Antimicrobial activity of *Lactobacillus* cell free supernatant against *Salmonella* Enteritidis and Infantis

Research Article

ABSTRACT

Cell-free supernatants (CFS) produced by lactic acid bacteria (LAB) have been characterized as natural antagonists of important pathogens, including *Salmonella*. Their bacteriostatic or bactericidal properties have been reported to serve as an alternative to antibiotics by minimizing problems related to antimicrobial resistance. This study aimed to evaluate the antimicrobial activity of CFS of 4 selected LAB strains belonging to *Lacticaseibacillus paracasei* (2 strain), *Limosilactobacillus reuteri* (1 strain), and *Lacticaseibacillus rhamnosus* (1 strain) species against *Salmonella* Enteritidis and *S.* Infantis serovars by the agar-well diffusion method. Cell-free culture media of lactic acid bacteria were used in either crude CFS (acidic) and neutralized form (NCFS) to also understand non-pH-dependent antimicrobial potential. All crude CFSs were found to exhibit antimicrobial activity against pathogens, ranging from moderate to strong. After pH neutralization, the crude CFS of *L. paracasei* (2 strains) lost their antimicrobial activity, except for the crude CFS produced by *L. reuteri* and *L. rhamnosus*. However, there was a significant decrease in the level of anti-*Salmonella* activity of *L. rhamnosus* NCFS. It was determined that *L. reuteri* NCFS continued to show antimicrobial activity at levels similar to the effects of crude CFS. It is thought that the antimicrobial activity of *L. reuteri* and *L. rhamnosus* CFS determined in the research does not depend only on their acidity and that the chemical characterization of the postbiotics, which is the source of this antimicrobial activity, should be evaluated.

Keywords: Antimicrobial activity, cell-free supernatant**,** lactic acid bacteria, *Salmonella* Infantis, *Salmonella* Enteritidis.

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INTRODUCTION

Postbiotics are formulations containing non-living microbes or their elements that offer health benefits to the host (Zołkiewicz et al., 2020). Exopolysaccharides and peptidoglycans are examples of extracellular postbiotics secreted by lactic acid bacteria (LAB), while intracellular postbiotics include organic acids, short-chain fatty acids (SCFAs), indole from amino acids, and peptides like acidophilin, bifidin, reuterin, and lactocepin (Thorakkattu et al., 2022). Cell-free supernatant (CFS) is a sort of postbiotic fluid that remains after living cells are removed from bacteria cultured in a culture medium via centrifugation and filtering, and it contains the bacteria's biological metabolites. Studies show that these metabolites can be isolated and used independently (Żółkiewicz et al., 2020). Although the mechanisms underlying their health advantages are not entirely understood, investigations have indicated that CFS has bacteriostatic or bactericidal properties, competing with harmful microorganisms and reducing their growth (Thorakkattu et al., 2022).

Salmonella is one of the most important zoonotic bacteria, ranking second globally in terms of foodborne gastrointestinal illnesses. It is predicted that the

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eating of *Salmonella*-contaminated food causes 86% of these infections (EFSA,2022). Salmonellosis in humans is caused by contaminated items such as dairy, seafood, poultry, eggs, and meat. *Salmonella* Typhimurium and *Salmonella* Enteritidis are known to be among the most frequently isolated serotypes in human salmonellosis cases, but the prevalence of *Salmonella* Infantis in salmonellosis cases has been reported to be rapidly increasing in recent years (Alzahrani et al., 2023).

Probiotics, prebiotics, essential oils, and bacteriophages have all been developed in recent years as alternatives to antimicrobials for *Salmonella* infection control. According to studies, CFS could be used as a feed addition to manage *Salmonella* in livestock farming, providing an alternative to antibiotics and alleviating concerns about antimicrobial resistance (Abramov et al., 2023; Dobreva et al., 2022; Evangelista et al., 2021).

Many LAB produce CFS, which have been identified as natural antagonists of key pathogens such as *Salmonella*, highlighting the need to research their anti-pathogenic activities (Dobreva et al., 2022). In this context, the present study focuses on the antimicrobial effects of CFS from selected LAB against *Salmonella* Enteritidis and Infantis serovars.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The study used LAB strains that had previously been isolated from conventional fermented dairy items (Yilmaz and Turkyilmaz, 2022). *Limosilactobacillus reuteri* (I-3) (n:1), *Lacticaseibacillus rhamnosus* (I-4) (n:1) and two strains of *Lacticaseibacillus paracasei* (I-2 and I-7) were selected for the study. *S.* Enteritidis (n:3; Lab. Code: N12, N13, N24) and *S.* Infantis (n:3; Lab. Code: N9, N10, N11) serovars, which were previously isolated from poultry litter and were found multidrug-resistant (MDR) bacteria in antimicrobial screening tests, were used as test microorganisms. All bacterial strains were used among the bacterial culture collection of the Microbiology Department, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydın, Türkiye.

Prior to preparing the CFS, LAB strains were cultivated for 24 hours at 37°C on De Man, Rogosa, and Sharpe Agar (MRS Agar, Merck, Darmstadt, Germany). Before conducting antimicrobial assays, *Salmonella* strains were cultured on Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany), and Tryptic Soy Broth (TSB, Merck, Darmstadt, Germany). Nutrient Agar (NA, Thermo Fisher Scientific) was employed for the agar well diffusion technique. Pathogenic strains were revitalized in TSB with a 10% inoculum at 37°C overnight. Each bacterial strain underwent two subcultures before experimentation and was stored at -20°C in Brain Heart Infusion broth (BHI, Merck, Darmstadt, Germany) supplemented with 20% glycerol.

CFS preparation

CFSs were generated utilizing the agar well diffusion technique, following the method outlined by Shokryazdan et al., 2014, with slight adjustments. Briefly, LAB strains from overnight cultures were introduced into MRS Broth at a concentration of 1% (v/v) and were left to grow at 37°C for 24 hours. Following the overnight incubation period, CFSs were prepared by centrifuging the broth at 4000 × g for 20 min at 4°C. The supernatant was sterilized using membrane filters (0.22 µm pore size, Sartorius, Göttingen, Germany) and were named as crude CFS (acidic CFS). To test the acid-dependent antimicrobial activities of CFS, pH-adjusted CFS-free media; MRS broth were used as negative controls. Neutralized CFS samples were prepared with 2 M NaOH and was called neutralized cell-free supernatant (NCFS) to test acid-independent antimicrobial activity. The pH

of the crude CFSs and NCFSs were measured with a pH meter.

Antimicrobial screening assay

Each strain of pathogen, initially cultured on TSA and left to grow overnight at 37°C, was adjusted to a concentration corresponding to 0.5 McFarland standard (~108 CFU/mL). These pathogens were then evenly spread onto NA plates using a cotton swab, and the plates were allowed to air-dry at room temperature. Subsequently, wells of 6 mm in diameter were created on the plates using a sterile cork borer, into which 100 µL of the CFS was carefully pipetted. Following this, the petri dishes were placed in an incubator at 37°C overnight. The effectiveness of both crude CFS and NCFS were assessed separately to determine their antimicrobial properties.

Statistical analysis

The quantitative data of the experimental results were presented as mean ± standard deviation (SD) of two independent experiments, tested in triplicate. The significance of differences ($p<0.05$) was determined using one-way ANOVA with the statistical package for Social Sciences (SPSS) software (Version 21, SPSS Inc., Chicago, IL. USA).

RESULTS

Anti-Salmonella activity of crude CFSs

The investigation revealed that the pH levels of the CFSs ranged from 4 to 5 prior to undergoing neutralization. The pH values of CFSs *L. paracasei* (I-2), *L. reuteri* (I-3), *L. rhamnosus* (I-4), and *L. paracasei* (I-7) were 4.07, 4.13, 4.43 and 4.07, respectively. Table 1 displays the pH values of the media containing varying concentrations of crude CFSs.

Table 1: The pH values of crude CFSs.

CFS-producing strains	Before pH neutralization Crude CFS
	Mean±SD
Lacticaseibacillus paracasei (I-2)	4.07±0.12
Limosilactobacillus reuteri (I-3)	4.13±0.12
Lacticaseibacillus rhamnosus (I-4)	4.43±0.12
Lacticaseibacillus paracasei (I-7)	4.07±0.12

Mean: Arithmetic mean of the CFSs, SD: Standard Deviation

In the current study, significantly different antimicrobial activities were found in all CFSs at acidic pH (Crude CFS) ($p<0.05$) (Table 1-4). The *L. paracasei* (I-2) CFS exhibited strong antimicrobial activity against *S.* Infantis (N9, N10 and N11) and *S.* Enteritidis N24, with mean inhibition zone diameters of 22.17, 20.10, 20.27 and 25.13mm, respectively. CFS showed moderate activity against the pathogens *S.* Enteritidis (N12 and N13) with zones of inhibition that varied between 18.20 and 18.13mm, respectively (Table 2). The *L. reuteri* (I-3) CFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11), with mean inhibition zone diameters of 10.13, 10.50, 11.23, 13.73, 10.13 and 14.03mm, respectively (Table 3). The *L. rhamnosus* (I-4) CFS exhibited strong antimicrobial activity against *S.* Enteritidis (N24) and *S.* Infantis (N11), with mean inhibition zone diameters of 26.03 and 21.17mm, respectively. CFS showed moderate activity against the pathogens *S.* Enteritidis (N12 and N13) and *S.* Infantis (N9 and N10) with zones of inhibition that varied between 17.03, 19.10, 18.17 and 18.23mm, respectively (Table 4). The *L. paracasei* (I-7) CFS exhibited strong antimicrobial activity against *S.* Enteritidis (N24) and *S.* Infantis (N11), with mean inhibition zone diameters of 25 and 21.03mm, respectively. CFS showed moderate activity against the pathogens *S.* Enteritidis (N12 and N13) and *S.* Infantis (N9 and N10) with zones of inhibition that varied between 16.23, 18.27,

18.03 and 15.10mm, respectively (Table 5). CFS-free media: MRS used as a negative control did not show any antimicrobial activities as expected.

Table 2: Anti-*Salmonella* activity of crude CFS of *Lacticaseibacillus paracasei* I-2.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* Enteritidis N12**	18.20±0.26*	Moderate
***S.* Enteritidis N13**	18.13±0.15*	Moderate
***S.* Enteritidis N24**	25.13±0.15*	Strong
***S.* Infantis N9**	22.17±0.15*	Strong
***S.* Infantis N10**	20.10±0.10*	Strong
***S.* Infantis N11**	20.27±0.15*	Strong
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS

Table 3: Anti-*Salmonella* activity of crude CFS of *Limosilactobacillus reuteri* I-3.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* EnteritidisN12**	10.13±0.15*	Moderate
***S.* EnteritidisN13**	10.50±0.50*	Moderate
***S.* EnteritidisN24**	11.23±0.25*	Moderate
***S.* Infantis N9**	13.73±0.25*	Moderate
***S.* Infantis N10**	10.13±0.15*	Moderate
***S.* Infantis N11**	14.03±0.15*	Moderate
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS.

Table 4: Anti-*Salmonella* activity of crude CFS of *Lacticaseibacillus rhamnosus* I-4.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* EnteritidisN12**	17.03±0.15*	Moderate
***S.* EnteritidisN13**	19.10±0.10*	Moderate
***S.* EnteritidisN24**	26.03±0.15*	Strong
***S.* Infantis N9**	18.17±0.15*	Moderate
***S.* Infantis N10**	18.23±0.21*	Moderate
***S.* Infantis N11**	21.17±0.15*	Strong
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS.

Table 5: Anti-*Salmonella* activity of crude CFS of *Lacticaseibacillus paracasei* I-7.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)	
	Crude CFS	**Inhibition effect**
***S.* EnteritidisN12**	16.23±0.25*	Moderate
***S.* EnteritidisN13**	18.27±0.21*	Moderate
***S.* EnteritidisN24**	25.00±0.10*	Strong
***S.* Infantis N9**	18.03±0.15*	Moderate
***S.* Infantis N10**	15.10±0.10*	Moderate
***S.* Infantis N11**	21.03±0.15*	Strong
Control: CFS-free media	0.00	Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by crude CFS.

Anti-Salmonella activity of NCFS

To mitigate the influence of organic acid compounds such as lactic, acetic, and formic acid, as well as bacteriocins present in crude CFSs that hinder pathogen growth, neutralization was performed. After pH neutralization, crude CFSs from the *L. paracasei* I-7 and *L. paracasei* I-2 lost their antimicrobial activity (Table 6-7).

Table 6: Anti-*Salmonella* activity of NCFS of *Lacticaseibacillus paracasei* I-7.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)		
	NCFS	NCFS (Neutralized by NaOH)	Inhibition effect
***S.* EnteritidisN12**	-*	6.93±0.06	Negative
***S.* EnteritidisN13**	-*	6.83±0.06	Negative
***S.* EnteritidisN24**	-*	6.87±0.06	Negative
***S.* Infantis N9**	-*	6.83±0.06	Negative
***S.* Infantis N10**	-*	6.93±0.06	Negative
***S.* Infantis N11**	-*	6.93±0.06	Negative
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. The inhibition zone is not determined: - (Negative).

Table 7: Anti-*Salmonella* activity of NCFS of *Lacticaseibacillus paracasei* I-2.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)		
	NCFS	NCFS (Neutralized by NaOH)	Inhibition effect
***S.* Enteritidis N12**	-*	6.80±0.10	Negative
***S.* Enteritidis N13**	-*	6.53±0.06	Negative
***S.* Enteritidis N24**	-*	6.67±0.15	Negative
***S.* Infantis N9**	-*	6.80±0.10	Negative
***S.* Infantis N10**	-*	6.87±0.06	Negative
***S.* Infantis N11**	-*	6.87±0.06	Negative
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. The inhibition zone is not determined: - (Negative).

The *L. rhamnosus* (I-4) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11) strains, with mean inhibition zone diameters of 15.34, 16.17, 14.17, 16.20, 15.23 and 14.27mm, respectively (Table 8). The *L. reuteri* (I-3) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11), with mean inhibition zone diameters of 10.00, 10.17, 10.27, 12.10, 10.07 and 12.17mm, respectively (Table 9). Neutralizing CFSs from the *L. rhamnosus* (I-4) and *L. reuteri* (I-3) strains with NaOH lowered anti-*Salmonella* activity, but it was not totally abolished (Table 8-9). There was a significant decrease in the level of anti-*Salmonella* activity of *L. rhamnosus* NCFS ($p<0.05$). It was determined that *L. reuteri* NCFS continued to show antimicrobial activity at levels similar to the effects of crude CFS ($p<0.05$).

Table 8: Anti-*Salmonella* activity of NCFS of *Lacticaseibacillus rhamnosus* I-4.

Salmonella Strain	*Salmonella* Strain Growth Inhibition Zone (mm)		
	NCFS	NCFS (Neutralized by NaOH)	Inhibition effect
***S.* EnteritidisN12**	15.34±0.28*	6.80±0.10	Moderate
***S.* EnteritidisN13**	16.17±0.15*	6.53±0.06	Moderate
***S.* EnteritidisN24**	14.17±0.15*	6.67±0.15	Moderate
***S.* Infantis N9**	16.20±0.17*	6.80±0.10	Moderate
***S.* Infantis N10**	15.23±0.21*	6.87±0.06	Moderate
***S.* Infantis N11**	14.27±0.12*	6.87±0.06	Moderate
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. Moderate inhibition effect: 10 mm ≤ Inhibition zone < 20 mm.

Table 9: Anti-*Salmonella* activity of NCFS of *Limosilactobacillus reuteri* I-3.

***Salmonella* Strain**	***Salmonella* Strain Growth Inhibition Zone (mm)**		
	NCFS	**NCFS (Neutralized by NaOH)**	**Inhibition effect**
***S.* EnteritidisN12**	10.00±0.00*	6.77±0.06	Moderate
***S.* EnteritidisN13**	10.17±0.15*	6.63±0.06	Moderate
***S.* EnteritidisN24**	10.27±0.25*	6.77±0.06	Moderate
***S.* Infantis N9**	12.10±0.10*	6.83±0.06	Moderate
***S.* Infantis N10**	10.07±0.12*	6.83±0.06	Moderate
***S.* Infantis N11**	12.17±0.29*	6.87±0.06	Moderate
Control: CFS-free media	-		Negative

Data are represented as means ± SD of two independent experiments, tested in triplicate. **Salmonella* strain growth inhibition zone induced by NCFS. Moderate inhibition effect: 10 mm ≤ Inhibition zone < 20 mm.

DISCUSSION

Salmonella Enteritidis and Infantis serovars are reported as the most commonly reported *Salmonella* serovars in broilers, implicated in cases of salmonellosis in humans (Alzahrani et al., 2023). Given the presence of strains exhibiting multidrug resistance in these scenarios, it is evident that a serious public health issue may arise. LAB and their metabolites have recently been regarded as important possibilities for managing *Salmonella* pathogens due to their proven bio-preservative and antimicrobial properties (Evangelista et al., 2021; Yilmaz and Turkyilmaz, 2022).

CFS produced by LAB is fluid containing byproducts of microbial proliferation and residual nutrients from the cultivation medium that were not absorbed (Thorakkattu et al., 2022). The number of studies studying CFS's anti-infective, antimicrobial, and antioxidant capabilities, such as their function in pathogen inhibition, has increased in recent years (Lee et al., 2022). It is expected that additional research into CFS characteristics and mechanisms of action will lead to a better knowledge of their bioactivities (Cuevas-Gonzalez et al., 2020). The antimicrobial activities of LAB CFS were evaluated in this research using strains of *S.* Enteritidis and *S.* Infantis serovars.

The research discovered that the pH levels of CFSs ranged from 4 to 5 before neutralization. The pH values of the CFSs *L. paracasei* (I-2), *L. reuteri* (I-3), *L. rhamnosus* (I-4), and *L. paracasei* (I-7) were 4.07, 4.13, 4.43, and 4.07. Although pH values fluctuate depending on the LAB strains utilized, pH values of around 4 have been reported in the literature (Abramov et al., 2023).

The research indicated that all crude CFSs had moderate to strong antimicrobial efficacy against microorganisms. All LAB strains' crude CFSs had high antimicrobial activity against *S.* Infantis N11 and *S.* Enteritidis N24, with the exception of *L. reuteri* (I-3). In addition, crude CFS of *L. paracasei* (I-2) demonstrated strong antimicrobial action against *S.* Infantis N9 and N10. The inhibitory impact of *L. reuteri* (I-3) crude CFS on all pathogens was found to be moderate, with no strong activity identified.

There are various studies in the literature demonstrating the antimicrobial activity of CFS against *Salmonella* strains (Arrioja-Bretón et al., 2020; Divyashree et al., 2021; Goa et al., 2022; Lando et al., 2023). A study reported that the CFSs of *Limosilactobacillus fermentum* LBF 233, *Limosilactobacillus fermentum* LBF 433, and *Lacticaseibacillus casei* LBC237 strains exhibited antimicrobial activity against *S.* Enteritidis and *S.* Typhimurium pathogens at concentrations of 10% and 20% (Lando et al., 2023). Another study examined the antimicrobial activity of LAB isolates against clinical isolates of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* spp.

using the agar well diffusion method. It was determined that all 12 LAB isolates used, including *Lactococcus lactis* subsp. *lactis* (6), *Lactobacillus acidophilus* (2), *Lactiplantibacillus plantarum* (1), *L. fermentum* (2), and *Leuconostoc lactis* (1), exhibited antimicrobial activity against the tested bacterial strains (Goa et al., 2022). Shi et al. (2022) reported that the CFS and cells of *L. rhamnosus* SQ511 exhibited antagonistic activity against *S.* Enteritidis ATCC13076 and were able to inhibit the growth of the pathogen. The researchers determined the average diameters of the inhibition zones to be 21.82, 18.45, and 12.34mm. In line with the literature, the *L. rhamnosus* (I-4) crude CFS utilized in this study has strong antimicrobial activity against *S.* Enteritidis (N24) and *S.* Infantis (N11), with mean inhibition zone widths of 26.03 and 21.17mm, respectively. Crude CFS shown moderate effectiveness against the pathogens *S.* Enteritidis (N12 and N13) and S. Infantis (N9 and N10). The zones of inhibition measured 17.03, 19.10, 18.17, and 18.23mm, respectively.

Abramov et al., (2023) found that the low pH of the environment enhanced the antimicrobial efficacy of *Ligilactobacillus salivarius* CFSs against the *S.* Typhimurium pathogen. Additionally, it was discovered that the antimicrobial activity of the neutralized CFS diminished following the neutralizing treatment. Evangelista et al., (2021) investigated the in vitro efficacy of LAB CFS against *Salmonella* and discovered that all acidic CFS suppressed the pathogen's development. The researchers performed pH neutralization on the crude CFS. After pH neutralization, they reported that the neutralized CFS of *L. acidophilus* Llorente, *L. fermentum* CCT 1629, *L. plantarum* PUCPR44, *L. reuteri* BioGaia, *L. rhamnosus* ATCC 7469, and *Pediococcus pentosaceus* UM116 strains partially retained their antimicrobial activities. In line with this research, crude CFSs that hinder pathogen growth, such as lactic, acetic, and formic acid, or bacteriocins, were neutralized. PH neutralization assays were important to determine whether the antimicrobial activity of CFS was acidity dependent. In this study, it was also determined that the CFSs of *L. reuteri* and *L. rhamnosus* continued to exhibit activity after neutralization. The *L. rhamnosus* (I-4) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11) strains, with mean inhibition zone diameters of 15.34, 16.17, 14.17, 16.20, 15.23 and 14.27mm, respectively. The *L. reuteri* (I-3) NCFS exhibited moderate antimicrobial activity against *S.* Enteritidis (N12, N13 and N24) and *S.* Infantis (N9, N10 and N11), with mean inhibition zone diameters of 10.00, 10.17, 10.27, 12.10, 10.07 and 12.17mm, respectively. However, after pH neutralization, crude CFSs from the *L. paracasei* (I-7) and *L. paracasei* (I-2) lost their antimicrobial activity.

It is known that the CFS produced by LAB mostly contains hydrogen peroxide, organic acids (mainly lactic acid), fatty acids, and proteins/peptides, and has mildly to strongly acidic profiles (ranging from pH 2.2 to 6.0) (Lim et al., 2018). Studies have determined that the production of hydrogen peroxide, organic acids, and bacteriocins are the main strategies by which *Lactobacillus* inhibits the growth of *Salmonella* (Ayeni et al., 2019). In the study, pH neutralization led to a decrease in the efficacy of some CFSs and resulted in the loss of antimicrobial activity in others. This finding underscores the importance of pH for pathogen inhibition. It is also known that pH neutralization reduces the activity of fatty acids because fatty acids at neutral pH are ionized, preventing them from penetrating bacterial cells (Shehata et al., 2019).

According to certain research, the presence of non-acidic antimicrobial substances such as bacteriocins causes the CFS of certain LAB strains to retain some of their antimicrobial activities following pH neutralization. Although these chemicals prefer low pH for best action, they can still be active at neutral pH (Prudêncio

et al., 2016; Shi et al., 2022). The evaluation suggests that the antimicrobial impact is predominantly derived from lactic acid and acetic acid, with additional metabolites such as organic acids, bacteriocins, and short and long-chain fatty acids all contributing to antimicrobial activity (Mani-Lopez et al., 2022).

In the research where moderate/good and strong activity against *S.* Typhimurium was determined for CFS of some LAB strains, it was reported that the identified antimicrobial activity might be attributed to the formation of organic acids. Researchers determined that the neutralized and catalase-treated supernatants had no effect on the tested Gram-negative pathogenic bacteria (Bahri et al., 2014). Parallel to this research, it was found that the antimicrobial effects of *L. paracasei* (I-2 and I-7) CFSs disappeared after the neutralization process. The disappearance of this effect suggests that the antimicrobial activities of these CFSs may stem from organic acids.

CONCLUSION

The antimicrobial activities of neutralized CFSs from *L. rhamnosus* (I-4) and *L. reuteri* (I-3) strains against pathogens might be associated with the presence of other antimicrobial agents, different from the predominant acidic components. The disappearance of the antimicrobial effects of *L. paracasei* (I-2 and I-7) CFSs after the neutralization process suggests that the antimicrobial activity may originate from organic acids. Analysis of the compound responsible for antimicrobial activity may provide information on the potential uses of CFS.

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