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Inhibitory effect of cell-free supernatants of *Pedicoccous acidilactici* and *Latilactobacillus sakei/Staphylococcus xylosus* in combination with ethylenediaminetetraacetic acid against *Escherichia coli* O157:H7 strains

Gökhan Kürşad İncili[1]*, Osman İrfan İlhak[2], Mehmet Çalıcıoğlu[1]

[1] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Fırat University, Elaziğ, Türkiye; [2] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Balıkesir University, Balıkesir, Türkiye.

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Abstract

The current research was carried out to evaluate *in vitro* anti-microbial properties of the cell-free supernatants (CFSs) derived from *Pediococcus acidilactici* (PA) and *Latilactobacillus sakei/ Staphylococcus xylosus* (LS) against *Escherichia coli* O157:H7 American Type Culture Collection strains (35150, 43894, and 43985). For this purpose, the diameters of zone of inhibition of the CFSs against *E. coli* O157:H7 strains were measured. In addition, a time-kill assay was conducted to determine the inhibitory effect of the CFSs alone or in combination with ethylenediaminetetraacetic acid (EDTA) during incubation at 37.00 ˚C for 24 hr. In the time-kill assay, *E. coli* O157:H7 was subjected to three concentrations of CFSs (1.00, 5.00, and 10.00%) and EDTA (0.02 M) in tryptic soy broth and the *E. coli* O157:H7 count was determined at 0, 6, and 24 hr intervals. The CFS of LS had a lower pH and higher titratable acidity compared to the PA. The CFS of LS displayed higher zones of inhibition than the CFS of PA against *E. coli* O157:H7 American Type Culture Collection 35150 and 43894 strains. The concentrations of 5.00 and 10.00% CFSs in combination with EDTA provided a 5.00 $\log_{10}$ decline in *E. coli* O157:H7 count over a 24-hr period. The results of this study indicated that the combination of CFSs (5.00 and 10.00%) and EDTA (0.02 M) exhibited an enhanced anti-bacterial effect against *E. coli* O157:H7 strains, which are substantial foodborne pathogenic bacteria.

Introduction

Autochthonous lactic acid bacteria (LAB) have been utilized for centuries in fermented foods, such as dairy and meat products, sourdough, and vegetables.[1] Scientific and technological developments have elucidated the characteristics of LAB; thus, commercial starter cultures are now available to enable standardized production of fermented foods. In addition, LAB are the most widely preferred microorganisms among probiotics and have been used for a long time.[2] Numerous studies have been conducted on probiotics and many benefits have been identified for hosts.[3] The LAB have been increasingly used for bio-preservation of foods against pathogenic and spoilage microorganisms in the recent decades.[4] In the bio-preservation approach, LAB exert anti-microbial effects by competing for nutrients and adherence surfaces, as well as synthesizing anti-microbial compounds during their growth.[5] However, to achieve these benefits, it is necessary to control the microbiota and provide the suitable temperature and nutrients for growth.[5] To overcome these drawbacks, various approaches have been attempted to maintain bacterial stability and dominate the microbiota under environmental stress conditions, including the use of higher numbers of bacteria or encapsulation.[6] As an alternative to use of live LAB, there has been remarkable interest in the use of metabolites derived from these bacteria and the determination of their bioactive properties in recent years, which is a novel field of research.[5]

The term postbiotic has been established as non-living or inactivated microorganisms and their produced or released compounds that provide some benefits to the health of the host.[7] The term cell-free supernatants (CFSs) refers to microbial metabolites or compounds obtained after separating microorganisms from the liquid medium following their growth.[5] To date, it has been shown that CFSs contain various compounds, including organic acids,

***Correspondence:**

Gökhan Kürşad İncili. PhD
Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Fırat University, Elazığ, Türkiye
E-mail: gkincili@firat.edu.tr

amino acids, fatty acids, peptides, including bacteriocins, and exopolysaccharides.[8,9] Bacteriocins are one of the well-studied compounds present in the CFSs.[5] However, it is well known that bacteriocins do not display a broad spectrum of anti-microbial activity against Gram-negative microorganisms.[10-12] Overcoming this limitation requires the presence of chelating agents, such as ethylenediaminetetraacetic acid (EDTA), to broaden the anti-microbial spectrum. In this regard, the combination of chelators and bacteriocins has been shown to have a synergistic anti-microbial effect against Gram-negative bacteria.[10-12] Although, as mentioned above, CFSs contain a variety of bioactive compounds. Little is known about the potential synergy between EDTA and CFSs. Therefore, there is a need to elucidate the anti-microbial effect of the combination of CFS with EDTA.

The most commonly employed bacterial strains for the production of CFSs are belong to the *Lactobacillus* spp., *Lactococcus* spp., *Pediococcus* spp., and *Staphylococcus* spp.[13] Due to the presence of a wide variety of compounds, CFSs exhibit various bioactivities, including anti-microbial, anti-oxidant, anti-carcinogenic, immunostimulant, and anti-inflammatory effects.[14-18] However, it is noteworthy that the bioactive properties of CFSs are significantly strain-dependent.[17] Hence, further research is needed to fully clarify the bioactive properties of different LAB strains.

Escherichia coli O157:H7, also known as Shiga toxigenic *E. coli*, belongs to zoonotic pathotype causing a worldwide burden of food- and water-borne diseases.[19] The *E. coli* O157:H7 serotype has a number of genes encoding various virulence factors that can contribute to the onset of hemorrhagic colitis and hemolytic uremic syndrome. Also, this serotype has the capability to endure harsh environments, including those with high acidity and low pH levels. It is well known that this pathogen can survive in various acidic foods, such as fruit juices and fermented dairy and meat products.[20,21] So far, many decontamination approaches and procedures have been attempted to eliminate this pathogen from foods, while recent studies indicate that Shiga toxigenic *E. coli* strains continue to pose a threat to public health.[22] Thus, there is still a need to evaluate the new anti-microbial compounds and/or decontamination approaches to eliminate *E. coli* O157:H7.

The aim of this study was to test the *in vitro* anti-bacterial effect of CFSs obtained from *Pedicoccous acidilactici* (PA), *Latilactobacillus sakei/Staphylococcus xylosus* (LS), and their combination with EDTA against *E. coli* O157:H7 American Type Culture Collection (ATCC) 3510, 43894, and 43895 strains.

Materials and Methods

Preparing cell-free supernatants. The PA (BLC-20) and LS (B-FM), which are commercial bio-protective cultures manufactured by Chr. Hansen (Copenhagen, Denmark), were employed in this research. The bacteria were incubated in tryptic soy broth (TSB; Biokar, Beauvais, France) at 37.00 ˚C for 24 hr.[17] Following activation, centrifugation (4,000 *g* for 10 min) was performed to separate the pellet from the supernatant, and the supernatant was passed through a 0.45 µm pore size filter. The liquid CFSs were collected into sterilized jars and held at 4.00 ± 1.00 ˚C until use.

Preparation of *E. coli* O157:H7 inoculum. Three strains of *E. coli* O157:H7 were obtained from ATCC (American Type Culture Collection, Manassas, USA). Bacterial propagation was performed by overnight incubation in TSB at 35.00 ± 1.00 ˚C. After incubation, centrifugation (4,000 *g* for 10 min) was performed to obtain pellets. The pellets were then rehydrated in 0.10% peptone water. The absorbance of the rehydrated pellets was adjusted to ~0.100 at a wavelength of 600 nm to achieve an inoculation level of ~8.00 $\log_{10}$ colony-forming unit (CFU) mL^{-1}.[13] Subsequently, the bacteria were diluted decimally with 0.10% peptone water and a spiking level of 5.00 ± 0.50 $\log_{10}$ CFU mL^{-1} was employed in the study.

Determination of pH and titratable acidity. The pH measurements were carried out using a pH meter (HI 11310, Hanna Instruments, Woonsocket, RI, USA). The titratable acidity of the CFSs was measured by the method outlined in the previous research.[23] Briefly, a mixture of CFS and distilled water (1/9 v/v) was prepared and 1.00 mL phenolphthalein (1.00% w/v) (Merck, Darmstadt, Germany) was then added to the CFS solution. This solution was titrated with 0.10 N NaOH (Merck). The volume of NaOH (mL) consumed was calculated as titratable acidity by the following formula:

$$\% \text{ lactic acid equivalent} = [(v \times 0.10 \times 90) / m \times 1{,}000] \times 100$$

where, *v* is volume (mL) of NaOH and *m* is volume (mL) of CFS.[23]

Inhibition zone assay. In this assay, the pathogen inoculum was prepared as outlined above, and Mueller-Hinton agar (Merck) plates were separately inoculated with *E. coli* O157:H7 strains. The inoculated Petri dishes were kept at room temperature for 10 min to allow bacterial attachment. Following this, 20.00 µL of CFS was impregnated onto the sterilized filter papers (6.00 mm diameter), which were then placed on the plates. The Petri dishes were then kept at 35.00 ± 1.00 ˚C for 24 hr and the resulting diameters of zone of inhibitions (mm) were measured.[23]

Time-kill assay. During the experiment, three concentrations of CFSs (1.00, 5.00, and 10.00% v/v each of PA and LS) were used alone or combined with 0.02 M EDTA.[17] Briefly, the tubes containing TSB were inoculated with the aforementioned concentrations of CFSs and EDTA. In addition, a control tube containing only TSB was used in the experiment. All tubes were inoculated with *E. coli* O157:H7 strains at a concentration of approximately

5.00 ± 0.50 log_{10} CFU mL^{-1} and held at 35 .00 ˚C. The number of *E. coli* O157:H7 was determined at time 0 (after inoculation), 6, and 24 hr. For this purpose, cefixime-tellurite sorbitol MacConkey (Merck) agar was used. The Petri dishes were held at 35.00 ± 2.00 ˚C for 24 hr and the bacteria counts were then determined.[17] Additionally, the pH and titratable acidity were determined using the methods described above.

Statistical analyses. Three independent replicates were performed for each experiment. An independent *t*-test was employed to compare the pH, titratable acidity, and diameter of inhibition zone values of the CFSs. The microbiological, pH, and titratable acidity data in the time-kill assay were compared among groups and sampling times using ANOVA, and comparisons among the groups were determined using Tukey's test with a significance threshold of 0.05. The SPSS Software (version 12.0; SPSS Inc., Chicago, USA) was used for the statistical analyses.

Results

As illustrated in Figure 1, the amount of lactic acid caused a remarkable decline in the pH value and elevation in the titratable acidity values of the CFSs. The differences between the CFSs were found to be found significant ($p < 0.05$). In this context, the pH of the CFS obtained from LS was found to be lower than that obtained from PA ($p < 0.05$). Furthermore, the titratable acidity of the LS was higher than that of the PA ($p < 0.05$).

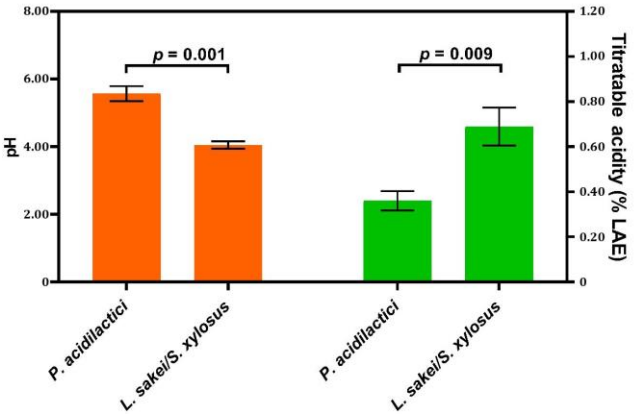

Fig. 1. The pH and titratable acidity (% lactic acid equivalent [LAE]) values of the cell-free supernatants of *Pediococcus acidilactici* and *Latilactobacillus sakei/Staphylococcus xylosus* (mean ± standard deviation). The *p* values indicate the statistical differences between the PA and LS.

The diameters of the inhibition zone caused by the LAB tested against *E. coli* O157:H7 strains ranged from 9.42 to 12.35 mm (Table 1). The diameters of the inhibition zones against *E. coli* O157:H7 ATCC 35150 and 43894 exhibited notable differences between the CFSs ($p < 0.05$). Nevertheless, no significant differences were observed between the CFSs in terms of inhibition zones against *E. coli* O157:H7 ATCC 43895 ($p > 0.05$). The largest diameter of the inhibition zone was observed in the CFS of LS against *E. coli* O157:H7 ATCC 43984. Nevertheless, the lowest diameter of inhibition zones was observed in the CFS of LS against *E. coli* O157:H7 ATCC 43895 (Fig. 2).

The result of the time-kill assay revealed that in the absence of EDTA, CFSs at all concentrations resulted in approximately 1.00 log_{10} decrease in the number of *E. coli* O157:H7 after 24 hr incubation (Table 2). The combination of EDTA and CFSs at concentrations of 5.00% and 10.00% demonstrated a minimum of 5.00 log_{10} CFU mL^{-1} inhibition over a 24-hr period ($p < 0.05$). Besides, the application of EDTA and 1.00% CFSs resulted in a nearly 4.00 log_{10} CFU mL^{-1} reduction in the number of *E. coli* O157:H7 over a 24-hr period ($p < 0.05$).

As shown in Tables 3 and 4, the pH and titratable acidity values of the tubes containing TSB changed significantly with increasing CFS concentration at time 0 ($p < 0.05$). In the majority of the groups, the pH values of the TSBs exhibited a notable decline at the 6th hr in comparison with the initial measurements ($p < 0.05$).

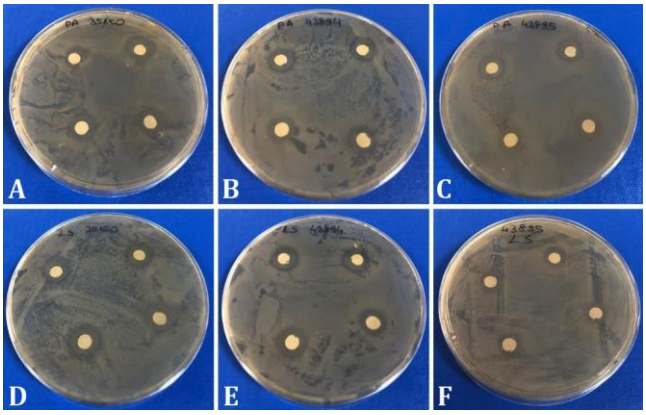

Fig. 2. The diameter of inhibition zones of the cell-free supernatant of *Pediococcus acidilactici* against *Escherichia coli* O157:H7 American Type Culture Collection (ATCC) **A)** 35150, **B)** ATCC 43894, **C)** ATCC 43895, and *Latilactobacillus sakei/ Staphylococcus xylosus* against *E. coli* O157:H7 **D)** ATCC 35150, **E)** ATCC 43894, and **F)** ATCC 43895.

Table 1. Inhibition zone diameters (mm) of cell-free supernatants obtained from *Pediococcus acidilactici* and *Latilactobacillus sakei/ Staphylococcus xylosus* against *Escherichia coli* O157:H7 strains (mean ± standard deviation).

E. coli O157:H7 strain	*Pediococcus acidilactici*	*Latilactobacillus sakei/ Staphylococcus xylosus*	*p*-value
ATCC 35150	10.01 ± 0.61^{a}	10.90 ± 0.51^{b}	0.021
ATCC 43984	10.27 ± 0.97^{a}	12.35 ± 0.53^{a}	0.008
ATCC 43985	9.46 ± 0.88^{a}	9.42 ± 0.64^{c}	0.924

ATCC: American type culture collection.
^{abc} Statistical differences are shown by different superscripts in the same columns ($p < 0.05$).

Table 2. The number of *Escherichia coli* O157:H7 incubated at 35.00 ± 1.00 ˚C for 24 hr in tryptic soy broth containing different concentrations of cell-free supernatant (CFS) with or without 0.02 M ethylenediaminetetraacetic acid (EDTA; mean Log_{10} colony-forming unit mL^{-1} ± standard deviation).

Groups	0 hr	6 hr	24 hr
Control	5.55 ± 0.22^{Ab}	8.34 ± 0.66^{Aa}	8.31 ± 0.23^{Aa}
PA 1.00%	5.49 ± 0.34^{Ab}	8.38 ± 0.49^{Aa}	4.41 ± 0.25^{Bc}
PA 5.00%	5.49 ± 0.49^{Ab}	8.27 ± 0.31^{Aa}	4.70 ± 0.28^{Bc}
PA 10.00%	5.54 ± 0.25^{Ab}	7.04 ± 0.35^{Ca}	4.37 ± 0.20^{Bc}
LS 1.00%	5.45 ± 0.35^{Ab}	8.56 ± 0.22^{Aa}	4.61 ± 0.27^{Bc}
LS 5.00%	5.36 ± 0.40^{Ab}	8.06 ± 0.24^{ABa}	4.81 ± 0.38^{Bb}
LS 10.00%	5.64 ± 0.15^{Ab}	7.37 ± 0.40^{BCa}	4.35 ± 0.53^{Bc}
PA 1.00% + EDTA	5.54 ± 0.10^{Ab}	6.87 ± 0.33^{CDa}	1.73 ± 0.68^{Cc}
PA 5.00% + EDTA	5.44 ± 0.26^{Aa}	6.12 ± 0.46^{Da}	0.00 ± 0.00^{Db}
PA 10.00% + EDTA	5.26 ± 0.21^{Aa}	4.67 ± 0.15^{Eb}	0.00 ± 0.00^{Dc}
LS 1.00% + EDTA	5.26 ± 0.08^{Ab}	6.70 ± 0.59^{CDa}	1.55 ± 0.53^{Cc}
LS 5.00% + EDTA	5.44 ± 0.01^{Aa}	6.15 ± 0.52^{Da}	0.00 ± 0.00^{Db}
LS 10.00% + EDTA	5.48 ± 0.35^{Aa}	4.49 ± 0.20^{Eb}	0.00 ± 0.00^{Dc}
EDTA	5.61 ± 0.28^{Aa}	6.65 ± 0.41^{CDa}	1.84 ± 0.53^{Cb}

PA: CFS of *Pediococcus acidilactici*; LS: CFS of *Latilactobacillus sakei/Staphylococcus xylosus*.
Different superscripts in the same column ($^{A-E}$) and same row ($^{a-c}$) indicate statistical differences ($p < 0.05$).

Table 3. Changes in the pH of tryptic soy broth tubes inoculated with *Escherichia coli* O157:H7 and different concentrations of cell-free supernatants (CFSs) with or without 0.02 M ethylenediaminetetraacetic acid (EDTA) during incubation at 35.00 ± 1.00 ˚C for 24 hr (mean ± standard deviation).

Groups	0 hr	6 hr	24 hr
Control	7.32 ± 0.14^{Aa}	5.93 ± 0.04^{Cb}	6.50 ± 0.21^{Ab}
PA 1.00%	7.20 ± 0.01^{Aa}	5.91 ± 0.06^{Cb}	6.35 ± 0.19^{Ab}
PA 5.00%	7.25 ± 0.14^{Aa}	5.94 ± 0.04^{Cb}	6.47 ± 0.30^{Ab}
PA 10.00%	7.16 ± 0.11^{ABa}	5.95 ± 0.04^{Cb}	6.33 ± 0.16^{Ab}
LS 1.00%	7.18 ± 0.01^{ABa}	5.93 ± 0.02^{Cb}	6.39 ± 0.40^{Ab}
LS 5.00%	7.02 ± 0.01^{Ba}	5.88 ± 0.01^{Cb}	5.71 ± 0.03^{Bc}
LS 10.00%	6.82 ± 0.01^{Ca}	5.78 ± 0.01^{Db}	5.24 ± 0.23^{Cc}
PA 1.00% + EDTA	6.28 ± 0.04^{DEa}	6.12 ± 0.02^{ABb}	5.69 ± 0.04^{Bc}
PA 5.00% + EDTA	6.25 ± 0.01^{DEa}	6.14 ± 0.04^{ABa}	5.57 ± 0.09^{BCb}
PA 10.00% + EDTA	6.15 ± 0.03^{EFa}	6.05 ± 0.01^{Bb}	5.70 ± 0.01^{Bc}
LS 1.00% + EDTA	6.29 ± 0.12^{DEa}	6.14 ± 0.09^{ABa}	5.59 ± 0.04^{BCb}
LS 5.00% + EDTA	6.16 ± 0.02^{EFa}	6.08 ± 0.02^{Bb}	5.72 ± 0.03^{Bc}
LS 10.00% + EDTA	6.01 ± 0.01^{Fa}	5.94 ± 0.07^{Ca}	5.83 ± 0.09^{Ba}
EDTA	6.38 ± 0.02^{Da}	6.19 ± 0.01^{Ab}	5.62 ± 0.02^{BCc}

PA: CFS of *Pediococcus acidilactici*; LS: CFS of *Latilactobacillus sakei/Staphylococcus xylosus*.
Different superscripts in the same column ($^{A-F}$) and same row ($^{a-c}$) indicate statistical differences ($p < 0.05$).

Table 4. Changes in the titratable acidity (% lactic acid equivalent) of tryptic soy broth tubes inoculated with *Escherichia coli* O157:H7 and different concentrations of cell-free supernatant (CFS) with or without 0.02 M ethylenediaminetetraacetic acid (EDTA) during incubation at 35.00 ± 1.00 ˚C for 24 hr (mean ± standard deviation).

Groups	0 hr	6 hr	24 hr
Control	0.16 ± 0.03^{Db}	0.26 ± 0.03^{Aa}	0.25 ± 0.01^{Da}
PA 1.00%	0.16 ± 0.03^{Da}	0.25 ± 0.03^{Aa}	0.21 ± 0.04^{Da}
PA 5.00%	0.19 ± 0.01^{CDa}	0.27 ± 0.01^{Aa}	0.20 ± 0.03^{Da}
PA 10.00%	0.24 ± 0.04^{Ca}	0.25 ± 0.02^{Aa}	0.22 ± 0.04^{Da}
LS 1.00%	0.18 ± 0.01^{CDa}	0.25 ± 0.04^{Aa}	0.23 ± 0.01^{Da}
LS 5.00%	0.19 ± 0.01^{CDb}	0.29 ± 0.05^{Ab}	0.33 ± 0.02^{Ca}
LS 10.00%	0.18 ± 0.03^{CDc}	0.27 ± 0.02^{Ab}	0.36 ± 0.01^{BCa}
PA 1.00% + EDTA	0.37 ± 0.01^{ABab}	0.29 ± 0.06^{Ab}	0.43 ± 0.03^{ABa}
PA 5.00% + EDTA	0.35 ± 0.05^{ABa}	0.31 ± 0.06^{Aa}	0.42 ± 0.05^{ABa}
PA 10.00% + EDTA	0.37 ± 0.05^{ABb}	0.34 ± 0.03^{Ab}	0.43 ± 0.01^{Aa}
LS 1.00% + EDTA	0.32 ± 0.01^{Bb}	0.33 ± 0.01^{Ab}	0.40 ± 0.02^{ABa}
LS 5.00% + EDTA	0.33 ± 0.01^{Bab}	0.31 ± 0.02^{Ab}	0.41 ± 0.03^{ABa}
LS 10.00% + EDTA	0.41 ± 0.04^{Aa}	0.32 ± 0.06^{Aa}	0.42 ± 0.03^{ABa}
EDTA	0.33 ± 0.02^{Bb}	0.31 ± 0.01^{Ab}	0.41 ± 0.00^{ABa}

PA: CFS of *Pediococcus acidilactici*; LS: CFS of *Latilactobacillus sakei/Staphylococcus xylosus*.
Different superscripts in the same column ($^{A-D}$) and same row ($^{a-c}$) indicate statistical differences ($p < 0.05$).

Following the 6th hr, a notable decline was observed in the groups containing 5.00 and 10.00% CFSs, as well as their combinations with EDTA ($p < 0.05$). In contrast to the pH values, the titratable acidity values exhibited a notable increase during the incubation period in the majority of groups ($p < 0.05$). Nevertheless, slight increases were noted in the groups containing 5.00 and 10.00% CFSs and EDTA compared to the other groups.

Discussion

The LAB is able to produce organic acids *via* anaerobic glycolysis during fermentation. The organic acids are one of the primary metabolites of the CFSs.[13] The LAB is divided into two groups based on their ability to produce final products, including homo-fermentative and hetero-fermentative strains. Homo-fermentative LAB mainly produce lactic acid, while hetero-fermentative can produce organic acids, gas, and other organic compounds, such as ethanol.[24,25] In the current research, the used LAB strains belonged to the homo-fermentative group, and produced mainly lactic acid *via* Embden-Meyerhof-Parnas pathway.[26-28] Besides, it is well known that coagulase-negative strains of *Staphylococci*, including *S. xylosus*, are often used in combination with LAB strains as starter cultures in the production of meat and meat products. Furthermore, the capacity of *S. xylosus* to produce bacteriocins and organic acids, including lactic acid, has been well-documented.[29] The amount of lactic acid caused a remarkable decline in the pH value and an elevation in the titratable acidity values of the CFSs in this study. The observed anti-microbial activity against *E. coli* O157:H7 strains is thought to be due to the pH and acidity.

The diameters of inhibition zone caused by the tested CFSs against *E. coli* O157:H7 strains ranged from 9.42 to 12.35 mm. In our previously published research, CFS of PA after growth in de Mann, Rogosa, and Sharpe (MRS) broth showed inhibition zones between 8.50 and 8.79 mm against the same *E. coli* O157:H7 strains.[23] These results show that the medium has a major influence on the composition of CFS. In line with this finding, Md Sidek *et al.* reported that PA produced higher bacteriocin-like inhibitory substances when grown in TSB compared to the MRS broth.[30] This information may explain the larger inhibition zones observed against *E. coli* O157:H7 strains in the current research. However, it should be noted that the CFS tested in this research had a higher pH and lower acidity levels compared to our previous research.[23] Also, significant differences in pH and titratable acidity values were observed between PA and LS. This finding suggests that using multiple species (LS) seems more effective than using a single species (PA). This is supported by the higher inhibition zones observed in LS compared to the PA. On the other hand, the literature review regarding the anti-microbial activity of CFS against *E. coli* strains revealed quite different results. In this context, the CFS (100µL of 100%) of PA (NRRL B-1116) produced using MRS broth exhibited no inhibition against *E. coli* ATCC 25922.[31] A similar observation was also reported in a research conducted by González-Pérez *et al.*, outlined that no inhibition zones were recorded against *E. coli* O157:H7 K3999 with 50.00 µL of *Pediococcus pentosaceus* CFS.[32] In another study, inhibition zones of the CFS (80.00 µL) of *P. pentosaceus* (strain KMP) were ranged between 18.50 to 26.50 mm against ten different *E. coli* strains.[33] Reportedly, that inhibition zones (27.00 - 32.00 mm) were obtained by applying CFS of PA against *E. coli*.[34] In this study, a 20.00 µL of CFS obtained from LS exhibited 9.42 to 12.35 mm zone diameters. However, no inhibitory activity against *E. coli* was reported by applying CFS of *S. xylosus*.[29] Moreover, CFS of *L. sakei* exhibited approximately 21.00 mm inhibition zone against *E. coli* ATCC 25922.[31] The aforementioned studies may suggest that the anti-microbial properties of CFSs vary by the LAB strain, growth medium, and target bacterial species.

The differences between the CFSs in terms of inhibition zones may be attributed to the variations in the amount of lactic acid and pH levels. Also, differences in susceptibility to acid resistance among the *E. coli* O157:H7 strains tested in this research may have contributed to these differences. It is known that mechanisms, such as the glutamate decarboxylase system, being encoded by the *gadA* and *gadB* genes, are involved in the acid adaptation of *E. coli* O157:H7.[35] In line with that, Wi *et al.* outlined that the expression of *GadAB* in *E. coli* O157:H7 ATCC 43894 strain was significantly suppressed, leading to a decrease in acid resistance compared to the other strains.[35] Our findings are in agreement with this data regarding the variation in inhibition zones among *E. coli* O157:H7 strains.

The result of the time-kill assay revealed that in the absence of EDTA, CFSs at all concentrations resulted in approximately 1.00 $\log_{10}$ decrease in the number of *E. coli* O157:H7 after 24 hr incubation. However, studies that have examined the effect of CFSs of different LAB on the number of pathogenic bacteria have reported results with a wide range of variation. In a study, Divyashree *et al.* reported a 7.00 $\log_{10}$ reduction in *Salmonella paratyphi* count after 18 hr of treatment with 15.00 mL of CFS (100%) from *Lactobacillus plantarum*.[36] Accordingly, it was reported that the CFSs of different *Lactobacillus* strains produced using MRS broth exhibited 7.00 $\log_{10}$ decrease in *Salmonella typhimurium* ATCC 14028 count.[37] However, according to Arena *et al.*, CFSs from *L. plantarum* strains at a concentration of 25.00% resulted in a reduction of approximately 2.00 $\log_{10}$ in the *Salmonella enteritidis* CECT 409 count.[38] The literature summarized above shows that the anti-bacterial properties of CFSs are greatly strain-dependent. It was found that CFSs were less effective in the absence of EDTA. While, the concentrations of 5.00 and 10.00% of CFSs combination with ETDA

completely inhibited *E. coli* O157:H7 by 24 hr. It has been established that some of the metabolites present in the CFSs, particularly bacteriocins or bacteriocin-like substances, do not exhibit anti-bacterial effect against Gram-negative species.[23] Therefore, to extend the anti-bacterial spectrum of the CFSs against these strains, a chelating agent, like EDTA, is required.[39] It has been documented that EDTA alters the permeability of the cell wall by interacting with cations, such as calcium and magnesium, that stabilize the cell wall, thus allowing macromolecules to enter the cell.[40] Consistent with this information, the results of this research show that the anti-bacterial effect of CFSs was significantly improved by combining them with EDTA. Similar results were also found in our previous study on *S. typhimurium* using the CFSs of the same LAB strains.[16] Other authors have also observed that EDTA and bacteriocin combination provides greater anti-microbial property than either alone.[9-11]

As expected, a remarkable decline and elevation in the pH and titratable acidity values, respectively, were observed in the groups during the 24 hr incubation period. In the control group, *E. coli* O157:H7 grew rapidly in the first 6 hr and then, no significant change was observed in the numbers of the pathogen between 6 and 24 hr. In this regard, acidity increased rapidly in the first 6 hr and then, decreased slightly, possibly due to the continuing metabolic activities of the bacterial cells. Regardless of the concentration, in the CFS-treated groups, a considerable increase in *E. coli* O157:H7 count occurred in the first 6 hr. Thereafter, the bacterial count decreased rapidly, particularly in the groups combined with EDTA. After the 6th hr, titratable acidity increased and pH decreased in the 5.00 and 10.00% CFSs combined with EDTA groups. The observed changes in these groups are believed to be caused by the lysis of bacterial cells resulting from the potent anti-bacterial effect of the combination of CFSs and EDTA. This may lead to the release of acidic components into the medium, including short-chain fatty acids in the cell wall and organic acids entering the cell.

In conclusion, the CFSs obtained from the PA and LS exhibited different zones of inhibition against the *E. coli* O157:H7 strains. This result suggests that the anti-microbial effects of the CFSs are strain-dependent. Moreover, the presence of EDTA has been shown to enhance the anti-microbial effect of the CFS against Gram-negative bacteria. Result of this study revealed that the combination of the CFSs obtained from PA and LS with EDTA provided significant anti-bacterial effect against *E. coli* O157:H7, and use of this combination can be considered to enhance the microbiological quality of food.

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Conflict of interest

Authors declare no conflict of interest in this research.

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