that of the other enzymes, indicating that there was hydrogen peroxide in the LCFS. As shown in Fig. 2C, LCFS had good antibacterial activity at pH 2–4, and the antibacterial activity gradually decreased at pH > 4.0, indicating that LCFS could better exhibit antibacterial activity in acidic environments. The decrease in antibacterial activity may be due to changes in the protein structure of antibacterial substances caused by neutral or alkaline conditions, which affect antibacterial activity [9,12,17].

3.3. Differentially abundant metabolite analysis in LCFS

Principal component analysis (PCA) was used to analyze the LCFS in seven time periods, and the results are shown in Fig. 3. Except for the 24 h, 36 h, and 48 h samples, the samples from the other periods could be distinguished. The difference between the LCFS of fermentation at 0 h and 4 h was significant. After 8 h of fermentation, the number of metabolites increased, and the antibacterial ability of LCFS continued to increase correspondingly, which was significantly different from that observed after 0 h of fermentation but not after 4 h. The number of metabolites in the LCFS increased continuously after 12 h of fermentation, which could be distinguished from that after 8 h. After 24 h of fermentation, the number of metabolites in the LCFS was balanced and could be distinguished after 12 h but not after 36 or 48 h. The above results were consistent with the results of the bacteriostatic test in **Section 3.1**.

The orthogonal partial least-squares discriminant analysis (OPLS-DA) model was used to screen for metabolites whose expression significantly changed between samples. As shown in Fig. 4A–C, the 3 groups could be distinguished considerably between 0 h and 8 h, 8 h and 12 h, and 12 h and 24 h, indicating significant changes in the metabolites in the LCFS liquid among the 3 groups. In the S-plot diagram (Fig. 5), each dot in the figure

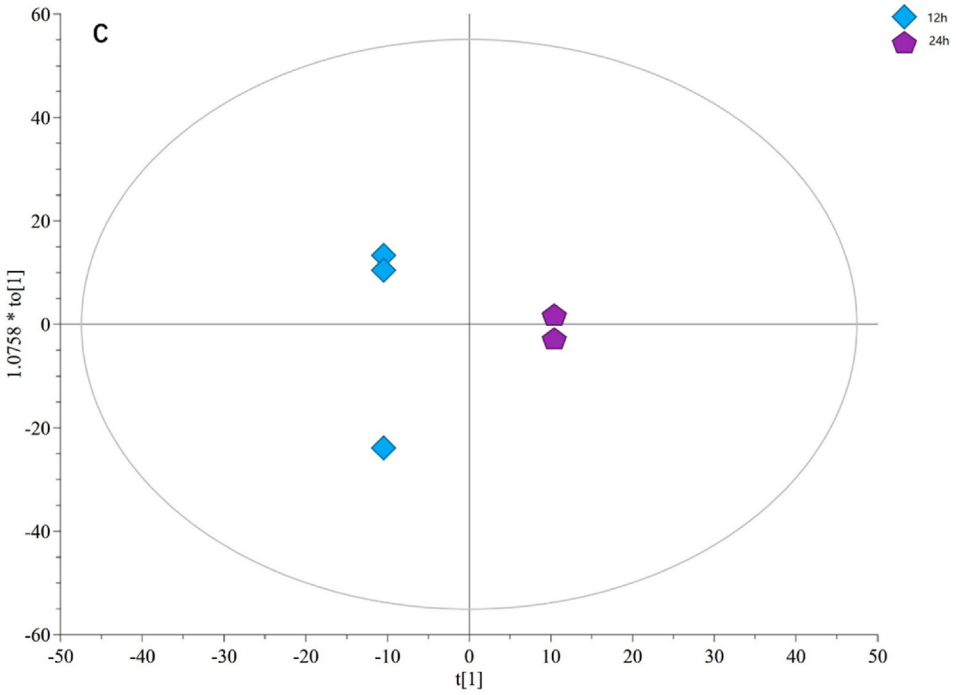


Fig. 4 (*continued*)

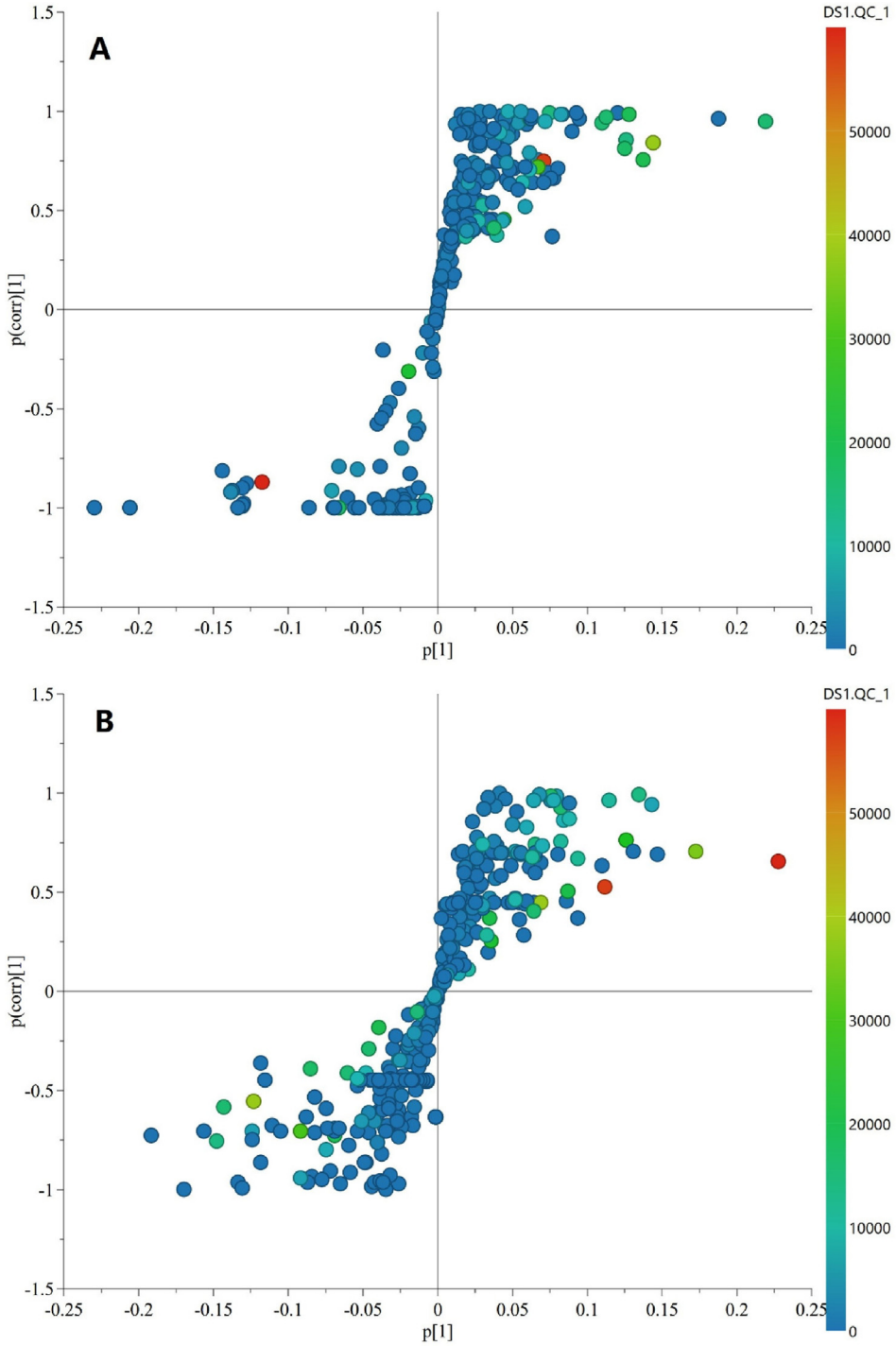


Fig. 5. S-plot diagram of LCFS at different fermentation times. A: 0 and 8 h; B: 8 and 12 h; C: 12 and 24 h. Each point in the diagram represents a metabolite. The further the point in the figure is from the center, the more significant the change in metabolites.

represents a metabolite. The further the point in the figure from the center was, the more significant the change in these metabolites was [9,18]. The variable importance in the projection (VIP) values of each variable in the metabolites of LCFS were calculated, and the variables with a VIP value > 1.0 (obtained from OPLS-DA models) and values of $p < 0.05^*$ were selected.

There were 19 differentially abundant metabolites at 0 h and 8 h of fermentation, 21 at 8 h and 12 h of fermentation, and 22 at 12 h and 24 h of fermentation (Table 2). Among them, undecenoic acid, phenyllactic acid, citric acid, α-ketoisovalerate, stearic acid, 2-hydroxybutyrate acid and acetic acid were upregulated by differentially abundant metabolites at 0 and 8 h after fermentation. Malic acid, L-aspartic acid, *trans*-2-undecenoic acid, clavulanic acid, L-leucine, citric acid, L-alanine, phenyllactic acid, glycine and L-isoleucine were upregulated by differentially abundant metabolites at 8 and 12 h after fermentation. Undecanoic acid, tyrosine, stearic acid, L-valine, L-serine, L-leucine, L-isoleucine, L-aspartic acid, lactic acid, propionic acid, adipic acid, hydroxyacetic acid, and dodecanoic acid were upregulated by differentially abundant metabolites at 12 and 24 h after fermentation. Combined with the above three groups of differentially abundant metabolites, it was speculated that the main antibacterial substances were organic acids, fatty acids and amino acids with antibacterial activity.

3.4. Isolation and identification of antimicrobial substances

The LCFS were separated by semi-preparative liquid chromatography, and the resulting chromatograms are shown in Fig. 6. The LCFS were divided into 24 parts, of which 4 had antibacterial activity. Table 3 shows that the maximum inhibitory diameter in Part 2 was 13.51 mm, and the minimum inhibitory diameter in Part 16 was 12.78 mm. As shown in Table 4, after identification by GC-MS (Fig. 7 and Fig. 8), it was found that the four parts mainly included organic acids such as lactic acid, glycolic acid and citric acid and fatty acids such as stearic acid and palmitic acid. Moreover, there are fatty glycerides, such as monopalmitin and glycerol monostearate. Finally, some amino acids have antibacterial properties, such as aspartic acid, glycine, and serine.

3.5. Determination of the antibacterial activity of organic acids and fatty acids

According to the concentrations of the main organic acids and fatty acids determined by high-performance liquid chromatography, pure acid solution and mixed acid solution with the same concentration were prepared to compare the antibacterial activity.

The results are shown in Table 5. The contents of lactic acid and citric acid were much greater than those of the other acids. Lactic acid had the strongest antibacterial activity, followed by glycolic acid and citric acid. The other acids have no obvious antibacterial ability due to their low content. In addition, the antibacterial activity of the mixed acids was less than that of LCFS. The above results confirmed the results in Section 3.4. In addition to organic acids and fatty acids, LPZN19 produces other antibacterial metabolites, such as fatty acid glycerides and amino acids.

4. Discussion

This study analyzed and explored the main antibacterial substances associated with the metabolites in the LPZN19 cell-free supernatant (LCFS). First, we determined the LCFS with the strongest antibacterial activity during the time period using the Oxford Cup method and explored its physicochemical properties. Then, we

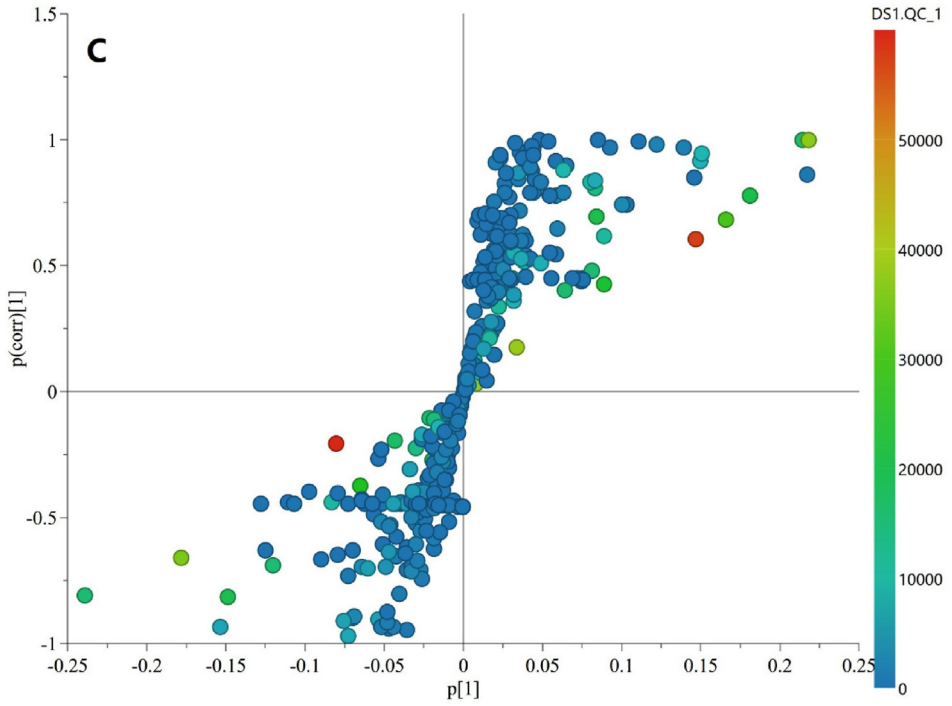


Fig. 5 (*continued*)

Table 2
Differential metabolites in fermentation broth at different fermentation time.

No.	RT (min)	Name	CAS#	Formula	VIP
1	7.765	α-Ketoisovalerate	759-05-7	C5H8O3	1.10557
2	9.112	4- Aminobutyric acid	56-12-2	$C_4H_9NO_2$	1.14304
3	9.557	Glycolic acid	79-14-1	$C_2H_4O_3$	1.11339
4	10.299	2-Hydroxybutyric acid	600-15-7	$C_4H_8O_3$	1.08791
5	10.489	L-Aspartic acid	56-84-8	$C_4H_7NO_4$	1.09466
6	10.571	Propionic acid	79-09-4	$C_3H_6O_2$	1.14424
7	12.442	Glycerol	56-81-5	$C_3H_8O_3$	1.03331
8	15.714	Pyroglutamic acid	98-79-3	$C_5H_7NO_3$	1.14392
9	16.809	Phenyllactic acid	7326-19-4	$C_9H_{10}O_3$	1.13429
10	17.003	Digitoxin	71-63-6	$C_{41}H_{64}O_{13}$	1.07711
11	18.999	Acetic acid	14190-17-1	$C_{14}H_{14}IO_2$	1.01696
12	19.428	Citric acid	77-92-9	$C_6H_8O_7$	1.11405
13	22.768	Undecanoic acid	112-37-8	$C_{11}H_{22}O_2$	1.14341
14	22.975	Octadecanoic acid	18748-91-9	$C_{21}H_{44}O_2Si$	1.12368
15	24.189	Inositol	2582-79-8	$C_{24}H_{60}O_6Si_6$	1.14421
16	24.781	Dodecanoic acid	55191-44-1	$C_{19}H_{34}O_6$	1.14429
17	26.716	Stearic acid	57-11-4	$C_{18}H_{36}O_2$	1.08884
18	29.006	β-Sitosterol	83-46-5	$C_{29}H_{50}O$	1.13827
19	32.958	Deoxyfructosazine	17460-13-8	$C_{12}H_{20}N_2O_7$	1.10533
20	7.384	L-Alanine	56-41-7	$C_3H_7NO_2$	1.07433
21	7.983	Glycine	56-40-6	$C_2H_5NO_2$	1.03183
22	9.976	4- Aminobutyric acid	56-12-2	$C_4H_9NO_2$	1.11372
23	10.489	L-Aspartic acid	56-84-8	$C_4H_7NO_4$	1.36499
24	10.673	L-Leucine	61-90-5	$C_6H_{13}NO_2$	1.13696
25	11.193	L-Isoleucine	73-32-5	$C_6H_{13}NO_2$	1.02081
26	11.571	DL-Norleucine	616-06-8	$C_6H_{13}NO_2$	1.03351
27	12.442	Glycerol	56-81-5	$C_3H_8O_3$	1.14608
28	12.857	L-Threonine	6028-28-0	$C_4H_9NO_3$	1.10987
29	15.353	Malic acid	6915-15-7	$C_4H_6O_5$	1.37343
30	16.088	Adipic acid	124-04-9	$C_6H_{10}O_4$	1.05584
31	16.169	Digitoxin	71-63-6	$C_{41}H_{64}O_{13}$	1.14549
32	16.792	Phenyllactic acid	7326-19-4	$C_9H_{10}O_3$	1.07348
33	19.316	Citric acid	77-92-9	$C_6H_8O_7$	1.11163
34	22.768	Undecenoic acid	112-37-8	$C_{11}H_{22}O_2$	1.09916
35	22.941	Dodecanoic acid	143-07-7	$C_{12}H_{24}O_2$	1.14394
36	22.975	Stearic acid	57-11-4	$C_{18}H_{36}O_2$	1.11488
37	23.591	Palmitic Acid	57-10-3	$C_{16}H_{32}O_2$	1.14793
38	24.768	Trans-2-undecenoic acid	15790-94-0	$C_{11}H_{20}O_2$	1.43453
39	29.006	β-Sitosterol	83-46-5	$C_{29}H_{50}O$	1.08318
40	32.658	Clavulanic acid	58001-44-8	$C_8H_9NO_5$	1.14997
41	8.755	Octyl thioglycolate	7664-80-4	$C_{10}H_{20}O_2S$	1.44534
42	9.064	Propionic acid	79-09-4	$C_3H_6O_2$	1.04973
43	9.112	Lactic acid	50-21-5	$C_3H_6O_3$	1.36196
44	9.483	Glycolic acid	79-14-1	$C_2H_4O_3$	1.02298
45	9.83	L-Valine	72-18-4	$C_5H_{11}NO_2$	1.42109
46	10.489	L-Aspartic acid	56-84-8	$C_4H_7NO_4$	1.27753
47	10.646	Hydracrylic acid	22788-18-7	$C_7H_{15}Cl_2N_2O_4P$	1.18195
48	11.193	L-Isoleucine	73-32-5	$C_6H_{13}NO_2$	1.48337
49	11.329	L-Leucine	61-90-5	$C_6H_{13}NO_2$	1.25671
50	13.088	Succinic acid	110-15-6	$C_4H_6O_4$	1.14557
51	14.652	Digitoxin	71-63-6	$C_{41}H_{64}O_{13}$	1.076
52	16.088	Adipic acid	124-04-9	$C_6H_{10}O_4$	1.06398
53	16.482	2-Methylglutaconic acid	53358-21-7	$C_6H_8O_4$	1.00723
54	16.533	Undecanoic acid	112-37-8	$C_{11}H_{22}O_2$	1.17517
55	16.792	2-Phenyllactic acid	7326-19-4	$C_9H_{10}O_3$	1.10738
56	16.799	3-Phenyllactic acid	7326-19-4	$C_9H_{10}O_3$	1.52075
57	21.428	Tyrosine	23277-41-0	$C_{10}H_{11}I_2NO_3$	1.07914
58	24.768	Trans-2-undecenoic acid	15790-94-0	$C_{11}H_{20}O_2$	1.4073
59	24.781	Dodecanoic acid	143-07-7	$C_{12}H_{24}O_2$	1.51153
60	26.699	Stearic acid	57-11-4	$C_{18}H_{36}O_2$	1.22635
61	35.539	Glycerol monostearate	123-94-4	$C_{21}H_{42}O_4$	1.44121

Note: No1-19, 20–40, 41–61 represent 0–8 h, 8–12 h, 12–24 h fermentation broth respectively.

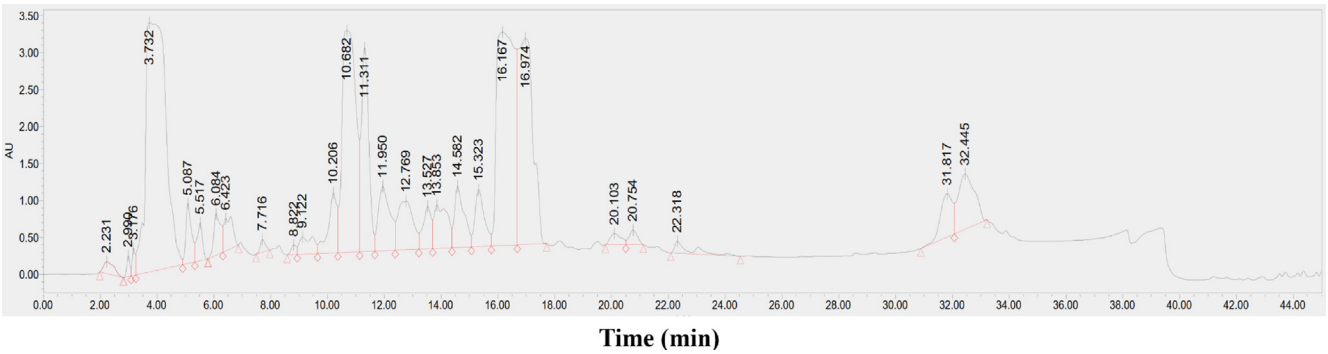


Fig. 6. Semi-preparative liquid chromatogram of LCFS.

Table 3
Antibacterial activity of different parts.

	Separate part			
	2	14	15	16
Time (min)	2.231–3.732	12.769–13.527	13.527–13.583	22.318–31.817
Bacteriostatic diameter (mm)	13.51 ± 0.38	13.28 ± 0.13	12.86 ± 0.15	12.78 ± 0.63

Table 4
Identification results of antibacterial parts.

2	14	15	16
Lactic acid	Palmitic acid	Palmitic acid	Lactic acid
Stearic acid	Lactic acid	Stearic acid	Palmitic acid
Palmitic acid	Stearic acid	Lactic acid	Stearic acid
Glycolic acid	Octanoic acid	Citric acid	Monopalmitin
Citric acid	N-nonoic acid	Monopalmitin	Glycerol monostearate
Malic acid	Monopalmitin	Glycerol monostearate	Glycine
Acetin	Glycerol monostearate		Serine
Monopalmitin	Glycine		
Glycerol monostearate	Serine		
Aspartic acid			

separated the LCFS by semi-preparative liquid chromatography, and the antibacterial activity of the LCFS was tested via the Oxford cup method. Finally, the parts with antibacterial activity were identified by GC-MS. The results of antibacterial activity with different fermentation times and physicochemical tests showed that after 24 h, the LCFS had the strongest antibacterial activity and that the LCFS had strong heat and acid resistance. LCFS was separated by semipreparative liquid chromatography to obtain 4 antibacterial components, which are mainly organic acids and include some amino acids with antibacterial activity.

Mao et al. [9] investigated the growth curve and antibacterial activity of *Lactobacillus plantarum* DY6 at different fermentation time points. The results showed that the growth performance and antibacterial activity of the supernatant of DY6 reached their maximum values within 24 h, which is completely consistent with the results of this study on LPZN19. Research on the physicochemical properties of the LCFS showed that the bacteriostatic activity of all the treatment groups was significantly lower than that of the control group, indicating that the proteins in the LCFS contributed to the bacteriostatic ability. The above results are not consistent with previous studies [9], which may be related to the specificity of the tested strain. In addition, the antibacterial activity of catalase was lower than that of other enzymes, indicating that there was hydrogen peroxide in the LCFS, which is similar to the findings of previous studies [17]. Organic acids and fatty acids are the main antibacterial components in the fermentation broth of lactic acid bacteria, and the antibacterial activity of fermentation broth is closely related to the acid content [18,19]. Additionally, glycerol, fatty glyceride and several amino acids are considered to have antibacterial activity. Fatty glyceride is an efficient preservative with a strong inhibitory effect on bacteria and fungi and little effect on food flavor [20]. Pan et al. [21] studied the metabolites of *Lactobacillus casei* and reported that the LCFS of *L. casei* contained a large amount of glycine and aspartic acid. There is little research on the antibacterial activity and mechanism of action of amino acids; therefore, it is worthwhile to carry out related research in this area. Moreover, the antibacterial test results for organic acids and fatty acids indicate that the antibacterial activity of the mixed acids was less than that of LCFS. As a result, we speculated that all the antimicrobial metabolites played synergistic roles, which is similar to previous research results [22,23].

5. Conclusions

The metabolites of *L. plantarum* LPZN19 were analyzed and identified on the basis of their antibacterial activity at different times. The results demonstrated that after 24 h, the LCFS had the strongest antibacterial activity. Lactic acid, phenyllactic acid, malic acid, aspartic acid, dodecanoic acid and propionic acid were the main differentially abundant metabolites. LCFS was separated by semi-preparative liquid chromatography to obtain 4 antibacterial components, mainly organic acids, fatty acids, fatty glycerides and amino acids, which have antimicrobial activity. Furthermore, we believe that all antimicrobial substances participate in this inhibition and that they play synergistic roles. These findings clarify the main antimicrobial metabolites of *L. plantarum* LPZN19 and provide an effective method for rapid screening of antimicrobial substances.

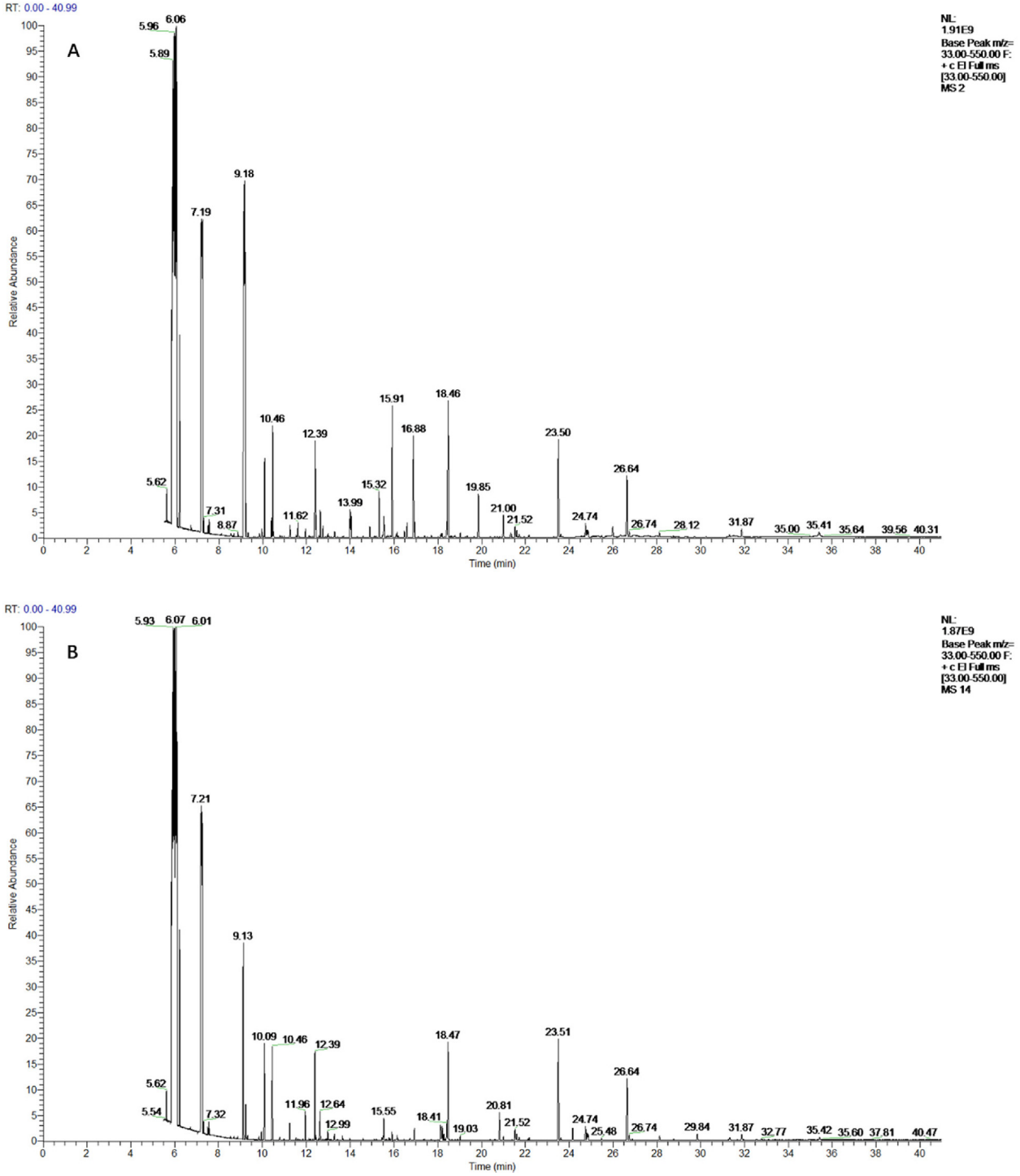
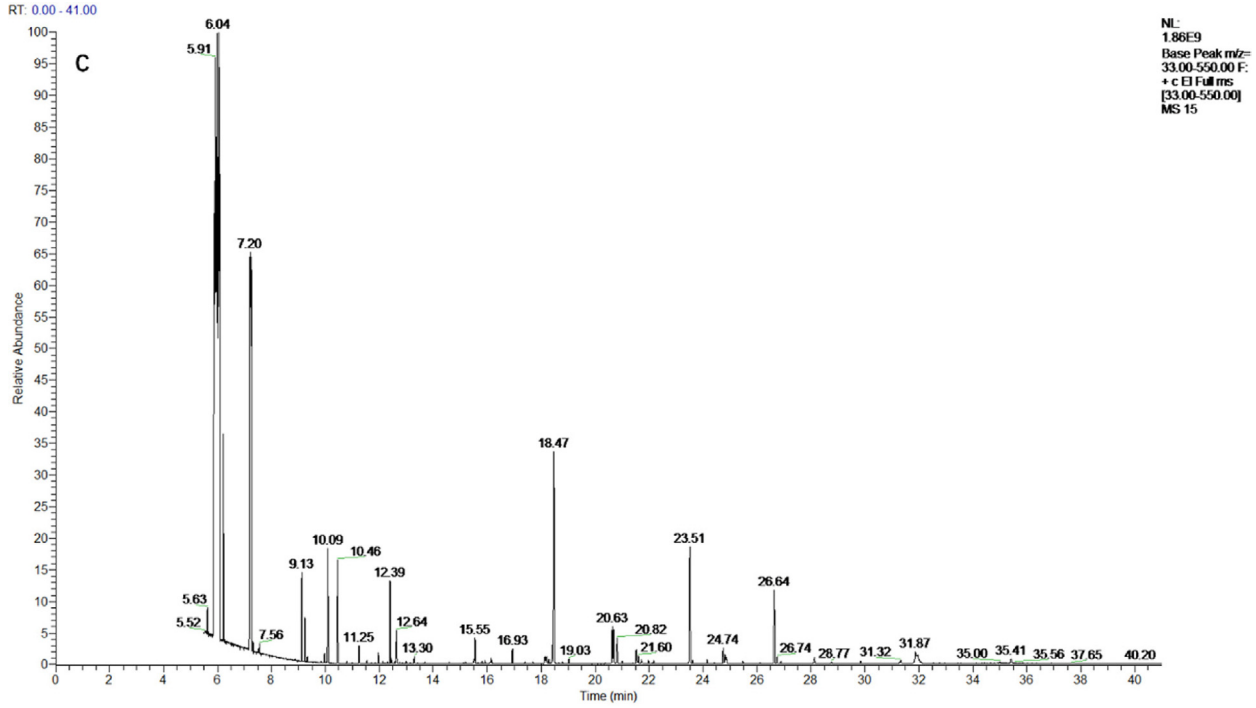


Fig. 7. GC chromatograms of antimicrobial parts. A: Part 2; B: Part 14; C: Part 15.; D: Part 16.



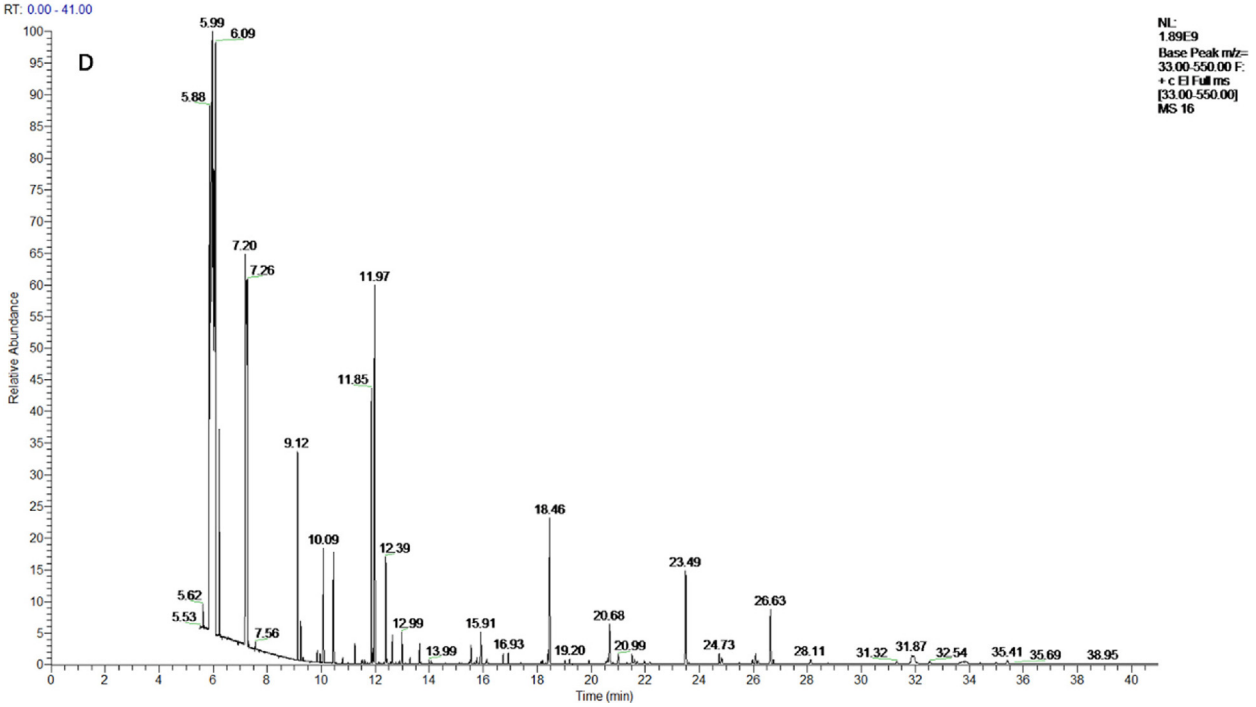


Fig. 7 (*continued*)

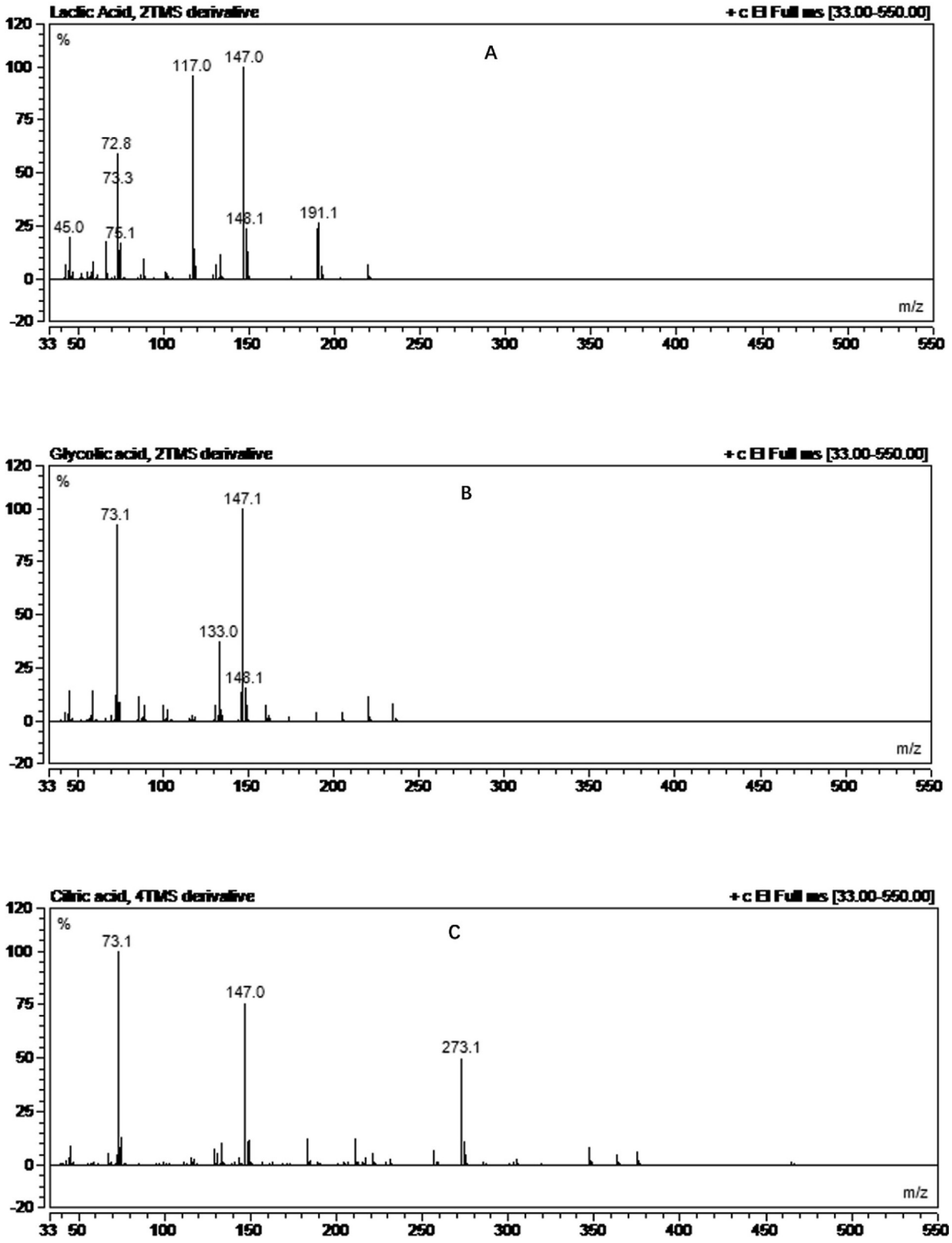


Fig. 8. Mass spectrograph of antimicrobial parts. A: Lactic acid; B: Glycolic acid; C: Citric acid; D: Stearic acid; E: Palmitic acid.

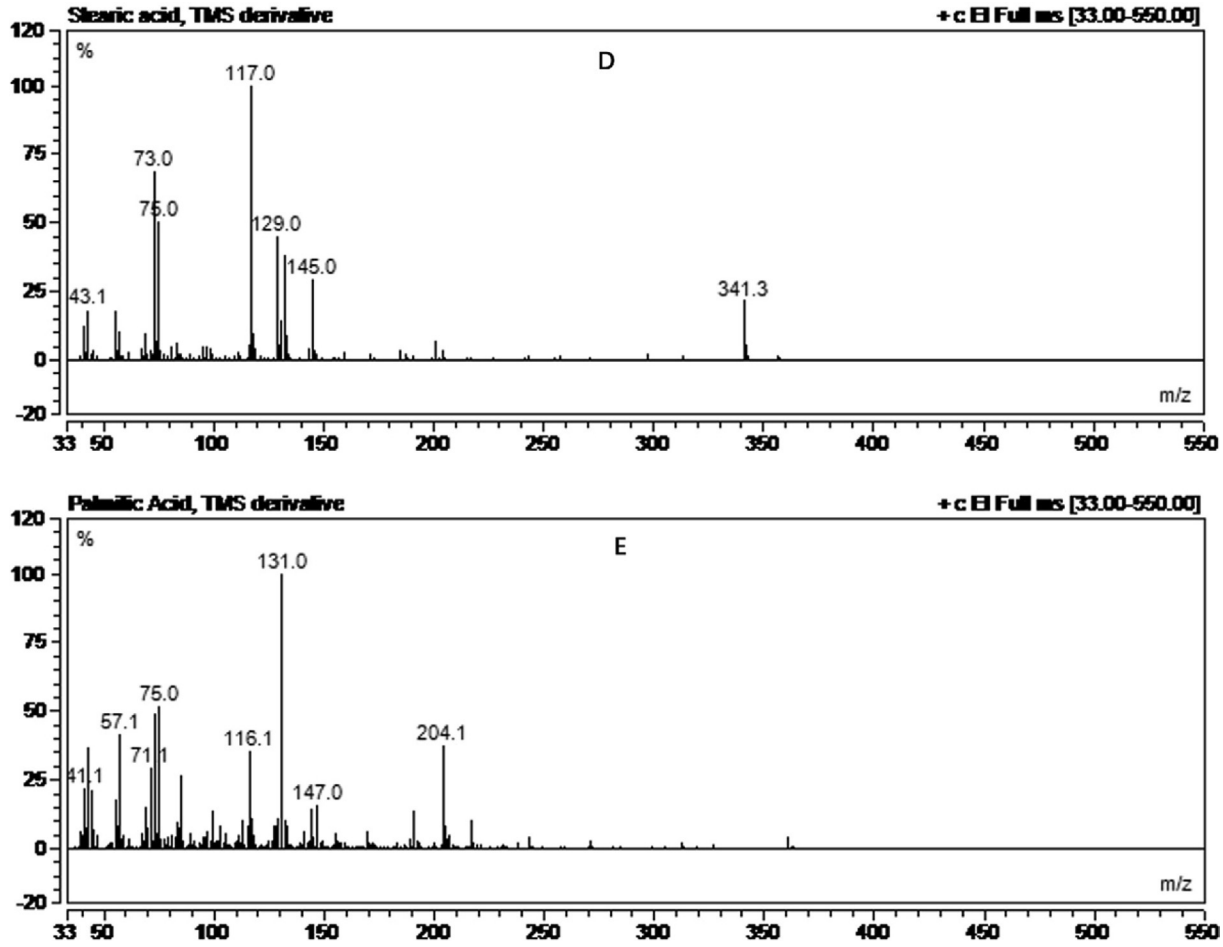


Fig. 8 (*continued*)

Table 5
Antibacterial activity of organic acid and fatty acids.

Metabolin	Content (mg/mL)	Bacteriostatic diameter (mm)
Lactic acid	16.71 ± 0. 97	11.97 ± 0.73
Glycolic acid	1.07 ± 0.10	10.28 ± 0.26
Citric acid	17.62 ± 0.24	8.24 ± 0.06
Malic acid	2.9 ± 0.44	–
Stearic acid	2.57 ± 0.43	–
Palmitic acid	2.92 ± 0.38	–
Mixed acid	43.79 ± 0.82	12.72 ± 0.35
LCFS		13.54 ± 0.12

Note: –: No antibacterial activity.

Conflict of interest

The authors declare no conflicts of interest in this study.

CRediT authorship contribution statement

Yilun Wang: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yuxiang Xu:** Supervision.

Supplementary material

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Data availability

Data will be made available on request.

References

[1] Ağagündüz D, Şahin TO, Ayten S, et al. Lactic acid bacteria as pro-technological, bioprotective and health-promoting cultures in the dairy food industry. Food Biosci 2022;47, 101617. https://doi.org/10.1016/j.fbio.2022.101617.
[2] Nisa M, Dar RA, Fomda BA, et al. Combating food spoilage and pathogenic microbes via bacteriocins: A natural and eco-friendly substitute to antibiotics. Food Control 2023;149, 109710. https://doi.org/10.1016/j.foodcont.2023.109710.
[3] Arrioja D, Mani-López E, Palou E, et al. Antimicrobial activity and storage stability of cell-free supernatants from lactic acid bacteria and their applications with fresh beef. Food Control 2020;115, 107286. https://doi.org/10.1016/j.fbio.2023.103196.
[4] Silva SPM, Teixeira JA, Silva CCG. Recent advances in the use of edible films and coatings with probiotic and bacteriocin-producing lactic acid bacteria. Food Biosci 2023;56, 103196. https://doi.org/10.1016/j.fbio.2023.103196.
[5] Ogawa J, Kishino S, Ando A, et al. Production of conjugated fatty acids by lactic acid bacteria. J Biosci Bioeng 2005;100(4):355–64. https://doi.org/10.1263/jbb.100.355. PMid: 16310724.
[6] Zha M, Li K, Zhang W, et al. Untargeted mass spectrometry-based metabolomics approach unveils molecular changes in milk fermented by *Lactobacillus plantarum* P9. LWT 2021;140, 110759. https://doi.org/10.1016/j.lwt.2020.110759.
[7] Park SE, Yoo SA, Seo SH, et al. GC-MS based metabolomics approach of Kimchi for the understanding of *Lactobacillus plantarum* fermentation characteristics. LWT-Food Sci Technol 2016;68:313–21. https://doi.org/10.1016/j.lwt.2015.12.046.
[8] Wang Y, Li B, Liu Y, et al. Investigation of diverse bacteria encoding histidine decarboxylase gene in Sichuan-style sausages by culture-dependent techniques, polymerase chain reaction–denaturing gradient gel

electrophoresis, and high-throughput sequencing. LWT 2021;139, 110566. https://doi.org/10.1016/j.lwt.2020.110566.
[9] Mao Y, Zhang X, Xu Z. Identification of antibacterial substances of *Lactobacillus plantarum* DY-6 for bacteriostatic action. Food Sci Nutr 2020;8(6):2854–63. https://doi.org/10.1002/fsn3.1585. PMid: 32566203.
[10] Wang Z, Zheng L, Li C, et al. Preparation and antimicrobial activity of sulfopropyl chitosan in an ionic liquid aqueous solution. J Appl Polym Sci 2017;134(26):44989. https://doi.org/10.1002/app.44989.
[11] Zhao M, Huang D, Zhang X, et al. Metabolic engineering of *Escherichia coli* for producing adipic acid through the reverse adipate-degradation pathway. Metab Eng 2018;47:254–62. https://doi.org/10.1016/j.ymben.2018.04.002. PMid: 29625225.
[12] Todorov SD. Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of the adsorption of bacteriocin AMA-K to *Listeria* sp. Braz J Microbiol 2008;39 (1):178–1118. https://doi.org/10.1590/S1517-83822008000100035. PMid: 24031200.
[13] Zhang Y, Li H, Hu T, et al. Metabonomic profiling in study hepatoprotective effect of polysaccharides from *Flammulina velutipes* on carbon tetrachloride-induced acute liver injury rats using GC-MS. Int J Biol Macromol 2018;110:285–93. https://doi.org/10.1016/j.ijbiomac.2017.12.149. PMid: 29305217.
[14] Tao Y, Zhang L. Intensity prediction of typical aroma characters of cabernet sauvignon wine in Changli County (China). LWT-Food Sci Technol 2010;43 (10):1550–6. https://doi.org/10.1016/j.lwt.2010.06.003.
[15] Becerra V, Odermatt J, Nopens M. Identification and classification of glucose-based polysaccharides by applying Py-GC/MS and SIMCA. J Anal Appl Pyrol 2013;103:42–51. https://doi.org/10.1016/j.jaap.2012.12.018.
[16] Luz C, Saladino F, Luciano FB, et al. *In vitro* antifungal activity of bioactive peptides produced by *Lactobacillus plantarum* against *Aspergillus parasiticus* and *Penicillium expansum*. LWT-Food Sci Technol 2017;81:128–35. https://doi.org/10.1016/j.lwt.2017.03.053.
[17] Zhang X, Esmail GA, Alzeer AF, et al. Probiotic characteristics of *Lactobacillus* strains isolated from cheese and their antibacterial properties against gastrointestinal tract pathogens. Saudi J Biol Sci 2020;27(12):3505–13. https://doi.org/10.1016/j.sjbs.2020.10.022. PMid: 33304162.
[18] Reis JA, Paula AT, Casarotti SN, et al. Lactic acid bacteria antimicrobial compounds: Characteristics and Applications. Food Eng Rev 2012;4 (2):124–40. https://doi.org/10.1007/s12393-012-9051-2.
[19] Ouiddir M, Bettache G, Leyva M, et al. Selection of Algerian lactic acid bacteria for use as antifungal bioprotective cultures and application in dairy and bakery products. Food Microbiol 2019;82:160–70. https://doi.org/10.1016/j.fm.2019.01.020. PMid: 31027770.
[20] Sutyak NK, Prichard MN, Khaykin A, et al. The natural antimicrobial peptide subtilosin acts synergistically with glycerol monolaurate, lauric arginate, and ε-poly-l-lysine against bacterial vaginosis-associated pathogens but not human lactobacilli. Antimicrob Agents Chemother 2012;56(4):1756–61. https://doi.org/10.1128/AAC.05861-11. PMid: 22252803.
[21] Pan L, Yu J, Ren D, et al. Metabolomic analysis of significant changes in *Lactobacillus casei* Zhang during culturing to generation 4,000 under conditions of glucose restriction. J Dairy Sci 2019;102(5):3851–67. https://doi.org/10.3168/jds.2018-15702. PMid: 30879813.
[22] Siedler S, Balti R, Neves AR. Bioprotective mechanisms of lactic acid bacteria against fungal spoilage of food. Curr Opin Biotechnol 2019;56:138–46. https://doi.org/10.1016/j.copbio.2018.11.015. PMid: 30504082.
[23] Desniar, Rusmana I, Suwanto A, et al. Organic acid produced by lactic acid bacteria from bekasam as food biopreservatives. IOP Conf Ser: Earth Environ Sci 2020;414, 012003. https://doi.org/10.1088/1755-1315/414/1/012003.