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Original Article

Antimicrobial activity of cell-free supernatant of lactic acid bacteria on spoilage bacteria of vacuum-packed sliced emulsion-type sausages

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Abstract

Background: Spoilage is very common in vacuum-packed sliced emulsion-type sausages during refrigerated storage. **Aims:** This study aimed to investigate the inhibitory effects of cell-free supernatant (CFS) of lactic acid bacteria (LAB) on the spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages as biological preservatives. **Methods:** These products from various companies were examined to find the spoilage bacteria. A total of 43 LABs were screened for inhibitory activity against the spoilage bacteria. The MIC_{90} of protective bacteria and the inhibitory effect of different components obtained from these bacteria was investigated. **Results:** Four LAB were confirmed as the predominant spoilage bacteria, including *Enterococcus mundtii*, *Latilactobacillus sakei*, *Latilactobacillus curvatus*, and *Weissella viridescens*. Six strains, including *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus helveticus* PTCC 1332, *Lactiplantibacillus plantarum* CEC 17484, *Lactiplantibacillus plantarum* LL441, *Lacticaseibacillus rhamnosus* ATCC 53103, and *Pediococcus acidilactici* DSM 20284 were able to produce proteinaceous antimicrobial metabolites against the four spoilage agents, which were selected as protective bacteria. CFS of the protective bacteria inhibited four spoilage bacteria by more than 88%. The MIC_{90} of all protective bacteria was less than 10 mg/ml against *E. mundtii* and *L. sakei*. After neutralizing acid and H_2O_2 of the CFS of *P. acidilactici*, it was still quite effective against *E. mundtii* and *L. sakei*. The sausage's pasteurization temperature did not affect the bacteria's active metabolites. **Conclusion:** The substances derived from these bacteria can be applied as biopreservatives in sliced sausage, even in the pre-pasteurization stage of these products.

Key words: Biopreservatives, CFS, LAB, Meat products, Protective culture

Introduction

Spoilage, ropy slime, and gas production are very common in vacuum-packed sliced emulsion-type sausages. Slime does not have a specific smell and often forms before the expiration date of the product and has an unpleasant appearance to the consumers. This issue may restrict the product's ability to be distributed and cause substantial financial loss for the manufacturers (Aymerich *et al*., 2002). According to several studies, cooked sausages become recontaminated during processing stages (after cooking) such as slicing and packing (Davies *et al*., 1999). Lactic acid bacteria (LAB) are considered major spoilage organisms on various types of vacuum-packed cooked sausages because they are generally resistant to several food preservation methods, such as microaerophilic conditions, the presence of curing salts and nitrite, and low storage temperatures (Dušková *et al*., 2013; Kalschne *et al*., 2015; Abhari *et al*., 2018). The bacteria mostly isolated from spoiled cooked cured meats are *Latilactobacillus sakei*, *Latilactobacillus curvatus*, and *Leuconostoc mesenteroides* (Iulietto *et al*., 2015; Kalschne *et al*., 2015; Khorsandi *et al*., 2019).

The growing concern of consumers regarding the safety and quality of food, as well as increasing dissatisfaction with the use of chemical food preservatives, has increased attention to biopreservatives. In biopreservation, by using natural or controlled microflora, mostly LAB, and/or their antimicrobial metabolites like lactic acid, bacteriocins, and others, shelf life and/or the safety of food products are enhanced (Devlieghere *et al*., 2004). The inhibitory effect of LAB against other microorganisms is based on the competition for nutrients and the production of antimicrobial metabolites such as organic acids, hydrogen peroxide, antimicrobial enzymes, low molecular weight bioactive compounds, and proteinaceous substances like

bacteriocins that inhibit the growth of a wide variety of pathogenic and spoilage bacteria and improve the safety of food (Ouwehand and Vesterlund, 2004). Cell-free supernatant (CFS) could act as appropriate antimicrobial agents since they contain a wide variety of antimicrobials (Beristain-Bauza *et al*., 2016; Drumond *et al*., 2023). Numerous studies have been conducted on the use of LABs and their metabolites in the inhibition of pathogenic bacteria like *Salmonella* spp., *Campylobacter* spp., *Clostridium botulinum*, and *Listeria monocytogenes* in a variety of meat and sausage products (Turgis *et al*., 2012; Chaillou *et al*., 2014; Rivas *et al*., 2014; Dokka *et al*., 2018; Bungenstock *et al*., 2021). There are a few studies on the use of LAB culture in the inhibition of the predominant spoilage bacteria, such as *Brochothrix thermosphacta* and LAB, in different types of sausages (Hu *et al*., 2008; Comi *et al*., 2016).

This study aimed to identify effective LABs and investigate the inhibitory effects of their CFS on the spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages as biological preservatives, as well as evaluate the potential of CFS for pre-pasteurization application in these products.

Materials and Methods

Isolation of spoilage bacteria of vacuum-packed sliced emulsion-type sausages

Ten packages of vacuum-packed sliced emulsion-type sausages produced by various local factories and weighing about 300-400 g were purchased from local stores. The collected samples contained different amounts of beef/chicken, potato starch, soy isolate protein, sodium chloride, sodium ascorbate, sodium nitrite (120 ppm), sucrose, water, and flavoring agents. The sausages were stored until signs of deterioration, such as package swelling, dripping, and discoloration, appeared. Each pack of sausages was ground under sterile conditions. 10 g of samples were then suspended in 90 ml of sterile normal saline (0.9% w/v) and homogenized for 2 min in a stomacher (Dl18B, Germany). Then, serial dilution was performed. The appropriately diluted samples were surface cultured on de Man, Rogosa, and Sharpe (MRS) agar (Merck, Darmstadt, Germany) in duplicate, and incubated at 37°C for 48 h under anaerobic conditions using Anaerocult A gas packs (Merck, Darmstadt, Germany) (Khorsandi *et al*., 2019). In each plate, dominant colonies were sub-cultured on MRS agar medium for purification, and they were identified based on biochemical and molecular characteristics.

Characterization and identification of spoilage bacteria

The purified colonies were identified based on morphological characteristics and Gram staining. On colonies, oxidase and catalase tests, and carbohydrate fermentation patterns were determined using the methods described by Islam *et al*. (2016). According to the results of morphological and biochemical tests, 13 isolates were suspected to be LAB; *16S rRNA* sequencing was performed to accurately identify the isolates. Their DNA was extracted based on the classic heat-thaw method (Salehi *et al*., 2005). The amplification of *16S rRNA* was performed according to Frank *et al*. (2008) with some modifications. The PCR was conducted with universal primers, 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 1492R (5´-GGT TAC CTT GTT ACG ACT T-3´), which span nearly the full length of the *16S rRNA* gene, with an expected amplicon of ~ 1500 bp.

The PCR reactions were carried out at a final volume of 25 µL in an Eppendorf Master Cycler Gradient PCR system (Eppendorf, Germany). The reaction mixture contained 12.5 µL of master mix (Ampliqon, Denmark), 1 µL of each primer, 2 µL of DNA sample, and 8.5 µL of nuclease-free water. The reactions were performed on a thermal cycler PCR (TC-XP-G, China) under the following conditions: initial denaturation for 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, with a final extension step at 72°C for 15 min. Aliquots of 5 µL of each reaction were analyzed on a 1% (w/v) agarose gel in TBE buffer (Frank *et al*., 2008) and visualized under a UV transilluminator. The bacterial *16S rRNA* band was excised from the gel and purified for subsequent sequencing using the GeneJET Gel Extraction Kit (Thermo Scientific, Lithuania), following the manufacturer's recommendations. The purified bands were sequenced at the Microsynth Company (Switzerland). Approximate taxonomic identification of the sequences obtained from the *16S rRNA* of the spoilage bacteria isolates was achieved through the BLASTn server (http://www.ncbi.nlm.nih.gov/BLAST/) (Dos Santos *et al*., 2019).

According to the results of sequencing, *Entrococcus mundtii*, *Latilactobacillus sakei*, *Latilactobacillus curvatus*, and *Weissella viridescens* were identified as specific spoilage bacteria in vacuum-packed sliced emulsion-type sausages. These bacteria were sub-cultured in MRS broth, and after 24 h of incubation, the bacterial cells were stored in the presence of 25% glycerol at -80°C. These four LABs were used as target bacteria in further steps.

Investigating the inhibitory activity of treated CFS of LAB against the spoilage bacteria

To find suitable bacteria that can produce inhibitory compounds (in addition to producing acid and hydrogen peroxide) against spoilage bacteria, in the first step, 43 strains of LAB from different sources were cultured and their supernatant was neutralized by adding NaOH and catalase enzyme as follows.

The single pure colony of each strain was cultured in MRS broth and incubated anaerobically at 37°C for 48 h. To prepare the CFS, cells were removed by centrifugation at 10,000 × g for 10 min at 4°C (Hettich Zentrifugen D-78532 Tuttlingen, Germany) (Zhang *et al*., 2018) and the supernatant was filter-sterilized through 0.22 µm membrane filters (Millipore, United States). Neutralizing acid and H_2O_2 of CFSs were

performed according to Zhang *et al*. (2018) with some modifications. The pH of the CFSs was adjusted to 7.0 using a 4 M NaOH and pH meter (SCHOTT, CG824, Nederland). To remove hydrogen peroxide, neutralized CFSs were treated with 0.1 mg/ml bovine catalase (Sigma-Aldrich Corporation, USA) at 25°C for 2 h. The catalase was then inactivated by boiling treated CFSs for 10 s, and finally sterilized by a filter.

In the second step, the antimicrobial activity of the treated CFSs was determined using microplate assay (Lim, 2016). For this purpose, 80 µL of MRS broth double strength, 100 µL of treated CFS of the inhibitor strains, and 20 µL suspension (1.0×10^6 CFU/ml) of the spoilage organism (*E. mundtii*, *L. sakei*, *L. curvatus*, and *W. viridescens*) were poured on each well. A negative control containing MRS broth, a positive control containing MRS broth and each spoilage bacteria, and a CFS control containing each CFS and MRS broth were also considered. After that, the microplate was incubated at 37°C for 24 h under anaerobic conditions using Anaerocult A gas packs. The optical density (OD) at 600 nm was measured using a microplate reader (BioTek's Power Wave XS, USA). Each experiment was performed three times. For the evaluation of the inhibition of treated CFS, the percentage of growth at each strain's CFS was calculated using the following equation: (Jalaei *et al*., 2014)

$$\%\,\text{Inhibition} = 100 - \left[\frac{\text{OD of sample} - \text{OD of negative control}}{\text{OD of the positive control} - \text{OD of nagative control}} \times 100\right]$$

Finally, six strains that had the most inhibitory effect on four spoilage bacteria were selected as protective cultures and subjected to further investigation.

Determination of minimum inhibitory concentration (MIC) of crude CFS of the protective bacteria against spoilage bacteria

The concentration of the crude CFS of each bacteria (mg/ml) was calculated by measuring their dry matter percentage. The MIC of CFS for protective bacteria was determined using the conventional broth microdilution technique (Lambert and Pearson, 2000). Therefore, 100 µL of double-strength MRS broth was poured into each well, and then 100 µL of the CFS was poured into the first well. After pipetting, 100 µL of suspension was transferred from the first well to the second well. It was thus diluted to 5 wells in this manner. The final volume of each well reached 100 µL. Finally, 10 µL of the spoilage bacterial suspension (10^6 CFU/ml) was added to each well. A negative control containing MRS broth, a positive control containing MRS broth and each spoilage bacteria, and a CFS control containing each CFS and MRS broth were also considered. The microplates were incubated in anaerobic conditions at 37°C for 24 h. The percent of the inhibitory effect of each concentration of CFSs was determined as described above. Based on the inhibition percentage of different concentrations, the equation line was obtained and MIC_{90} (the lowest CFS concentration that inhibited 90% of bacterial growth) was calculated. Each assay was carried out in triplicate.

Investigating the inhibitory effect of different components of the CFS of protective bacteria

To investigate the inhibitory effect of the complete CFS of each selected protective bacteria and the stability of the CFS at sausage pasteurization temperature for the possibility of adding these protective CFSs to the sausage before the pasteurization process, their CFSs were tested. Further tests were performed on CFS to investigate the inhibitory effects of various components including acids, H_2O_2, proteinaceous compounds sensitive to trypsin, pepsin, and the sausage pasteurization process (Zhang *et al*., 2018). The method of performing each step is described in Table 1.

Each treated sample was sterilized through a 0.22 µm membrane filter before examination. The antibacterial activity of each treated CFS against the spoilage bacteria was assessed by the microplate method as described above.

Table 1: Seven-step treatments performed on the cell-free supernatant (CFS) of protective bacteria

Step	Goal of investigation	Type of samples	Type of CFS treatment
1	Effect of the complete CFS	CFS	-
2	Sensitivity of CFS to pasteurization temperature	CFS	Heating at 85°C for 10 min
3	Effect of acids	CFS	Adjust to pH 7.0 using 4 M NaOH and the pH meter
4	Effect of H_2O_2	Sample of step 3	Addition of 0.1 mg/ml bovine catalase (3000 U/mg); Incubation at 25°C for 2 h; Inactivation of the enzyme by boiling for 10 s
5	Sensitivity of proteinaceous compounds to trypsin	Sample of step 4	Addition of 5 mg/ml trypsin (264 U/mg); Incubation at 37°C for 2 h; Inactivation of the enzyme by boiling for 3 min
6	Sensitivity of proteinaceous compounds to pepsin	Sample of step 4	Adjust to pH 2.0 using 4 M HCl; Addition of 10 mg/ml pepsin (19 U/mg); Incubation at 37°C for 2 h; Adjust to pH 7.0 using 4 M NaOH; Inactivation of enzyme by boiling for 3 min
7	Sensitivity of proteinaceous compounds to heat	Sample of step 4	Heating at 85°C for 10 min

Statistical analysis

All experiments were done in triplicate. The analysis of variance (ANOVA) and Duncan's multiple range tests were performed by the statistical package for SPSS (version 16, SPSS Inc., Chicago, IL). A significance level of less than 0.05 was considered in the analysis.

Results

Isolation, characterization, and identification of spoilage bacteria

The vacuum-packed sliced emulsion-type sausages from various companies were examined to find the predominant spoilage bacteria. According to the results of morphological and biochemical tests, 13 isolates were suspected to be LAB. Finally, four bacteria were confirmed as LAB using *16S rRNA* sequencing (Table 2; Fig. 1). The spoilage bacteria were identified as *Enterococcus mundtii*, *Latilactobacillus sakei* strain, *Latilactobacillus curvatus*, and *Weissella viridescens*. The sequence of *Enterococcus mundtii* and *Weissella viridescens* were deposited in NCBI with the accession numbers of OR286473 and OR288169, respectively. Table 2 shows the results of biochemical tests of them.

Investigation of the inhibitory activity and selection of protective bacteria

A total of 43 LABs were tested for inhibitory activity against four spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages. Their results are shown in Table 3.

Out of the 43 tested strains, six that had the most inhibitory effect based on their ability to produce antimicrobial substances (in addition to producing acid and hydrogen peroxide) on four spoilage bacteria were selected for further investigation. As a result, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus helveticus* PTCC 1332, *Lactiplantibacillus plantarum* CEC 17484, *Lactiplantibacillus plantarum* LL441, *Lacticaseibacillus rhamnosus* GG ATCC 53103, and *Pediococcus acidilactici* DSM 20284 were selected as protective bacteria for further study (Table 3).

Determination of MIC_{90} of CFS of protective bacteria against sausage spoilage bacteria

The results of the MIC_{90} of each CFS of protective bacteria are shown in Table 4. *E. mundtii* and *L. sakei* are inhibited by 90% when exposed to a concentration of less than 10 mg/ml of all CFS of protective bacteria. The MIC of CFS of protective bacteria varied from 10.49 to 35.16 mg/ml, 3.00-7.90 mg/ml, and 3.14-8.71 mg/ml against *L. curvatus*, *L. sakei*, and *E. mundtii*, respectively. *W. viridescens* is inhibited by CFS of protective bacteria in the concentration range of 34.92 to 41.85 mg/ml. *L. rhamnosus* affected three spoilage bacteria, including *E. mundtii*, *L. sakei*, and *L. curvatus*, at the lowest concentration (3.1, 3.0, and 10.5 mg/ml, respectively).

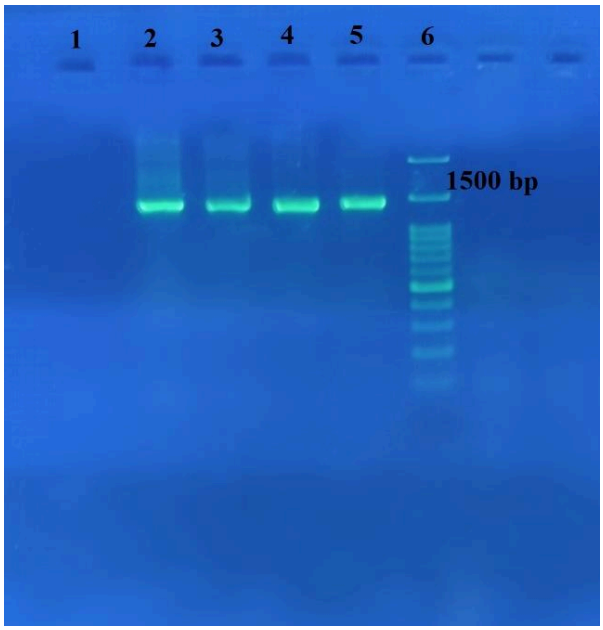

Fig. 1: Gel picture of the *16S rRNA* gene amplified with primers 27F and 1492R on extracted DNA from four spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages. Lane 1: Negative control, Lane 2: *L. sakei*, Lane 3: *L. curvatus*, Lane 4: *W. viridescens*, Lane 5: *E. mundtii*, and Lane 6: 100 bp DNA ladder

Table 2: Characteristics and identification of four spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages

Tests	Suspected isolates			
	1	2	3	4
Gram staining	+	+	+	+
Oxidase test	-	-	-	-
Catalase test	-	-	-	-
Cell morphology	Cocci	Rod	Rod	Rod
Fermentation of Dextrose	+	+	+	+
Fermentation of Sucrose	+	+	+	+
Fermentation of Raffinose	-	-	-	-
Fermentation of Lactose	+	+	+	-
Fermentation of Galactose	+	+	+	-
Fermentation of Ribose	+	+	+	-
Fermentation of Fructose	+	+	+	+
Fermentation of Mannose	+	+	-	+
Strain detection based on *16S rRNA*	*E. mundtii*	*L. sakei*	*L. curvatus*	*W. viridescens*

Table 3: Percentage of inhibition of cell free supernatant (CFS) of 43 lactic acid bacteria (without acid and H_2O_2) against four spoilage bacteria of vacuum-packed sliced emulsion-type sausages

No.	Strain	Strain number	Spoilage bacteria			
			E. mundtii	*L. sakei*	*L. curvatus*	*W. viridescens*
1	*Carnobacterium divergens*	NCDO 2739 240998	30.4	15.4	0.0	0.0
2	*Companilactobacillus kimchii*	LMG 19822	0.0	0.0	0.0	19.0
3	*Enterococcus faecium*	CTC 492 05C03	0.0	0.0	0.0	18.6
4	*Enterococcus faecium*	Wild type	0.0	0.0	0.0	5.01
5	*Lacticaseibacillus casei*	ATCC 393	0.0	12.2	20.8	5.8
6	*Lacticaseibacillus casei*	NCDO151	0.0	0.0	3.9	13.1
7	*Lacticaseibacillus casei*	ATCC 334	0.0	0.0	0.0	8.0
8	*Lacticaseibacillus casei*	Shirota	0.9	2.7	7.3	5.7
9	*Lacticaseibacillus rhamnosus* GG	ATCC 53103	0.0	11.0	12.8	12.1
10	*Lactiplantibacillus plantarum*	arizonpsis 13273	0.0	0.0	0.0	47.2
11	*Lactiplantibacillus plantarum*	NCDO 1193	0.0	8.3	0.0	29.6
12	*Lactiplantibacillus plantarum*	PTCC 1058	0.0	0.0	16.9	16.0
13	*Lactiplantibacillus plantarum*	LP 299	0.0	0.0	15.8	13.4
14	*Lactiplantibacillus plantarum*	CEC 17484	23.3	25.2	18.1	11.3
15	*Lactiplantibacillus plantarum*	LL441 E01 B56	17.3	20.3	30.0	10.8
16	*Lactobacillus acidophilus*	ATCC 4356	15.7	25.2	16.5	10.0
17	*Lactobacillus alimentarius*	LMG 4187	0.0	0.0	0.0	41.2
18	*Lactobacillus brevis*	L40 E05C27	0.0	0.0	0.0	5.0
19	*Lactobacillus coryniformis*	DSM 20004	0.0	0.0	19.7	49.8
20	*Latilactobacillus curvatus*	LMG 9198	0.0	0.0	0.0	39.7
21	*Lactobacillus delbrueckii*	PTCC 1743	0.0	5.6	13.0	8.7
22	*Lactobacillus gasseri*	Wild type	0.0	0.9	22.9	15.0
23	*Lactobacillus gasseri*	OMNI	0.0	0.0	16.1	16.8
24	*Lactobacillus helveticus*	PTCC 1332	14.2	20.8	18.6	11.1
25	*Lactobacillus helveticus*	Wild type	0.0	6.0	1.4	3.3
26	*Lactobacillus iners*	DSM 13335	0.0	0.0	0.0	5.6
27	*Lactobacillus intestinalis*	DSM 6629	0.0	15.4	12.5	0.0
28	*Lactobacillus kefiranofaciens*	ATCC 43761	0.0	14.2	0.0	12.1
29	*Lactobacillus mali*	DSM 20444	0.0	19.0	20.1	2.3
30	*Lactobacillus paralimentarius*	DSM 13138	0.0	0.0	0.0	21.9
31	*Lactococcus lactic*	662 05D36	42.2	27.9	0.0	0.0
32	*Latilactobacillus sakei*	LTH 673 05C06	40.8	41.7	0.0	0.0
33	*Latilactobacillus sakei*	LB 706 05C07	0.0	0.0	4.9	10.9
34	*Leuconostoc mesenteroides*	DB1275	0.0	0.0	0.0	4.94
35	*Ligilactobacillus animalis*	DSM 20602	0.0	0.0	0.0	41.7
36	*Ligilactobacillus salivarius*	NCFB 2747	0.0	0.0	0.0	1.0
37	*Limosilactobacillus colehominis*	DSM 14060	0.0	0.0	0.0	100
38	*Limosilactobacillus ruteri*	Wild type	0.0	0.0	0.0	0.0
39	*Limosilactobacillus ruteri*	DSM 20016	0.0	0.0	14.7	8.3
40	*Limosilactobacillus ruteri*	DSM 47391	4.1	6.7	12.3	16.8
41	*Limosilactobacillus ruteri*	DSM 11939	0.0	0.0	17.6	14.6
42	*Pediococcus acidilactici*	DSM 20284	99.7	99.5	10.4	9.3
43	*Pediococcus pentosaceus*	FBB 61 05C79	0.0	0.0	0.0	8.6

The data presented are the result of duplicate experiments

Table 4: MIC_{90} (mg/ml) of each cell-free supernatant (CFS) of the protective bacteria against four spoilage bacteria of vacuum-packed sliced emulsion-type sausages

CFS of protective bacteria	Initial pH of the CFS	Spoilage bacteria			
		E. mundtii	*L. sakei*	*L. curvatus*	*W. viridescens*
L. acidophilus ATCC 4356	3.7 ± 0.1	7.74	6.96	17.08	34.92
L. helveticus PTCC 1332	3.7 ± 0.0	7.28	5.74	17.48	35.19
L. plantarum CEC 17484	3.7 ± 0.1	3.62	7.90	19.74	39.11
L. plantarum LL441	3.7 ± 0.2	5.52	6.10	19.17	41.85
L. rhamnosus GG ATCC 53103	3.7 ± 0.1	3.14	3.00	10.49	39.46
P. acidilactici DSM 20284	4.0 ± 0.2	8.71	4.98	35.16	38.16

The data presented are means of triplicate experiments

Antibacterial activity of different components in the supernatant of protective bacteria

Figure 2 shows the percentages of CFS and treated CFS that inhibit spoilage bacteria. All crude CFS strongly inhibited spoilage bacteria (more than 88.0%), except *P. acidilactici* metabolites, which did not

significantly inhibit *W. viridescens* (56.9%). All CFS of protective bacteria were stable at sausage pasteurization temperature (85°C for 10 min).

When the organic acids of CFSs were neutralized, their antibacterial effect was significantly reduced to about 6.5 to 45% (P<0.05). After acid neutralization, the CFS of *L. rhamnosus* completely lost its inhibitory effect on *E. mundtii* (P<0.05), so it has been completely ineffective against this spoilage agent in subsequent treatments. On the other hand, the antimicrobial activity of CFS from *P. acidilactici* against *L. sakei* and *E. mundtii* was retained after eliminating the effects of organic acid. *L. plantarum* CEC 17484 retained half of its inhibitory power against *E. mundtii* after acid neutralization (45.0%) (P<0.05).

After removing hydrogen peroxide from neutral CFS, its inhibitory effect compared to neutral CFS in some cases decreased slightly (such as the effect of *L. plantarum* LL441 against *E. mundtii*); the *L. plantarum* CEC 17484's performance against *E. mundtii* decreased in half (P<0.05). After H_2O_2 elimination, the effectiveness of *L. helveticus* and both *L. plantarum* against *L. sakei* significantly increased (P<0.05), and in other cases, it remained relatively stable. The CFS derived from *P. acidilactici* completely inhibited *L. sakei* and *E. mundtii* after eliminating the effects of organic acid and H_2O_2.

After neutralizing acid and H_2O_2, *P. acidilactici* is still quite effective against *E. mundtii* and *L. sakei* (P<0.05). The antibacterial action of CFSs without acid and H_2O_2 against *L. sakei*, *L. curvatus*, and *E. mundtii* after treatment with pepsin and trypsin was completely lost (P<0.05), demonstrating the proteinaceous nature of the active substance. All of the bacteria's active metabolites against *W. viridescens* were resistant to pepsin and trypsin enzymes (P>0.05). *L. helveticus* showed a slight inhibitory effect on *L. sakei* after treatment with trypsin (8%).

The results of heat-treated samples showed that the antimicrobial substances besides acid and H_2O_2 of each protective bacterium (except *L. plantarum* LL441) against *L. curvatus* and *W. viridescens* had good stability at 85°C for 10 min (sausage pasteurization temperatures), while the activities of all bacterial metabolites besides acid and H_2O_2 against *E. mundtii* and *L. sakei* were heat-sensitive (P<0.05). Antimicrobial metabolites without acid and H_2O_2 of *L. plantarum* LL441 against *W. viridescens* were stable at 85°C for 10 min (P>0.05), while these effective metabolites against other spoiled bacteria were heat-sensitive (P<0.05).

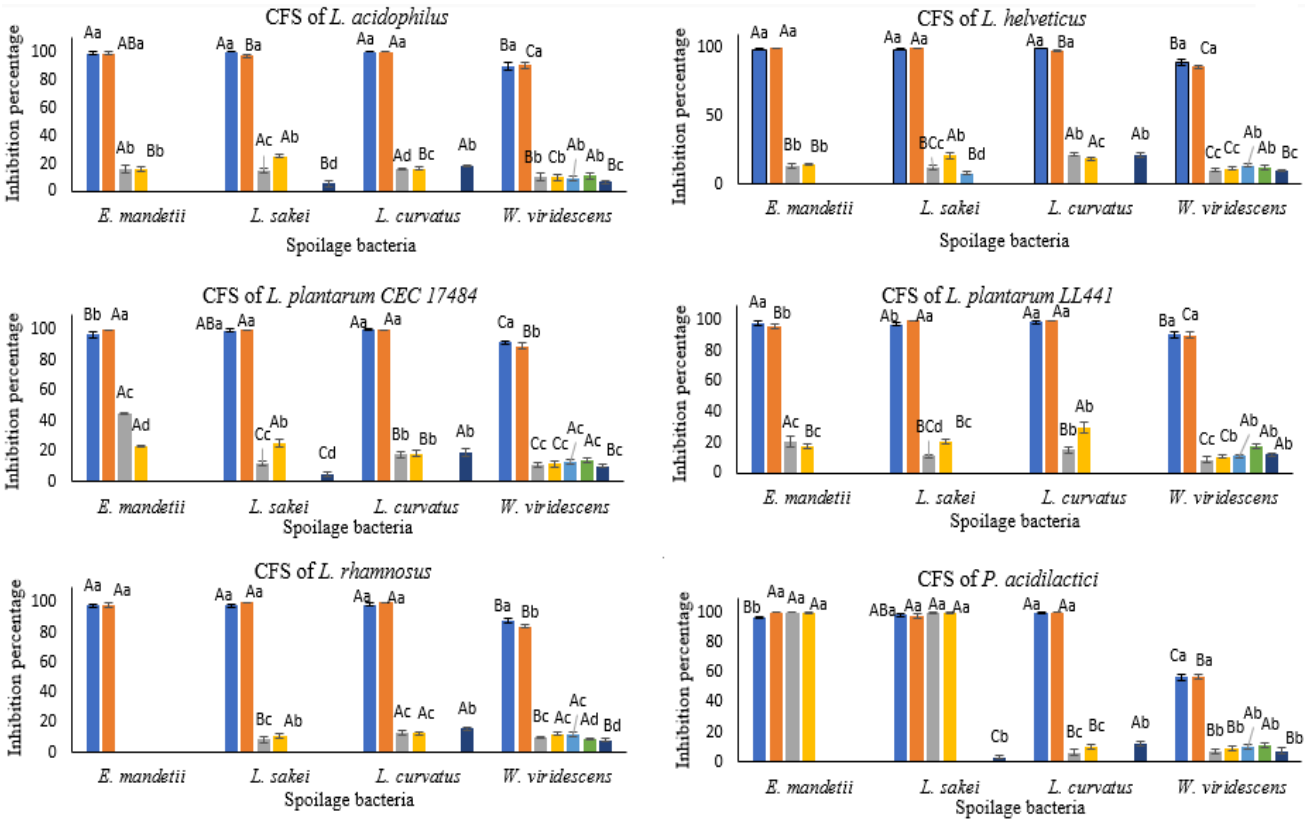

Fig. 2: Percentage inhibition of treated cell-free supernatant (CFS) of protective bacteria against four spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages (mean±SD). ■: Crude CFS (step 1), ■: Heating the CFS at 85°C for 10 min (step 2), ■: Neutralized CFS (step 3), ■: Step 3 + catalase treatment (step 4), ■: Step 4 + trypsin treatment (step 5), ■: Step 4 + pepsin treatment (step 6), and ■: Step 4 + heating at 85°C for 10 min (step 7). Different small letters indicate a significant difference between different steps of CFSs on a spoilage bacterium, and different capital letters indicate a significant difference between one step of CFSs on four spoilage bacteria

Discussion

Isolation, characterization, and identification of spoilage bacteria

In this study, the spoilage bacteria of vacuum-packed sliced emulsion-type sausages were identified by 16S rRNA sequence. The spoilage bacteria included *Latilactobacillus curvatus*, *Latilactobacillus sakei*, *Weissella viridescens*, and *Entrococcus mundtii*. Lactic acid bacteria are classified as specific spoilage organisms (SSO) on various types of vacuum-packed cooked meat products, because they have a relatively high resistance to some of the food preservation hurdles used, such as nitrite and curing salts, microaerophilic conditions, and low storage temperatures (Dušková *et al*., 2013; Kalschne *et al*., 2015; Abhari *et al*., 2018). The LABs most involved in meat spoilage consist of heterofermentative leuconostocs (*Leuconostoc* spp.), heterofermentative lactobacilli (*Lactobacillus* spp., mainly *L. curvatus* and *L. sakei*), *Carnobacterium* spp. (Hu *et al*., 2009), and, to a lesser extent, the homofermentative *Lactobacillus* spp. and *Pediococcus* spp. (Iulietto *et al*., 2015; Kalschne *et al*., 2015; Khorsandi *et al*., 2019). *L. sakei* was identified as a major source of LAB spoilage in vacuum-packed sliced cooked ham (Hu *et al*., 2009; Han *et al*., 2011; Kalschne *et al*., 2014), and *W. viridescens* was determined as the cause of spoilage of vacuum-packed cooked sausages (Iacumin *et al*., 2014). Three LAB including *L. curvatus*, *L. mesentroides*, and *L. sakei* were found as specific spoilage bacteria in vacuum-packed cooked cured emulsion-type sausages collected from various local companies in Iran by Khorsandi *et al*. (2019). According to Kalschne *et al*. (2015), at 45 days of storage, *Latilactobacillus curvatus*, *Leuconostoc mesentereoides*, and *Lactobacillus sakei* were the three major species of LAB associated with the spoilage of vacuum-packed sliced cooked ham.

Investigation of the inhibitory activity and selection of protective bacteria

LAB occurs naturally in different food sources and has been used for centuries in food fermentation. They became a part of the human diet without any adverse health effects, which procured their generally recognized as safe (GRAS) status. The long history of using LAB in food and feed, backed up by a recent scientific understanding of its antimicrobial efficacy and improved health effects, suggests that they are preferable alternatives to chemical preservatives (Barcenilla *et al*., 2022). Their inhibitory effect against other microorganisms is based on the competition for nutrients and the production of antimicrobial metabolites such as organic acids, antimicrobial enzymes, hydrogen peroxide, low molecular weight bioactive compounds, and proteinaceous substances like bacteriocins (Ouwehand and Vesterlund, 2004).

The CFS of LAB contains a variety of antimicrobial substances, making it an appropriate antimicrobial agent (Beristain-Bauza *et al*., 2016). It has been applied as a biopreservative of food in some studies, including the application of the CFS of *P. acidilactici* B-LC-20 combination with chitosan edible coating, which extended the shelf life of frankfurters by 14 days (İncili *et al*., 2023). Sliced sausages treated with concentrated LAB bacteriocin showed a considerable reduction in the development of applied *L. monocytogenes* (Bungenstock *et al*., 2021).

Considering that a significant portion of LAB's antimicrobial effect is caused by the production of acids and hydrogen peroxides, in this study, the selection of protective bacteria was considered based on their ability to produce compounds that inhibit spoilage bacteria apart from acid and hydrogen peroxide production. The six strains that were selected as protective bacteria had the most inhibitory effect beyond the effect of acid and hydrogen peroxide on four spoilage bacteria. All the selected species were probiotic bacteria and were considered safe. These bacteria, besides producing acids and hydrogen peroxide, were able to produce bacteriocins such as pediocin, helveticin, plantaricin, and lactacin, or bacteriocin-like substances (Penderup Jensen *et al*., 2009; Sanni *et al*., 2013; Beristain-Bauza *et al*., 2016; Flórez and Mayo, 2018).

Determination of MIC_{90} of CFS of protective bacteria against sausage spoilage bacteria

All CFS of selected protective bacteria were capable of inhibiting 90% of four spoilage bacteria. Depending on the concentration of inhibitory compounds in the supernatant, each of the protective bacteria was effective at a different concentration. *E. mundtii* and *L. sakei* were more sensitive to metabolites of protective bacteria than other spoilage bacteria and inhibited at a lower concentration of their CFS (<10 mg/ml). These results agree with the findings of Ammor *et al*. (2005), who found an *Enterococcus* spp. sensitive to acids and unable to grow at pH 4.2. *L. rhamnosus* inhibited *E. mundtii*, *L. sakei*, and *L. curvatus* at lower concentrations of CFS than other protective bacteria (3.1, 3.0, and 10.5 mg/ml, respectively). Because the effect of *L. rhamnosus* supernatant was greatly reduced and even ineffective in inhibiting *E. mundtii* and *L. sakei* after acid removal (Fig. 2), the inhibition effect of this bacterium was attributed to producing a high concentration of undissociated acids. *Weissella* spp. was resistant to the accumulation of H_2O_2, because it was able to produce H_2O_2 in conditions with a lower redox potential (Dušková *et al*., 2013). This ability might render *Weissella* spp. less susceptible to the CFS's antimicrobial effects, which are mostly caused by the presence of H_2O_2.

Antibacterial activity of different components in the supernatant of protective bacteria

In this study, the protective bacteria supernatants successfully inhibited four spoilage bacteria (more than 88%). No decrease in antibacterial activity was recorded after 10 min at 85°C, similar to that recorded for the supernatant of *P. acidilactici* PFC69 even at 121°C for

15 min (Kaya and Simsek, 2020). As a result, adding these supernatants to sausages before pasteurization had no negative effects on the inhibitory substances. An important part of the production of LAB is a complex of acids, including lactic, propionic, phenyllactic, formic, succinic, and hydroxyl fatty acids (Mani-López *et al*., 2022). Organic acid can pass through the cell membrane in an undissociated state. Proton ions of acids move the internal pH down, which interrupts the proton motive force from working and stops substrate transport processes that destroy bacteria (Saelim *et al*., 2017). So, by removing the acid from the CFS of protective bacteria, the effects on spoilage bacteria have been significantly reduced. Antimicrobial metabolites of *L. rhamnosus* without the presence of acid were not able to inhibit *E. mundtii*, which shows that the effect of *L. rhamnosus* on this spoilage agent was only due to the presence of acids. Beristain -Bauza *et al*. (2016) demonstrated that when CFS of *L. rhamnosus* was tested in its natural state, its antimicrobial activity against some pathogenic bacteria was attributed to lactic acid and a bacteriocin-like substance, but when the supernatant was neutral (pH 6.5), the antimicrobial activity was assumed to be due to a bacteriocin-like substance. In the present study, this bacteriocin-like substance had no effect on *E. mundtii*, despite its effect on three other spoilage agents. On the contrary, *P. acidilactici* was still able to completely inhibit *L. sakei* and *E. mundtii* after neutralizing acid, similar to complete CFS, which indicates that their effective metabolites on these two spoilage agents were other than acid. There have been various reports of the production of bacteriocin (pediocin and pediocin-like) by *Pediococcus* spp. with antimicrobial properties against various bacteria, including *Enterococci* spp. (Albano *et al*., 2007; Kaya and Simsek, 2020).

The CFSs of protective bacteria were neutralized and treated with catalase to exclude any action arising from organic acids or hydrogen peroxide, respectively. In some cases, no change in the inhibitory effect was recorded when compared to neutral CFS, indicating that H_2O_2 has few inhibitory effects. *P. acidilactici* is still quite effective against *E. mundtii* and *L. sakei*. Similar results were reported by Albano *et al*. (2007) and Kaya and Simsek (2020). The inhibitory effect of some samples was slightly reduced. Penderup Jensen *et al*. (2009) found that the antimicrobial effect on *L. lactis* spp. and *L. mesenteroides* disappeared when the acid and hydrogen peroxide from the CFS of *L. helveticus* spp. were removed.

After neutralizing the acid and H_2O_2, the metabolites of protective bacteria still have an antimicrobial effect. Because these inhibitory compounds are deactivated after treatment with trypsin, pepsin, or both proteolytic enzymes and have lost their inhibitory effect, it is likely that the antimicrobial metabolite has a protein structure and is bacteriocin or bacteriocin-like (Albano *et al*., 2007). Bacteriocins such as plantaricin C, which is generated by *L. plantarum* LL441, are pore-forming antimicrobial peptides that interfere with the development of bacteria's cell walls through their interaction with the peptidoglycan precursor lipid II (Flórez and Mayo, 2018). The CFS of *L. rhamnosus* against *E. mundtii* is completely ineffective after neutralizing the acid, and it does not produce any other inhibitory compound, except acid, against these bacteria. All the active metabolites of the bacteria against *W. viridescens* were resistant to pepsin, trypsin, and pasteurization heating, which may be resistant to these two proteolytic enzymes despite their protein nature or to another antimicrobial compound that is highly heat stable, likely not of a protein nature, and is neither an acid nor hydrogen peroxide. This result is in agreement with Penderup Jensen *et al*. (2009), who proposed that *L. helveticus* 481, besides producing the bacteriocin Helveticin J, also produced another antimicrobial compound that is highly heat stable, likely not of a protein nature, and is neither an acid nor hydrogen peroxide. The bacteriocins of the protective bacteria that were effective on *L. curvatus* were resistant to heat pasteurization. Furthermore, Beristain-Bauza *et al*. (2016) reported that the antimicrobial activity of *L. sakei* against *E. coli* and *L. monocytogenes* was lost when CFS was treated with proteinase K but maintained its activity at neutral pH, when thermal treatment, or treated with peroxidase. Another researcher has also mentioned that the antimicrobial activity of *L. helveticus* DGCC176 and LHC2 was heat sensitive and that no inhibitory activity remained after incubation for 30 min at 55°C against other strains of *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (Penderup Jensen *et al*., 2009).

As a biopreservative, living LAB or their CFS can increase the safety and shelf life of food goods. Protective cultures can reduce the severity of cooking to maintain the quality of heat-sensitive components, organoleptic quality, and nutritional value of food. The primary obstacles to the commercialization of LAB culture biopreservation are the culture's sensitivity to heat, the potential spoilage at high temperatures, and the requirement for LAB culture to be specifically selected to produce high quantities of bacteriocins at low temperatures. Even though the use of CFS of LAB in food does not have these issues, it should be noted that the amount consumed has an inhibitory effect but does not affect organoleptic factors like color and taste; consumption can be decreased by using concentrated, more effective metabolites like bacteriocins.

In this study, six LAB strains were selected whose CFSs had more than 88% inhibitory effect on spoilage bacteria isolated from vacuum-packed sliced emulsion-type sausages. In addition to producing organic acids and hydrogen peroxide, these LABs were able to produce effective antimicrobial substances with a protein nature, such as bacteriocin or bacteriocin-like substances, with different sensitivity to protease enzymes and heat. Since these CFSs were resistant to sausage pasteurization temperature, they can be used as biopreservatives even in the pre-pasteurization stage of sausage production. Of course, the *in vivo* effect of these compounds on sausages needs further investigation.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

Abhari, K; Jafarpour, D and Shekarforoush, SS (2018). Effects of in-package pasteurization on preventing spoilage in emulsion vacuum packaged sausages during refrigerated storage. Foods Raw Mater., 6: 40-46.

Albano, H; Todorov, SD; van Reenen, CA; Hogg, T; Dicks, LM and Teixeira, P (2007). Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. Int. J. Food Microbiol., 116: 239-247.

Ammor, S; Rachman, C; Chaillou, S; Prévost, H; Dousset, X; Zagorec, M; Dufour, E and Chevallier, I (2005). Phenotypic and genotypic identification of lactic acid bacteria isolated from a small-scale facility producing traditional dry sausages. Food Microbiol., 22: 373-382.

Aymerich, MT; Garriga, M; Costa, S; Monfort, JM and Hugas, M (2002). Prevention of ropiness in cooked pork by bacteriocinogenic cultures. Int. Dairy J., 12: 239-246.

Barcenilla, C; Ducic, M; López, M; Prieto, M and Álvarez-Ordónez, A (2022). Application of lactic acid bacteria for the biopreservation of meat products: A systematic review. Meat Sci., 183: 108661.

Beristain-Bauza, SC; Mani-López, E; Palou, E and López-Malo, A (2016). Antimicrobial activity and physical properties of protein films added with cell-free supernatant of *Lactobacillus rhamnosus*. Food Control. 62: 44-51.

Bungenstock, L; Abdulmawjood, A and Reich, F (2021). Suitability of lactic acid bacteria and deriving antibacterial preparations to enhance shelf-life and consumer safety of emulsion type sausages. Food Microbiol., 94: 103673.

Chaillou, S; Christieans, S; Rivollier, M; Lucquin, I; Champomier-Verges, MC and Zagorec, M (2014). Quantification and efficiency of *Lactobacillus sakei* strain mixtures used as protective cultures in ground beef. Meat Sci., 97: 332-338.

Comi, G; Andyanto, D; Manzano, M and Iacumin, L (2016). *Lactococcus lactis* and *Lactobacillus sakei* as bio-protective culture to eliminate *Leuconostoc mesenteroides* spoilage and improve the shelf life and sensorial characteristics of commercial cooked bacon. Food Microbiol., 58: 16-22.

Davies, EA; Milne, CF; Bevis, HE; Potter, RW; Harris, JM; Williams, GC; Thomas, LV and Delves-Broughton, J (1999). Effective use of nisin to control lactic acid bacterial spoilage in vacuum-packed bologna-type sausage. J. Food Prot., 62: 1004-1010.

Devlieghere, F; Vermeiren, L and Debevere, J (2004). New preservation technologies: possibilities and limitations. Int. Dairy J., 14: 273-285.

Dokka, A; Reddy, YK; Spoorthy, GS and Reddy, IS (2018). Protective cultures -A review. IJCMAS., 7: 228-238.

Dos Santos, HRM; Argolo, CS; Argôlo-Filho, RC and Loguercio, LL (2019). A 16S rDNA PCR-based theoretical to actual delta approach on culturable mock communities revealed severe losses of diversity information. BMC Microbiol., 19: 1-14.

Drumond, MM; Tapia-Costa, AP; Neumann, E; Nunes, ÁC; Barbosa, JW; Kassuha, DE and Mancha-Agresti, P (2023). Cell-free supernatant of probiotic bacteria exerted antibiofilm and antibacterial activities against *Pseudomonas aeruginosa*: A novel biotic therapy. Front. Pharmacol., 14: 1152588.

Dušková, M; Kameník, J and Karpíšková, R (2013). *Weissella viridescens* in meat products–a review. Acta Vet. Brno., 82: 237-241.

Flórez, AB and Mayo, B (2018). Genome analysis of *Lactobacillus plantarum* LL441 and genetic characterisation of the locus for the lantibiotic plantaricin C. Front. Microbiol., 9: 01916.

Frank, JA; Reich, CI; Sharma, S; Weisbaum, JS; Wilson, BA and Olsen, GJ (2008). Critical evaluation of two primers commonly used for amplification of bacterial *16S rRNA* genes. Appl. Environ. Microbiol., 74: 2461-2470.

Han, Y; Jiang, Y; Xu, X; Sun, X; Xu, B and Zhou, G (2011). Effect of high pressure treatment on microbial populations of sliced vacuum-packed cooked ham. Meat Sci., 88: 682-688.

Hu, P; Xu, XL; Zhou, GH; Han, YQ; Xu, BC and Liu, JC (2008). Study of the *Lactobacillus sakei* protective effect towards spoilage bacteria in vacuum packed cooked ham analyzed by PCR–DGGE. Meat Sci., 80: 462-469.

Hu, P; Zhou, G; Xu, X; Li, C and Han, Y (2009). Characterization of the predominant spoilage bacteria in sliced vacuum-packed cooked ham based on 16S rDNA-DGGE. Food Control. 20: 99-104.

Iacumin, L; Andyanto, D; Manzano, M; Comuzzo, P and Comi, G (2014). A case of spoilage in wurstel sold in an Italian supermarket. Food Control. 43: 245-250.

İncili, GK; Akgöl, M; Karatepe, P; Üner, S; Tekin, A; Kanmaz, H; Kaya, B; Çalicioğlu, M and Hayaloğlu, AA (2023). Quantification of bioactive metabolites derived from cell-free supernatant of *Pediococcus acidilactici* and screening their protective properties in frankfurters. Probiotics Antimicrob. Proteins. 4: 1-18.

Islam, KN; Akbar, T; Akther, F and Islam, NN (2016). Characterization and confirmation of *Lactobacillus* spp. from selective regional yoghurts for probiotic and interference with pathogenic bacterial growth. Asian J. Biol. Sci., 9: 1-9.

Iulietto, MF; Sechi, P; Borgogni, E and Cenci-Goga, BT (2015). Meat spoilage: a critical review of a neglected alteration due to ropy slime producing bacteria. Ital. J. Anim. Sci., 14: 4011.

Jalaei, J; Fazeli, M; Rajaian, H and Shekarforoush, SS (2014). *In vitro* antibacterial effect of wasp (*Vespa orientalis*) venom. JVATiTD., 20: 01-06.

Kalschne, DL; Geitenes, S; Veit, MR; Sarmento, CM and Colla, E (2014). Growth inhibition of lactic acid bacteria in ham by nisin: A model approach. Meat Sci., 98: 744-752.

Kalschne, DL; Womer, R; Mattana, A; Sarmento, CMP; Colla, LM and Colla, E (2015). Characterization of the spoilage lactic acid bacteria in "sliced vacuum-packed cooked ham". Braz. J. Microbiol., 46: 173-181.

Kaya, Hİ and Şimşek, Ö (2020). Characterization of *Pediococcus acidilactici* PFC69 and *Lactococcus lactis* PFC77 bacteriocins and their antimicrobial activities in tarhana fermentation. Microorganisms. 8: 1083.

Khorsandi, A; Eskandari, MH; Aminlari, M; Shekarforoush, SS and Golmakani, MT (2019). Shelf-life extension of vacuum packed emulsion-type sausage

using combination of natural antimicrobials. Food Control. 104: 139-146.

Lambert, RJW and Pearson, J (2000). Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J. Appl. Microbiol., 88: 784-790.

Lim, ES (2016). Inhibitory effect of bacteriocin-producing lactic acid bacteria against histamine-forming bacteria isolated from Myeolchi-jeot. Fish Aquatic Sci., 19: 1-10.

Mani-López, E; Arrioja-Bretón, D and López-Malo, A (2022). The impacts of antimicrobial and antifungal activity of cell-free supernatants from lactic acid bacteria *in vitro* and foods. Compr. Rev. Food Sci. Food Saf., 21: 604-641.

Ouwehand, AC and Vesterlund, S (2004). Antimicrobial components from lactic acid bacteria. In: Salminen, S; Wright, A and Ouwehand, A (Eds.), *Lactic acid bacteria, microbiological and functional aspects.* (3rd Edn.), New York, Marcel Dekker Inc., PP: 375-395.

Penderup Jensen, M; Braun, A; Vogensen, FK and Ardö, Y (2009). Study of antimicrobial activity among *Lactobacillus helveticus* strains using three different assays. Ann. Microbiol., 59: 187-190.

Rivas, FP; Castro, MP; Vallejo, M; Marguet, E and Campos, CA (2014). Sakacin Q produced by *Lactobacillus curvatus* ACU-1: functionality characterization and antilisterial activity on cooked meat surface. Meat Sci., 97: 475-479.

Saelim, K; Jampaphaeng, K and Maneerat, S (2017). Functional properties of *Lactobacillus plantarum* S0/7 isolated fermented stinky bean (Sa Taw Dong) and its use as a starter culture. J. Funct. Foods. 38: 370-377.

Salehi, TZ; Mahzounieh, M and Saeedzadeh, A (2005). Detection of *inv*A gene in isolated *Salmonella* from broilers by PCR method. Int. J. Poult. Sci., 4: 557-559.

Sanni, A; Franz, C; Schillinger, U; Huch, M; Guigas, C and Holzapfel, W (2013). Characterization and technological properties of lactic acid bacteria in the production of "Sorghurt," a cereal-based product. Food Biotechnol., 27: 178-198.

Turgis, M; Stotz, V; Dupont, C; Salmieri, S; Khan, RA and Lacroix, M (2012). Elimination of *Listeria monocytogenes* in sausage meat by combination treatment: Radiation and radiation-resistant bacteriocins. Radiat. Phys. Chem., 81: 1185-1188.

Zhang, J; Yang, Y; Yang, H; Bu, Y; Yi, H; Zhang, L; Han, X and Ai, L (2018). Purification and partial characterization of bacteriocin Lac-B23, a novel bacteriocin production by *Lactobacillus plantarum* J23, isolated from Chinese traditional fermented milk. Front. Microbiol., 9: 2165.