Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst.

Antimicrobial activity of cell-free supernatants from probiotic lactic acid bacteria strains against intestinal opportunistic isolates and effects on their own producer strains

M. V. Ivashko, N. V. Boyko

Uzhhorod National University, Uzhhorod, Ukraine

Article info

Received 10.05.2025
Received in revised form 01.07.2025
Accepted 19.07.2025

Uzhhorod National University, Universytetska st., 14, Uzhhorod, 88000, Ukraine. Tel.: +38-096-676-04-56. E-mail: maksym.ivashko@uzhnu.edu.ua

Rivne State University of Humanities, Stepana Bandery st., 12, Rivne, 33028, Ukraine. Tel. +38-098-259-37-57. E-mail: irmail@gmail.com

Ivashko, M. V., & Boyko, N. V. (2025). Antimicrobial activity of cell-free supernatants from probiotic lactic acid bacteria strains against intestinal opportunistic isolates and effects on their own producer strains. Regulatory Mechanisms in Biosystems, 16(3), e25126. doi:10.15421/0225126

Metabolites produced by lactic acid bacteria (LAB) are involved in maintaining the balance of the human gut microbiota and play a role in inhibiting the growth of pathogens. The present study evaluated the antimicrobial activity of cell-free supernatants (CFS) obtained from ten probiotic strains of LAB. The antimicrobial activity was studied under varying pH conditions and after heat and proteolytic treatment. Activity was evaluated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella oxytoca*, and *Candida albicans* using the agar well diffusion method. The majority of the CFS exhibited a significant inhibitory effect against *S. aureus*, *E. coli*, and *K. oxytoca*, while inhibition of *E. faecalis* and *C. albicans* was predominantly observed for individual supernatants. Based on the results, the five most active CFS were selected for further research. The effect of CFS was also investigated in relation to probiotic strains of LAB, including their respective producer strains. It was found that the CFS of *Lacticaseibacillus casei* IMB 7412 strain exhibited the most pronounced inhibitory effect, both on its own viable cells and on other studied LAB strains. In contrast, *Lactiplantibacillus plantarum* IMB 7413 showed minimal changes in concentration under the influence of CFS. Additionally, the CFS of *Lactobacillus bulgaricus* A22 stimulated the growth of *L. plantarum* A. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the selected supernatants were determined by the micro-broth dilution method, which proved to be more sensitive than the agar well diffusion method, particularly in studies on *E. faecalis* and *C. albicans*. The antimicrobial activity was significantly dependent on pH, being highest under acidic conditions and decreasing or completely disappearing at neutral or alkaline pH. The heat resistance of the cell-free supernatants was relatively high, although heat treatment at 100 °C partially reduced their antimicrobial activity. Treatment with proteinase of the CFS of *L. casei* IMB 7412 resulted in a complete loss of antimicrobial activity, indicating the involvement of protein compounds in this effect. Thus, these results deepen our understanding of the antimicrobial effects of CFS from probiotic strains and their potential to modulate the intestinal microbiota.

Keywords: lactic acid bacteria; cell-free supernatant; intestinal opportunistic isolates; antimicrobial activity; pharmabiotics; metabolites; organic acids.

Introduction

Lactic acid bacteria (LAB) are microorganisms capable of synthesizing lactic acid as the end product of their fermentation process (König & Fröhlich, 2017). This group includes representatives from the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, and others (Goldstein et al., 2015). Due to their beneficial properties, LAB are classified as "Generally Recognized as Safe" (GRAS) by the U.S. Food and Drug Administration (FDA) and are widely used in the production of probiotic products and modern pharmabiotics.

The gut microbiota is considered a "vital superorganism" and encompasses over a thousand microbial species, forming a complex ecological community (Perler et al., 2023). It plays a key role in establishing colonization resistance, strengthening the intestinal barrier, regulating metabolic processes, and supporting the development of the immune system (Di Stefano et al., 2023). Disruption of the diversity of the gut microbiota, caused by antibiotic therapy or other detrimental influences on the human body, can lead to excessive growth of opportunistic pathogens (McDonnell et al., 2021; Walker et al., 2021). Dysbiosis of the gut microbiota is a significant factor in the development of intestinal diseases such as Crohn's disease, ulcerative colitis, and other inflammatory conditions, where microorganisms from the genera *Staphylococcus*, *Enterococcus*, *Escherichia*, *Klebsiella*, and *Candida* play a pivotal role in driving inflammatory processes and damaging the intestinal mucosa (Zhao et al., 2023).

It is known that the specific effect of LAB is determined by their metabolites, which drive the functional activity of probiotic strains. In addition to lactic acid, these bacteria produce a wide range of biologically active compounds, such as bacteriocins, amino acids, exopolysaccharides, and other metabolites, which can affect the growth of potentially pathogenic microorganisms (Tang et al., 2023). For example, Pompilio et al. (2024) demonstrated that cell-free supernatants (CFS) from various *Lactobacillus* strains exhibit antibacterial, antibiofilm, and antivirulence activity against *Pseudomonas aeruginosa*. In addition, Liu et al. (2024) showed that the CFS of *Pediococcus acidilactici* LWX 401 had a broad antibacterial spectrum against food-borne pathogens.

Despite extensive research into the nature and beneficial properties of probiotic strain metabolites, their specific role in regulating the balance of the gut microbiota remains unclear. Investigating the functional efficacy of metabolites produced by probiotic strains, which already serve as the basis for pharmabiotics, is essential for predicting changes in opportunistic members of gut microbiota, while also considering their impact on beneficial microbiota. The aim of this study is to assess the effect of cell-free supernatants from probiotic LAB strains on intestinal opportunistic isolates and to investigate their antimicrobial activity against probiotic LAB strains, including their respective producer strains.

Materials and methods

The strains used in the study are listed in Table 1. The study used LAB strains that are included in the probiotic products Liolact® (Selur Pharma, Bulgaria) and BioME Combi 10+1 (Ediens, Ukraine). *L. plantarum* A, *L. bulgaricus* A6, A22, S6, S19, and *L. rhamnosus* S25 strains and were kindly provided by Professor Albert Krastanov. All LAB strains and *C. albicans* were grown in de Man, Rogosa and

Sharpe broth (MRS broth; HiMedia, India), while *S. aureus*, *E. faecalis*, *E. coli*, and *K. oxytoca* were cultured in tryptone soy broth (TSB; Pharmaktiv, Ukraine).

Table 1
Tested microorganisms and their sources of isolation

Microorganisms	Source of isolation
LAB strains	
L. casei IMB 7412*	Sauerkraut
L. plantarum IMB 7414*	
L. plantarum IMB 7413*	Human intestine
E. faecalis M-4-II-I*	
L. plantarum A**	Sourdough starter
L. bulgaricus A6**	
L. bulgaricus A22**	
L. bulgaricus S6**	
L. bulgaricus S19**	
L. rhamnosus S25**	
Opportunistic isolates	
*S. aureus****	Human intestine
*E. faecalis****	
*E. coli****	
*K. oxytoca****	
*C. albicans****	

Note: * – D. K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine; ** – University of Food Technology, Plovdiv, Bulgaria; *** – Research Development and Educational Center for Molecular Microbiology and Mucosal Immunology, Uzhhorod National University.

To obtain cell-free supernatants, 24-hour LAB cultures were standardized to an optical density of 0.5 McFarland (approximately 1.5×10^8 CFU/mL). A 100 µL suspension was inoculated into 10 mL of sterile MRS broth and incubated at 37 °C for 24 hours. To separate the cells from the supernatants, the cultures were centrifuged at 2500 g for 10 minutes and the supernatants were collected and filtered through cellulose acetate membrane nanofilters with a pore size of 0.22 µm (Membrane Solutions, China). The CFS was spread on MRS agar and incubated at 37 °C for 24 hours to confirm the absence of viable cells. All supernatants were stored at –23 °C until further use in experiments. The initial pH values of the CFS are shown in Table 2.

Table 2
pH value of cell-free supernatants obtained from lactic acid bacteria strains after 24 hours of cultivation

CFS of LAB strains	pH
L. casei IMB 7412	3
L. plantarum IMB 7414	
L. plantarum A	
L. bulgaricus A6	
L. bulgaricus A22	
L. bulgaricus S6	
L. plantarum IMB 7413	4
L. bulgaricus S19	
L. rhamnosus S25	
E. faecalis M-4-II-I	5

The antimicrobial activity of the supernatants against intestinal opportunists was assessed using the agar well diffusion method, as described by Pompilio et al. (2024), with modifications by the authors. Indicator strains at a concentration of 10^6 CFU/mL were spread onto the surface of agar plates using a sterile cotton swab. Wells with a diameter of 8 mm were made aseptically using a sterile 1 mL pipette tip and filled with 100 µL of each supernatant. MRS broth was used as a control. The inoculated plates were incubated at 37 °C for 24 hours, after which the inhibition zone diameter was measured.

Sterile 96-well microplates (Biosigma, Italy) were used to evaluate the antimicrobial activity of CFS, specifically against their own producer strains and other probiotic LAB strains. Subsequently, 80 µL of bacterial suspension at a density of 0.5 McFarland was mixed with 20 µL of the respective supernatant. LAB suspensions added to MRS broth served as control samples. Aliquots were incubated at 37 °C for 24 hours. The suspensions were diluted tenfold and plated onto MRS agar plates, which were then incubated under anaerobic conditions using Oxoid Anaerogen® packets (Thermo Fisher Scientific, UK) for 24 hours. After incubation, colony counts were performed.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the supernatants were determined using the microdilution method in 96-well microplates, following the methodology described by Drumond et al., (2023), with some modifications. Briefly, the CFS were serially diluted in TSB using a two-fold dilution method. To each well containing 100 µL of the diluted supernatants, 25 µL of microorganism suspension of 10^6 CFU/mL was added. For the positive control, the indicator strain suspension and TSB were added to the microplate, and for the negative control, only TSB medium was added. The microplates were incubated at 37 °C for 24 hours. MIC values were determined as the lowest concentration of CFS required to prevent the visible growth of microorganisms. To determine the MBC, suspensions were plated onto the surface of agar plates, while the MBC was defined as the lowest CFS concentration capable of inhibiting more than 99% of the growth of indicator strains (Rodríguez-Melcón et al., 2021).

The antibacterial activity of the CFS was assessed under various conditions using the agar well diffusion method. To eliminate the influence of organic acids, CFS pH was neutralized to the values of 6 and 9 using 1M NaOH. The effect of temperature on CFS activity was tested by thermal treatment at different temperatures (60, 80, and 100 °C) for 30 minutes. Neutralized CFS (pH 6) were treated with Proteinase K (30 units/mg). Proteinase K was added to the CFS in a 1:1 ratio, followed by a two-hour incubation at 37 °C. CFS samples treated with an enzyme were heated to 100 °C for 10 minutes to inactivate the enzyme. Untreated CFS (pH 6) was used as a control.

All experiments were performed in triplicate (n = 3), and the data are presented as mean values (x) ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to determine significant differences between group means. Differences were considered statistically significant at $P < 0.05$.

Results

In this study, the antimicrobial activity of the CFS from LAB strains against opportunistic intestinal isolates was evaluated. The results obtained are presented in Table 3.

Table 3
Inhibition zones (mm) of the cell-free supernatants from lactic acid bacteria against opportunistic intestinal isolates (x ± SD; n = 3)

CFS of LAB strains	Opportunistic intestinal isolates				
	S. aureus	*E. faecalis*	*E. coli*	*K. oxytoca*	*C. albicans*
L. casei IMB 7412	18.80 ± 0.72^{a}	9.33 ± 0.58^{ab}	17.17 ± 0.76^{a}	14.47 ± 0.81^{a}	9.27 ± 0.25^{a}
L. plantarum IMB 7413	10.47 ± 0.50^{e}	n.i.	12.00 ± 0.00^{d}	10.07 ± 0.12^{d}	n.i.
L. plantarum IMB 7414	13.93 ± 0.60^{c}	n.i.	14.17 ± 0.29^{c}	11.50 ± 1.32^{cd}	n.i.
L. plantarum A	16.67 ± 0.58^{b}	n.i.	16.37 ± 0.55^{a}	14.10 ± 0.17^{a}	n.i.
L. bulgaricus A6	16.17 ± 0.29^{b}	n.i.	15.33 ± 0.58^{bc}	13.13 ± 0.23^{a}	n.i.
L. bulgaricus A22	18.20 ± 0.26^{a}	n.i.	15.17 ± 0.29^{bc}	11.67 ± 0.58^{bc}	n.i.
L. bulgaricus S6	17.23 ± 0.25^{ab}	9.17 ± 0.29^{b}	15.33 ± 0.58^{bc}	13.0 ± 0.00^{abc}	n.i.
L. bulgaricus S19	11.17 ± 1.04^{de}	10.9 ± 0.17^{a}	9.67 ± 0.58^{e}	n.i.	n.i.
L. rhamnosus S25	12.67 ± 0.58^{cd}	n.i.	13.00 ± 1.00^{cd}	10.17 ± 0.29^{bcd}	n.i.
E. faecalis M-4-II-I	13.33 ± 1.15^{c}	n.i.	12.83 ± 0.29^{cd}	10.20 ± 0.17^{bcd}	n.i.

Note: different letters ($^{a-e}$) indicate statistically significant differences between inhibition zones produced by different CFS for each test strain ($P < 0.05$, one-way ANOVA with Tukey's HSD test); n.i. – no inhibition.

The supernatants exhibited varying degrees of inhibitory activity against the indicator microorganisms. The most pronounced antibacterial activity against *S. aureus* and *E. coli* was observed in the CFS of *L. casei* IMB 7412, *L. plantarum* A, and *L. bulgaricus* A6, A22, and S6 strains, with maximum inhibition zones of 18.8 ± 0.72 mm and 17.17 ± 0.76 mm, respectively. In contrast, the CFS of *L. casei* IMB 7412 and *L. bulgaricus* S6 and S19 exhibited minimal inhibitory activity against *E. faecalis*, with inhibition zones of 9.33 ± 0.58 mm, 9.17 ± 0.29 mm, and 10.9 ± 0.17 mm, respectively. For *K. oxytoca*, the CFS of *L. casei* IMB 7412, *L. plantarum* A, and *L. bulgaricus* A6 and S6 demonstrated the most effective results, with inhibition zones ranging from 14.47 ± 0.81 to 13.00 ± 0.00 mm. Additionally, the CFS of *L. casei* IMB 7412 exhibited antifungal activity, with an inhibition zone of 9.27 ± 0.25 mm. In contrast, no antifungal activity was detected in the other CFS.

In light of these findings, the CFS from *L. casei* IMB 7412, *L. plantarum* A, and *L. bulgaricus* A6, A22, and S6 strains were selected for further experiments, as these strains exhibited the highest levels of antimicrobial activity against the indicator microorganisms.

The effect of the CFS on the growth of LAB strains was assessed by culturing them in 96-well microplates. As shown in Table 4, the supernatants exhibited varying effects on the LAB strains.

Table 4

Antibacterial activity of cell-free supernatants against probiotic strains of lactic acid bacteria, including their respective producer strains (log_{10} CFU/mL; x ± SD; n = 3)

LAB strains	Control	CFS of LAB strains				
		L. casei IMB 7412	*L. plantarum* A	*L. bulgaricus* A6	*L. bulgaricus* A22	*L. bulgaricus* S6
L. casei IMB 7412	8.00 ± 0.00^{a}	5.96 ± 0.04^{c}	6.26 ± 0.24^{c}	6.77 ± 0.07^{b}	6.27 ± 0.14^{c}	6.20 ± 0.17^{c}
L. plantarum IMB 7413	8.61 ± 0.01^{a}	8.25 ± 0.23^{a}	8.33 ± 0.06^{a}	8.26 ± 0.24^{a}	8.36 ± 0.10^{a}	8.37 ± 0.09^{a}
L. plantarum IMB 7414	7.68 ± 0.03^{a}	5.86 ± 0.13^{d}	6.78 ± 0.00^{c}	6.83 ± 0.08^{c}	5.85 ± 0.07^{d}	7.18 ± 0.17^{b}
L. plantarum A	7.21 ± 0.04^{a}	6.20 ± 0.17^{b}	7.11 ± 0.19^{a}	6.55 ± 0.10^{b}	7.40 ± 0.17^{a}	6.49 ± 0.20^{b}
L. bulgaricus A6	7.48 ± 0.05^{a}	6.47 ± 0.16^{c}	7.14 ± 0.09^{b}	7.30 ± 0.14^{a}	6.57 ± 0.05^{c}	6.79 ± 0.02^{c}
L. bulgaricus A22	8.20 ± 0.17^{a}	6.50 ± 0.17^{c}	6.50 ± 0.10^{c}	6.20 ± 0.17^{c}	7.01 ± 0.02^{b}	6.20 ± 0.17^{c}
L. bulgaricus S6	7.15 ± 0.05^{a}	5.95 ± 0.05^{b}	6.32 ± 0.28^{b}	5.91 ± 0.08^{b}	5.92 ± 0.03^{b}	6.32 ± 0.28^{b}
L. bulgaricus S19	7.43 ± 0.08^{a}	5.90 ± 0.06^{c}	6.50 ± 0.17^{b}	6.4 ± 0.17^{b}	6.73 ± 0.12^{b}	7.09 ± 0.05^{a}
L. rhamnosus S25	8.00 ± 0.00^{a}	6.43 ± 0.11^{c}	7.26 ± 0.24^{b}	7.19 ± 0.21^{b}	6.42 ± 0.10^{c}	$7.51 \pm 0.08^{a,b}$
E. faecalis M-4-II-I	8.00 ± 0.00^{a}	7.04 ± 0.07^{b}	6.65 ± 0.13^{c}	6.56 ± 0.07^{c}	7.39 ± 0.23^{b}	7.13 ± 0.16^{b}

Note: different letters ($^{a-f}$) in the same row indicate significant differences between the effects of different CFS on each strain ($P < 0.05$, one-way ANOVA with Tukey's HSD test).

The CFS of *Lbs. casei* 7412 exhibited the most pronounced inhibitory effect on the majority of probiotic strains. Specifically, strain *L. casei* 7412 demonstrated the greatest sensitivity to the cell-free supernatant obtained from its own producer strain. The quantitative value (5.96 ± 0.04) was statistically significantly lower than that of the control group (8.00 ± 0.00). Concurrently, strain *L. plantarum* 7413 exhibited resistance to all CFS.

For strain *L. plantarum* 7414, maximum growth inhibition was observed under the influence of CFS from *Lbs. casei* 7412 and *L. bulgaricus* A22, while the effect of the CFS from *L. bulgaricus* S6 was minimal. The strain *L. plantarum* A demonstrated increased susceptibility to the CFS of *L. casei* 7412. In contrast, the CFS obtained from *L. bulgaricus* A22 stimulated the growth of this strain, with the cell count increasing to 7.40 ± 0.17 compared to the control (7.21 ± 0.04). Meanwhile, *L. plantarum* A exhibited no sensitivity to its own CFS. CFS from *L. casei* 7412 and *L. bulgaricus* A22 also exhibited pronounced inhibitory activity against *L. bulgaricus* A6, whereas the CFS from *L. bulgaricus* A6 had no effect on the growth of its own producer strain.

A marked decrease in the viability of *L. bulgaricus* A22 was observed when cultivated with CFS from *L. casei* 7412, *L. plantarum* A, *L. bulgaricus* A6, and S6. In turn, *L. bulgaricus* S6 was the most sensitive to the action of CFS from *L. casei* 7412, *L. bulgaricus* A6, and A22.

Similar to most strains, *L. bulgaricus* S19 showed maximum growth inhibition when supplemented with CFS from *L. casei* 7412. For *L. rhamnosus* S25, the strongest inhibitory effect was recorded for CFS obtained from *L. casei* 7412 and *L. bulgaricus* A22. At the same time, a significant decrease in the growth of *E. faecalis* M-4-II-I was observed under the influence of CFS from *L. plantarum* A and *L. bulgaricus* A6.

The results of the MIC and MBC determination are shown in Table 5.

Table 5

Minimum inhibitory concentration and minimum bactericidal concentration of cell-free supernatants of lactic acid bacteria strains

CFS of LAB strains	Opportunistic intestinal isolates									
	S. aureus		*E. faecalis*		*E. coli*		*K. oxytoca*		*C. albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
L. casei IMB 7412	1:4	1:4	1:4	1:2	1:4	1:4	1:8	1:4	1:2	0
L. plantarum A	1:4	1:4	1:4	1:2	1:4	1:4	1:4	1:4	1:2	0
L. bulgaricus A6	1:4	1:4	1:4	1:4	1:2	1:2	1:8	1:4	1:4	1:2
L. bulgaricus A22	1:4	1:4	1:4	1:2	1:8	1:4	1:4	1:4	1:2	1:2
L. bulgaricus S6	1:4	1:4	1:2	0	1:4	1:4	1:4	1:4	0	0

Note: results are shown as dilution coefficients (1:x), where x is the dilution level at which the effect was observed; 0 – undiluted supernatant; MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration.

The MIC and MBC for the five supernatants against *S. aureus* coincided at the dilution level of 1:4. In the case of *E. faecalis*, the highest MIC level (1:4) was observed for the CFS of *L. casei* IMB 7412, *L. plantarum* A, and *L. bulgaricus* A6 and A22 strains. The highest MBC value (1:4) was recorded for the CFS of *L. bulgaricus* A6 strain. In the case of *E. coli*, the maximum MIC value (1:8) was demonstrated by the CFS of *L. bulgaricus* A22, while the MBC value (1:4) was identified for the CFS of *L. casei* IMB 7412, *L. plantarum* A, and *L. bulgaricus* A22 and S6 strains. Furthermore, the CFS of *L. casei* IMB 7412 and *L. bulgaricus* A6 strains demonstrated the highest MIC values against *K. oxytoca* (1:8). At the same time, the MBC values for the CFS of all strains were observed at a dilution of 1:4. In the case of *C. albicans*, the highest MIC values (1:4) were recorded for the CFS of *L. bulgaricus* A6, the MBC (1:2) was observed for *L. bulgaricus* A6 and A22.

The impact of pH, temperature, and Proteinase K on the antibacterial activity of the CFS was studied using *E. coli* strain. The antibacterial activity of the CFS under various conditions is shown in Figure 1.

The present study established that the antibacterial activity of the CFS was contingent on the pH level, with the CFS retaining activity in an acidic environment but showing reduced or complete inactivity at pH 6 and 9. The CFS from *L. casei* IMB 7412 strain was the only

one to exhibit antibacterial activity at pH 6 (10.83 ± 0.29 mm), while at pH 9, no activity was observed. Following thermal treatment, the antibacterial activity of the CFS was maintained at 60 and 80 °C; however, at 100 °C, a reduction in the activity of the supernatants was observed, although it remained high (Fig. 2). Furthermore, treatment of the CFS from LAB strains with Proteinase K completely abolished the antibacterial activity of the supernatants.

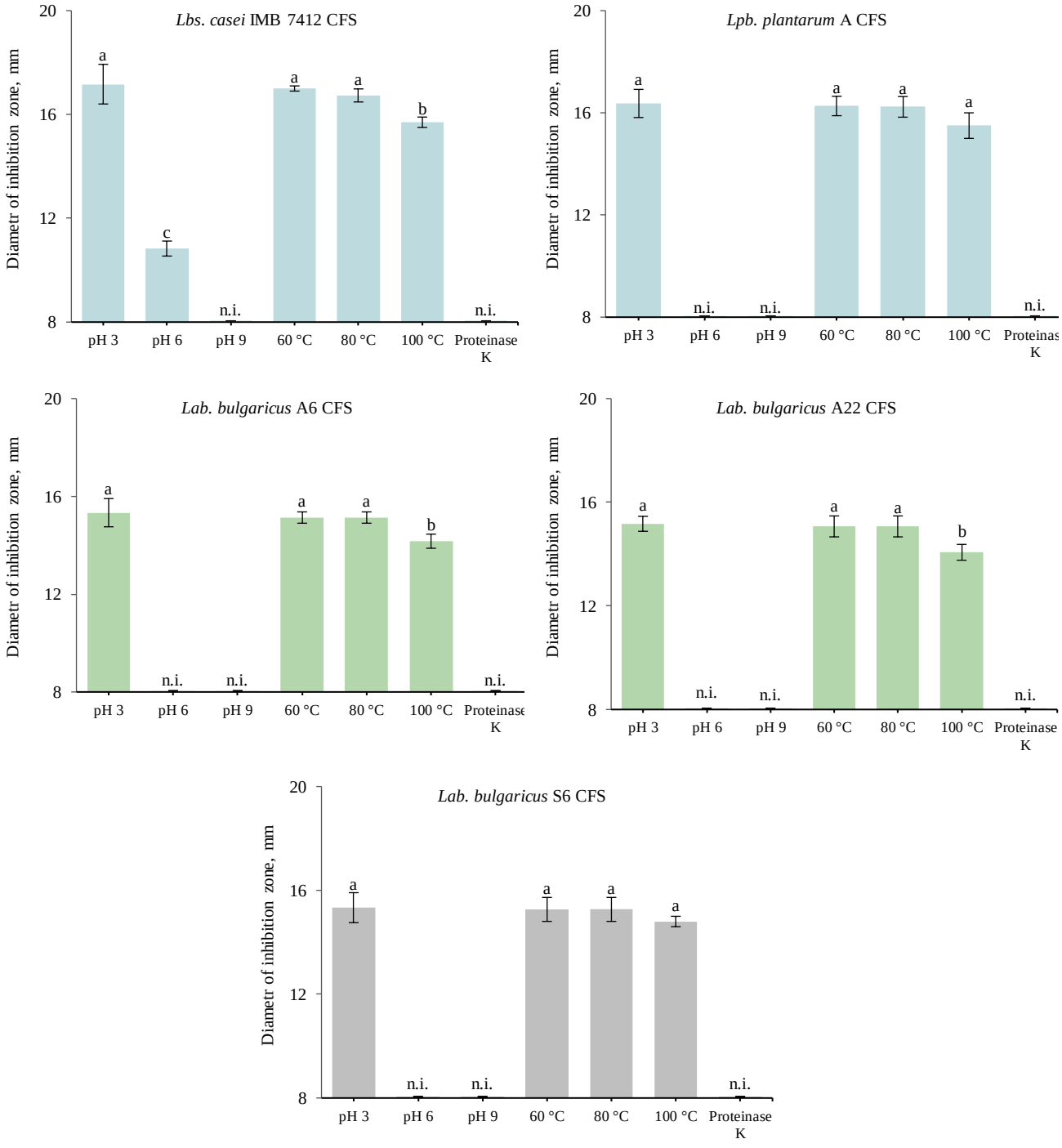

Fig. 1. Antibacterial activity of cell-free supernatants of lactic acid bacteria under different exposure conditions (x ± SD; n = 3): eight millimeters corresponds to the diameter of the wells in agar; different letters (a–c) indicate statistically significant differences for CFS of the same strain under different conditions (P <0.05, one-way ANOVA with Tukey's HSD test); n.i. – no inhibition

Discussion

Numerous studies indicate that the gut microbiota plays a significant role in the pathogenesis of both intestinal and extraintestinal disorders (Meleshko et al., 2021; Chang et al., 2022; Li et al., 2024). The effectiveness of probiotics in restoring or modulating the gut microbiota in various diseases is widely recognized. For example, Zhang et al. (2020) demonstrated that dysbiosis caused by prolonged travel can be mitigated through probiotic intake, as evidenced by an increase in beneficial species such as *Bifidobacterium longum*, *B. animalis*, and *L. plantarum*, along with a reduction in opportunistic pathogens like *Clostridium leptum*, *K. pneumoniae*, and *Prevotella copri*. Moreover, De Wolfe et al. (2018) found that oral administration of a probiotic combination of lactobacilli and bifidobacteria was associated with a significant reduction in the duration of diarrhea in individuals infected with *C. difficile*.

One of the key mechanisms of probiotics' influence on pathogens is the production of metabolites, which have garnered significant research interest. This study aimed to evaluate the antimicrobial properties of the CFS from probiotic LAB strains by testing them against intes-

tinal opportunistic isolates using the agar well diffusion method. It was found that the cell-free supernatants inhibited the growth of opportunistic pathogens, which is consistent with the results of previous studies (Rocha-Ramírez et al., 2023). Specifically, Scillato et al. (2021) observed that CFS inhibited the growth of multidrug-resistant urogenital pathogens, except *E. faecium*, *E. faecalis*, *C. albicans*, and *C. glabrata* (now *Nakaseomyces glabratus*). Our results also confirm that most of the supernatants were ineffective against *E. faecalis* and *C. albicans*. Based on the obtained data, for further experiments, the CFS from five strains (*L. casei* IMB 7412, *L. plantarum* A, and *L. bulgaricus* A6, A22, and S6) that demonstrated the best antimicrobial properties were selected.

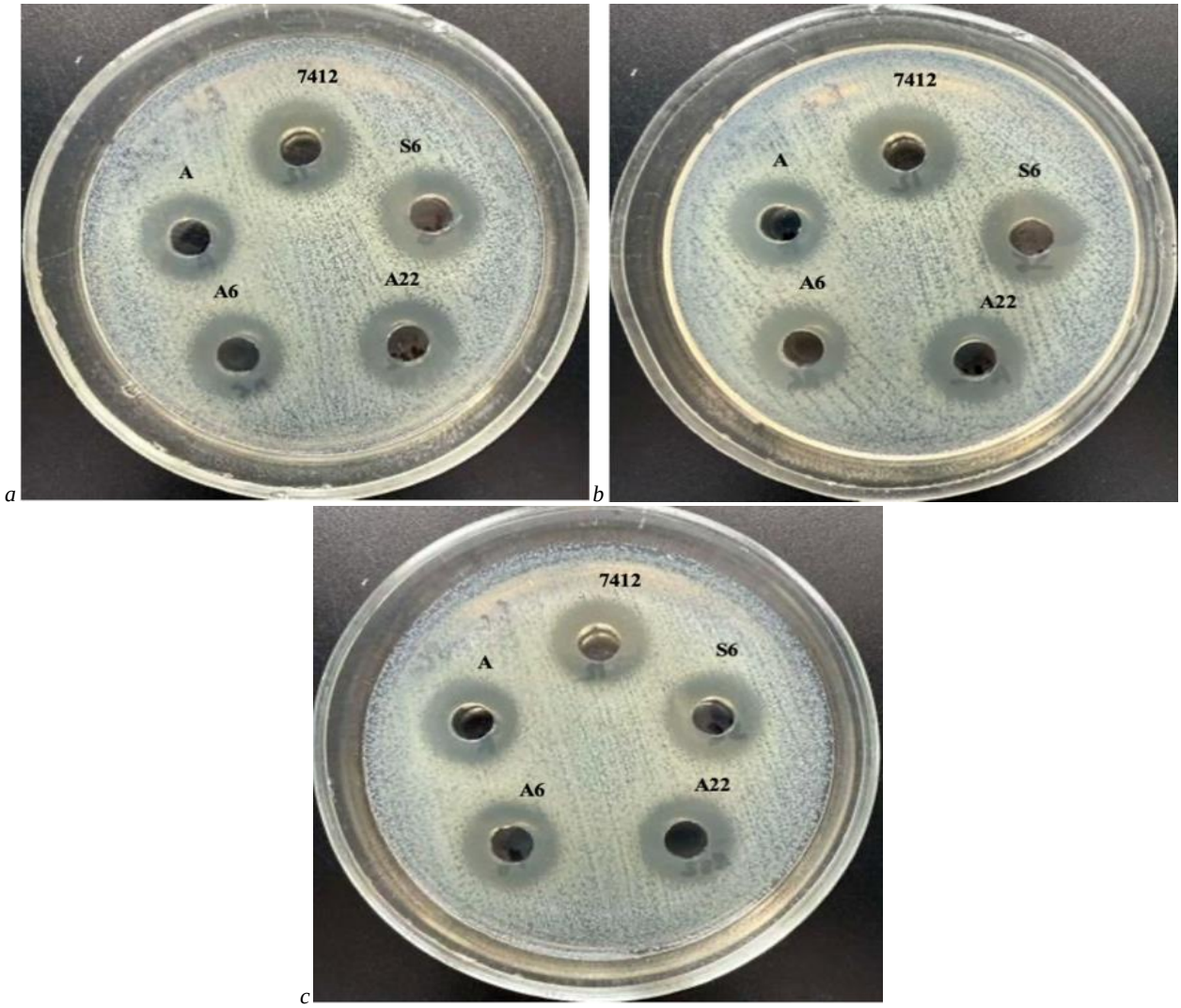

Fig. 2. Antibacterial activity of cell-free supernatants of lactic acid bacteria after heat treatment against *E. coli* (*a* – 60 °C, *b* – 80 °C, *c* – 100 °C): strain designations: 7412 – *L. casei*; A – *L. plantarum*; A6, A22, S6 – *L. bulgaricus*

It is imperative to acknowledge that each individual possesses a distinct diagnostic microbiome ratio, thereby emphasizing the necessity to comprehend how probiotic strains influence both opportunistic pathogens and beneficial microorganisms, to implement a targeted correction of the gut microbiota. The functional activity of microorganisms, which is facilitated through their metabolites, plays a pivotal role in this regard. Nevertheless, the impact of CFS on LAB strains still lacks research. This study sought to examine the response of beneficial LAB strains to selected CFS.

The results demonstrated that the CFS exhibit both interspecies and intraspecies antagonistic activity. Inhibition of the growth of LAB strains, particularly their own CFS-producing strains, may be attributed to the production of organic acids, such as lactic or acetic acid, as well as to bacteriocins, to which these strains were found to be sensitive. Our findings are particularly consistent with those of Fredua-Agyeman et al. (2017), who revealed that the CFS from *L. plantarum* and *L. rhamnosus* inhibits the growth of probiotic strains *L. acidophilus* and *E. faecium*. Furthermore, Janßen et al. (2020) demonstrated that *Latilactobacillus sakei* strain TMW 1.624 produces bacteriocins capable of inhibiting the growth of *L. curvatus* and *L. sakei* strains.

It was observed that *L. plantarum* IMB 7413 strain exhibited minimal fluctuations in concentration, which may suggest its resistance to the supernatants. Another noteworthy observation was that the CFS from *L. bulgaricus* A22 strain stimulated the growth of *L. plantarum* A. This phenomenon has also been documented by Lebas et al. (2020), who demonstrated that the CFS from lactobacilli and bifidobacteria modulated the growth of *F. prausnitzii* A2-165.

The stimulatory effect of the supernatants can be explained by the production of metabolites that support the growth of beneficial microorganisms. It is well known that probiotic strains and commensal bacteria are capable of breaking down undigested fibers, leading to the formation of short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate (Martin-Gallausiaux et al., 2021). These metabolites strengthen the intestinal barrier, possess anti-inflammatory properties, and modulate the immune response by regulating cytokine production (Tedelind et al., 2007; Parada Venegas et al., 2019). Moreover, SCFAs contribute to maintaining the balance of the gut microbiota by reducing the number of opportunistic pathogens and stimulating the growth of beneficial microorganisms (Peterson et al., 2022).

The study of antimicrobial activity of the supernatants by using the microdilution method in broth revealed that they inhibited the

growth of *E. faecalis* and *C. albicans*, in contrast to the agar diffusion method. These discrepancies may be attributed to limited diffusion or instability of active compounds in agar. Additionally, liquid media may better support the activity of certain metabolites or create conditions under which microorganisms become more sensitive. Studies confirm that the microdilution method is more sensitive for assessing antimicrobial activity, as it ensures uniform contact between microorganisms and metabolites (Scorzoni et al., 2007; Akhtar et al., 2022; Lazou & Chaintoutis, 2023).

Organic acids are the key metabolic by-products of LAB, playing an essential role in their antimicrobial activity. These acids lower the pH of the surrounding environment, creating conditions that are unfavourable for the survival of pathogenic microorganisms. Additionally, the undissociated forms of organic acids can cross bacterial membranes, dissociate in the cytoplasm, decrease intracellular pH, and disrupt vital cellular processes, leading to cell death (Peh et al., 2020). This study aimed to assess how pH changes influence the antimicrobial activity of the CFS from LAB strains. Current findings revealed that these supernatants exhibit the highest inhibitory activity against *E. coli* at acidic pH, with activity diminishing at pH 6 and 9. An exception was the CFS of *L. casei* IMB 7412, which showed minimal antimicrobial activity at pH 6 but lost it entirely at pH 9. This result emphasizes the crucial role of organic acids in the antimicrobial action of the supernatants. Similarly, Wang & Zeng (2022) found that the antibacterial activity of the CFS from *L. pentosus* L-36 was highest at pH 3.5, gradually decreasing as the pH rose to 4-6 and disappearing at pH 7 and 8.

The present study demonstrated that the CFS from LAB strains retained their antimicrobial activity after thermal treatment, although a slight reduction in activity was observed at 100 °C. Similar results were reported by Qadi et al. (2023), who confirmed the preservation of antimicrobial activity in CFS after heating. However, further treatment with Proteinase K led to the complete loss of antibacterial activity in the supernatants, particularly in the case of the CFS of *L. casei* IMB 7412, indicating the presence of protein molecules. These results are consistent with those reported by Moon et al. (2022), who demonstrated that after proteinase treatment, the antibacterial activity of the CFS from *L. paracasei* CH88 against *Gardnerella vaginalis* was abolished. A similar outcome was reported in the study by Yang et al. (2022), which demonstrated that the CFS of *Weissella viridescens* lost its activity against *Listeria monocytogenes* after proteolytic treatment.

Given the challenge of directly identifying the compounds responsible for antimicrobial activity, future research will focus on analyzing CFS composition. Furthermore, experimental research employing various approaches to studying supernatants will provide a deeper understanding of the mechanisms by which probiotic strains exert their effects. Another promising avenue of research is the analysis of CFS to determine their spectrum of action and the mechanisms through which they operate, particularly within the framework of mathematical models (Boyko et al., 2024).

Conclusion

The present study evaluated the antimicrobial mechanisms of probiotic LAB strains by examining the effects of their CFS against *S. aureus*, *E. faecalis*, *E. coli*, *K. oxytoca*, and *C. albicans*. The CFS of the five most active producer strains, namely *L. casei* IMB 7412, *L. plantarum* A, *L. bulgaricus* A6, A22, and S6, were selected due to their pronounced antimicrobial activity against the tested microorganisms. Furthermore, the impact of CFS on LAB strains, including their own producer strains, was investigated. The results revealed that CFS were capable of inhibiting probiotic strains, and in certain cases, they stimulated LAB growth. The broth microdilution method was found to be more sensitive for determining the MIC and MBC, as it detected the antimicrobial activity of the CFS against *E. faecalis* and *C. albicans*, whereas no activity was observed using the agar diffusion method. Furthermore, the antibacterial activity of the CFS was found to be pH-dependent (pH 3–9) and remained stable at elevated temperatures (60–100 °C). Additionally, proteolytic treatment of the CFS from *L. casei* IMB 7412 was found to result in the loss of its antimicrobial activity, indicating the presence of proteinaceous components. The findings of this study enhance the understanding of the antimicrobial properties of the probiotic strain-derived CFS, offering prospects for further research and their potential application in the gut microbiota modulation.

No external funding sources were received for this study.

The authors declare that they have no conflict of interest.

References

Akhtar, R., Akhtar, M. R., Arshad, R., & Masood, S. (2022). Evaluation of antibiotic susceptibility tests: Agar diffusion and broth dilution assay against *Enterococcus faecalis*. Pakistan Journal of Medicine and Dentistry, 11(3), 3–10.

Boyko, N., Polishchuk, V., Meleshko, T., Bilak, Y., Rukavchuk, R., Chendey, T., & Onufrei, O. (2024). Hybrid model for assessing the level of risk for the development of atherosclerosis. In: Babichev, S., & Lytvynenko, V. (Eds.). Lecture notes on data engineering and communications technologies. Springer, Cham. Pp. 28–50.

Chang, L., Wei, Y., & Hashimoto, K. (2022). Brain-gut-microbiota axis in depression: A historical overview and future directions. Brain Research Bulletin, 182, 44–56.

De Wolfe, T. J., Eggers, S., Barker, A. K., Kates, A. E., Dill-McFarland, K. A., Suen, G., & Safdar, N. (2018). Oral probiotic combination of *Lactobacillus* and *Bifidobacterium* alters the gastrointestinal microbiota during antibiotic treatment for *Clostridium difficile* infection. PloS One, 13(9), e0204253.

Di Stefano, M., Santonocito, S., Polizzi, A., Mauceri, R., Troiano, G., Lo Giudice, A., Romano, A., Mascitti, M., & Isola, G. (2023). A reciprocal link between oral, gut microbiota during periodontitis: The potential role of probiotics in reducing dysbiosis-induced inflammation. International Journal of Molecular Sciences, 24(2), 1084.

Drumond, M. M., Tapia-Costa, A. P., Neumann, E., Nunes, Á. C., Barbosa, J. W., Kassuha, D. E., & Mancha-Agresti, P. (2023). Cell-free supernatant of probiotic bacteria exerted antibiofilm and antibacterial activities against *Pseudomonas aeruginosa*: A novel biotic therapy. Frontiers in Pharmacology, 14, 1152588.

Fredua-Agyeman, M., Stapleton, P., Basit, A. W., & Gaisford, S. (2017). Microcalorimetric evaluation of a multi-strain probiotic: Interspecies inhibition between probiotic strains. Journal of Functional Foods, 36, 357–361.

Goldstein, E. J., Tyrrell, K. L., & Citron, D. M. (2015). *Lactobacillus* species: Taxonomic complexity and controversial susceptibilities. Clinical Infectious Diseases, 60(S2), S98–S107.

Janßen, D., Dworschak, L., Ludwig, C., Ehrmann, M. A., & Vogel, R. F. (2020). Interspecies assertiveness of *Lactobacillus curvatus* and *Lactobacillus sakei* in sausage fermentations. International Journal of Food Microbiology, 331, 108689.

König, H., & Fröhlich, J. (2017). Lactic acid bacteria. In: König, H., Unden, G., & Fröhlich, J. (Eds.). Biology of microorganisms on grapes, in must and in wine. Springer, Cham. Pp. 3–41.

Lazou, T. P., & Chaintoutis, S. C. (2023). Comparison of disk diffusion and broth microdilution methods for antimicrobial susceptibility testing of *Campylobacter* isolates of meat origin. Journal of Microbiological Methods, 204, 106649.

Lebas, M., Garault, P., Carrillo, D., Codoñer, F. M., & Derrien, M. (2020). Metabolic response of *Faecalibacterium prausnitzii* to cell-free supernatants from lactic acid bacteria. Microorganisms, 8(10), 1528.

Li, X., Li, X., Xiao, H., Xu, J., He, J., Xiao, C., Zhang, B., Cao, M., & Hong, W. (2024). Meta-analysis of gut microbiota alterations in patients with irritable bowel syndrome. Frontiers in Microbiology, 15, 1492349.

Liu, W.-X., Wang, J.-J., Xiao, X.-K., Chen, C.-R., Lu, X., Zhang, X.-Y., Lin, L.-B., & Wang, F. (2024). Antimicrobial effects and metabolomics analysis of cell-free supernatant produced by *Pediococcus acidilactici* LWX 401 isolated from Yunnan traditional pickles. Food Science and Technology, 191, 115626.

Martin-Gallausiaux, C., Marinelli, L., Blottière, H. M., Larraufie, P., & Lapaque, N. (2021). SCFA: Mechanisms and functional importance in the gut. The Proceedings of the Nutrition Society, 80(1), 37–49.

McDonnell, L., Gilkes, A., Ashworth, M., Rowland, V., Harries, T. H., Armstrong, D., & White, P. (2021). Association between antibiotics and gut microbiome dysbiosis in children: Systematic review and meta-analysis. Gut Microbes, 13(1), 1870402.

Meleshko, T., Petrov, V., Falalyeyeva, T., Kobyliak, N., & Boyko, N. (2021). Microbial and immune markers of patients with metabolic syndrome and cardiovascular diseases: Perspectives for early diagnostics. Minerva Biotechnology and Biomolecular Research, 33(2), 109–116.

Moon, E. C., Park, M. S., Lim, T., Kim, R. H., Ji, G. E., Kim, S. Y., & Hwang, K. T. (2022). Antibacterial effect of cell-free supernatant fraction from *Lactobacillus paracasei* CH88 against *Gardnerella vaginalis*. Scientific Reports, 12(1), 4763.

Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., Harmsen, H. J. M., Faber, K. N., & Hermoso, M. A. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Frontiers in Immunology, 10, 277.

Peh, E., Kittler, S., Reich, F., & Kehrenberg, C. (2020). Antimicrobial activity of organic acids against *Campylobacter* spp. and development of combinations – a synergistic effect? PloS One, 15(9), e0239312.

Perler, B. K., Friedman, E. S., & Wu, G. D. (2023). The role of the gut microbiota in the relationship between diet and human health. Annual Review of Physiology, 85(1), 449–468.

Peterson, C. T., Perez Santiago, J., Iablokov, S. N., Chopra, D., Rodionov, D. A., & Peterson, S. N. (2022). Short-chain fatty acids modulate healthy gut microbiota composition and functional potential. Current Microbiology, 79(5), 128.

Pompilio, A., Kaya, E., Lupetti, V., Catelli, E., Bianchi, M., Maisetta, G., Esin, S., Di Bonaventura, G., & Batoni, G. (2024). Cell-free supernatants from *Lactobacillus* strains exert antibacterial, antibiofilm, and antivirulence activity against *Pseudomonas aeruginosa* from cystic fibrosis patients. Microbes and Infection, 26(4), 105301.

Qadi, W. S. M., Mediani, A., Kasim, Z. M., Misnan, N. M., Sani, N. A., & Jamar, N. H. (2023). Biological characterization and metabolic variations among cell-free supernatants produced by selected plant-based lactic acid bacteria. Metabolites, 13(7), 849.

Rocha-Ramírez, L. M., Hernández-Chiñas, U., Moreno-Guerrero, S. S., Ramírez-Pacheco, A., & Eslava, C. A. (2023). *In vitro* effect of the cell-free supernatant of the *Lactobacillus casei* strain IMAU60214 against the different pathogenic properties of diarrheagenic *Escherichia coli*. Microorganisms, 11(5), 1324.

Rodríguez-Melcón, C., Alonso-Calleja, C., García-Fernández, C., Carballo, J., & Capita, R. (2021). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for twelve antimicrobials (biocides and antibiotics) in eight strains of *Listeria monocytogenes*. Biology, 11(1), 46.

Scillato, M., Spitale, A., Mongelli, G., Privitera, G. F., Mangano, K., Cianci, A., Stefani, S., & Santagati, M. (2021). Antimicrobial properties of *Lactobacillus* cell-free supernatants against multidrug-resistant urogenital pathogens. Microbiology Open, 10(2), e1173.

Scorzoni, L., Benaducci, T., Almeida, A. M. F., Silva, D. H. S., Bolzani, V. S., & Mendes-Giannini, M. J. S. (2007). Comparative study of disk diffusion and microdilution methods for evaluation of antifungal activity of natural compounds against medical yeasts *Candida* spp. and *Cryptococcus* sp. Revista de Ciências Farmacêuticas Básica e Aplicada, 28(1), 25–34.

Tang, H., Huang, W., & Yao, Y. F. (2023). The metabolites of lactic acid bacteria: Classification, biosynthesis and modulation of gut microbiota. Microbial Cell, 10(3), 49–62.

Tedelind, S., Westberg, F., Kjerrulf, M., & Vidal, A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: A study with relevance to inflammatory bowel disease. World Journal of Gastroenterology, 13(20), 2826–2832.

Walker, R. L., Vlamakis, H., Lee, J. W. J., Besse, L. A., Xanthakis, V., Vasan, R. S., Shaw, S. Y., & Xavier, R. J. (2021). Population study of the gut microbiome: Associations with diet, lifestyle, and cardiometabolic disease. Genome Medicine, 13(1), 188.

Wang, G., & Zeng, H. (2022). Antibacterial effect of cell-free supernatant from *Lactobacillus pentosus* L-36 against *Staphylococcus aureus* from bovine mastitis. Molecules, 27(21), 7627.

Yang, C., Zhang, Y., & Yu, T. (2022). Application of the cell-free supernatant from *Weissella viridescens* to control *Listeria monocytogenes*. Czech Journal of Food Sciences, 40(4), 290–297.

Zhang, J., Zhao, J., Jin, H., Lv, R., Shi, H., De, G., Yang, B., Sun, Z., & Zhang, H. (2020). Probiotics maintain the intestinal microbiome homeostasis of the sailors during a long sea voyage. Gut Microbes, 11(4), 930–943.

Zhao, M., Chu, J., Feng, S., Guo, C., Xue, B., He, K., & Li, L. (2023). Immunological mechanisms of inflammatory diseases caused by gut microbiota dysbiosis: A review. Biomedicine and Pharmacotherapy, 164, 114985.