




Bacteriocin Producing Bacteria Isolated from Turkish Traditional Sausage Samples

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Abstract

In this study, traditional sausage samples from different provinces of Turkey (Gaziantep, Antalya, Erzurum and Kahramanmaraş) were obtained and one hundred three isolates were collected. Using the (GTG)₅-PCR genomic fingerprint analysis method, seven of them were observed to be different and conventional tests of these isolates were performed. Molecular identification of two isolates carrying the bacteriocin gene and having antimicrobial activity by agar disc diffusion method was performed by 16S rRNA sequence analysis. As a result, the seven isolates were identified as *Aerococcus urinaeequi* (EK1), *Streptococcus salivarius* (EK2), *Leuconostoc mesenteroides* (EK3), *Macrococcus caseolyticus* (EK4), *Lactococcus garvieae* (EK5), *Staphylococcus saprophyticus* (EK6) and *Lactobacillus sakei* (EK7). Among these strains, it has been determined that *Ln. mesenteroides* and *L. sakei* carried the mecentericin and sacacin genes. When antimicrobial activity against different strains was examined, inhibition formations of *Ln. mesenteroides* and *L. sakei* on *Enterococcus faecalis*, *Shigella dysenteriae* and *Escherichia coli* O157: H7 were observed.

Keywords: Bacteriocin, (GTG)₅-PCR, 16S rRNA sequence analysis, antimicrobial activity

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INTRODUCTION

Bacteriocins are natural antimicrobial peptides which are used as bioprotectors in the food industry to protect human health and to increase the sustainability of foods¹. They are produced by both Gram positive and Gram negative bacteria. Most of the bacteriocins are 20-70 amino acid structure². Bacteriocins generally have cationic character and easily interact with Gram-positive bacteria that contain high amounts of anionic lipids in the cell membrane. This interaction causes the formation of pores in the cell membrane. The pores in the membrane show a bactericidal effect in the cells by changing the energy state of the cell^{3,4}.

Bacteriocins are basically divided into two main classes. Class I bacteriocins contain lantibiotics with a molecular weight less than 5 kDa and can be distinguished from class II bacteriocins due to post-translational modifications⁵. Lantibiotics are divided into two groups. Type A lantibiotics are positively charged, long and flexible molecules that act on the bacterial membranes by performing pore formation. The most well-known member of this group is nisin which was described in 1928⁶. Type B lantibiotics are smaller spherical peptides and are negatively charged or uncharged. It shows antimicrobial activity by inhibiting the specific enzymes of the host cell⁷. Class II bacteriocins are unmodified peptides. Class IIa (or pediosin PA1-like) peptides generally contain 37-48 amino acids⁵. Approximately 50 different class IIa bacteriocins have been isolated from fermented meat, fermented vegetables, dairy products, smoked salmon and the human gastrointestinal tract. Class IIb bacteriocins need two complementary peptides to show antimicrobial activity. Although some peptides of this class may exhibit antimicrobial activity separately, the addition of the complementary agent greatly enhances the activity. Class IIb bacteriocins contain amphiphilic and hydrophobic regions and are mostly cationic⁸. Apart from these two classes, there are class III and class IV. Class III contains heat-sensitive enzymes which degrade the cell wall⁹. Colicin produced by *Escherichia coli* is an example of class III bacteriocins, while helveticin M, helveticin J and enterolisin A produced by *Lactobacillus crispatus*, *L. helveticus* and *Enterococcus faecalis* are also examples of class

III bacteriocins. Helveticin M has recently been characterized and found to disrupt the cell wall of Gram-positive bacteria and the outer membrane of Gram-negative bacteria. In addition, it has been stated that class III bacteriocin is effective against both Gram-positive and Gram-negative bacteria¹⁰. Class IV bacteriocins contain spherical, heat-sensitive, helix and post-translationally modified proteins consisting of 35-70 amino acids⁹.

Genes related to bacteriocin biosynthesis are usually found collectively and are encoded in plasmids, chromosomes or transposons. Bacteriocins are synthesized as biologically inactive prepeptides, usually containing a N-terminal leader peptide bound to the C-terminal propeptide^{11,12}.

Bacteriocin-producing organisms are resistant to the bacteriocins they produce because they have specific immune proteins. Two separate bacteriological immune systems have been identified in the bacteriocin-producing cell which would be a special ABC carrier system consisting of immune protein and two or three subunits. These two immune systems work together to protect the bacteriocin-producing cells from their bacteriocin⁵.

The aim of the study was isolation and identification of lactic acid bacteria from traditional sausage samples collected from different locations of Turkey. Then, determination of whether the bacteria have bacteriocin gene or not and detection of antimicrobial effect of these isolates were aimed.

MATERIAL AND METHODS

Isolation and molecular characterization of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated from Turkish traditional sausages. The samples were collected from different provinces of Turkey; Gaziantep, Kayseri, Erzurum and Kahramanmaraş. Each sample (25 gr) was mixed with 225 mL physiological water (0.9% NaCl) and homogenized in stomacher. Samples were diluted (10^{-1} - 10^{-7}) and each dilution (100 μ L) were spread onto MRS and M17 agar plates and incubated at 37°C for 48 h. First of all, the growing colonies were spread on M17 and MRS agar to obtain pure cultures and were eliminated by morphology differences on plates. The pure, single and different colonies were stored in the Tryptic Soy Broth with 15% glycerol content at -86°C for further studies.

The total genomic DNA of each test strain was isolated according to the procedure of Promega™ Wizard® Genomic DNA Purification Kit. Afterwards, test isolates were subjected to rep-PCR [(GTG)₅-PCR] with the special primer of (GTG)₅ elements to obtain genomic fingerprinting. The 16S rRNA gene regions of the isolates which would be considered to be different according to the rep-PCR analysis were amplified with universal primers by PCR and were subjected to the cloning procedure¹³⁻¹⁷.

Conventional identification

The isolates which were selected according to genomic fingerprinting were subjected to conventional tests for pH, temperature and salt (NaCl) requirements in growth media. The pH, temperature and NaCl requirements for bacterial growth were measured in M17 and MRS broth by following optical density at 600 nm wavelength with spectrophotometer by the method of Prescott et al.¹⁸ Firstly, M17 and MRS broth media were prepared and, then a full loop of the test isolate on M17 and MRS agar was transferred to broth media and incubated at different temperatures (10-45°C) for 48 h. To measure the response to pH changes during growth, M17 and MRS broth media were prepared and pH of broth media was adjusted to different points at pH 3-11 range, before autoclaving. Then, a full loop of the test isolate on M17 and MRS agar was transferred to broth media and incubated at 37°C for 48 h. The salt (NaCl) requirement for growth was also tested in M17 and MRS broth medium containing 2-12 (w/v). For this, M17 and MRS broth media were prepared and salt at determined concentrations were added to broth media, before autoclaving. Then, a full loop of the test isolate on M17 and MRS agar was transferred to broth media and

incubated at 37°C for 48 h¹⁹.

Colony morphology, Gram staining, motility, the presence of catalase and oxidase reactions and gas productions were also investigated^{18,20,21}.

Determination of bacteriocin genes

The selected test isolates were evaluated according to bacteriocin genes with multiplex PCR reactions. 75 ng of purified DNA (2 µL) was used as the template in 30 µL total reaction mixture. Twenty-eight microliters of the reaction cocktail were prepared as follows: PCR Buffer (10X) 3 µL, MgCl₂ 1.2 µL (25mM), dNTPs (10 mM) 0.6 µL, bovine serum albumin 1.2 µL (20 mg/mL), primers (5 µM) 1 µL, Taq polymerase (250 U) 0.3 µL and water 13.7 µL. Primers were given in Table 1. A negative control (no DNA) was included in each PCR assay. PCR reactions were performed with a 96-well ProFlex™ PCR System, using the following conditions: an initial denaturation at 95°C for 5 min; 30 cycles consisting of 94°C for 0.5 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final polymerization at 72°C for 5 min before cooling at 4°C²².

For the multiplex-PCR reactions, products (2 µL) were mixed with 1.0 µL gel loading buffer (6X) and subjected to agarose (1% w/v) gel electrophoresis in Tris–Acetate–EDTA (TAE) buffer at 90 V and for 120 min. After separation, the fragments were stained with ethidium bromide solution (2 ml Etbr/100 ml 1X TAE buffer). The amplified DNA product was monitored using the Quantum Vilber Lourmat Gel Documentation System (Australia).

Agar disk diffusion method

In the agar disc diffusion test, pathogenic strains (*Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 ATCC 35150, *Shigella*

Table 1. Primers used in multiplex-PCR reaction for determination of bacteriocin genes

Gene	Primers
Garvicin A(IgnA)	IgnA-F: 5'-ATTTAATACGGACGGTATTGAT-3'
	IgnA-R: 5'-GGAGTAAAAAGATGGAAAACAA-3'
Mesentericin	MESY-F: 5'-AGTCTGTGGAAGCATATCAGCA-3'
	MESY-R: 5'-TACCAAATCCATTTCACCAT-3'
Sacacin	Sak A-F: 5'-ACAGAATTACAAACAATTACCGGC-3'
	Sak A-R: 5'-CATTCCAGCTAAACCACTAGCC-3'
Salivaricin	Salivaricin-F: 5'-GTAGAAAATATTTACTACATACT-3'
	Salivaricin-R: 5'-GTTAAAGTATTCGTAATAACTGATG-3'

dysenteriae ATCC 13313, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 13883, *Enterobacter cloacae* ATCC 13047, *Staphylococcus aureus* ATCC 29213) were inoculated on the Tryptic Soy Agar medium using the sterile swab. 100 µL of the test isolate, grown in M17 and MRS broth, were absorbed on discs with a diameter of 6 mm. Then, these discs were placed on the petri dishes where pathogenic bacteria were inoculated and incubated at 37°C for 24 h. At the end of the period, the petri dishes were removed to check if the inhibition zone was formed and the diameter of the zone was measured^{23,24}.

RESULTS AND DISCUSSION

Traditional sausage samples from different locations of Turkey were collected. Isolation of lactic acid bacteria were carried out and one hundred three isolates were selected according to colony differentiation on petri plates. (GTG)₅-PCR was performed to show distinction at species level (Fig.1) and according to differences, seven different isolates were decided to determine with 16S rRNA sequencing.

16S rRNA sequences of the seven isolates were amplified and cloned into *E. coli* JM101 strain. The obtained sequence was compared those in GenBank and EzTaxon. As given in Table 2; EK1, EK2, EK3, EK4, EK5, EK6

and EK7 were similar to *Aerococcus urinaeequi*, *Streptococcus salivarius*, *Macrococcus caseolyticus*, *Leuconostoc mesenteroides*, *Lactococcus garvieae*, *Staphylococcus saprophyticus* and *Lactobacillus sakei*, respectively. Also, phylogenetic analysis by using neighbour-joining method and data from 16S rRNA sequencing was carried out and given in Fig.2. As a result of the 16S rRNA gene sequences of the isolates, very close similarity rates (>98%) were obtained, as specified before in different experiments. Madigan and Martinko²⁵ reported that isolates having sequence similarity over 97% might belong to the same species.

These isolates were selected according to genetic polymorphism of (GTG)₅ region. Svec et al.²⁶ reported that (GTG)₅-PCR was highly effective in the identification of lactobacilli isolated from food samples, it enabled the rapid and reliable identification of lactobacilli and other lactic acid bacteria, which were important in the food fermentation industries.

As a result of conventional identification (Table 3), all the isolates were smooth and coccus (except EK7). All of them were Gram positive, non-motile, oxidase (except EK3) and catalase negative (except EK3 and EK6) and homofermentative (except EK4). To determine physicochemical requirements of the isolates, they were grown in different culture conditions (Table 4). The

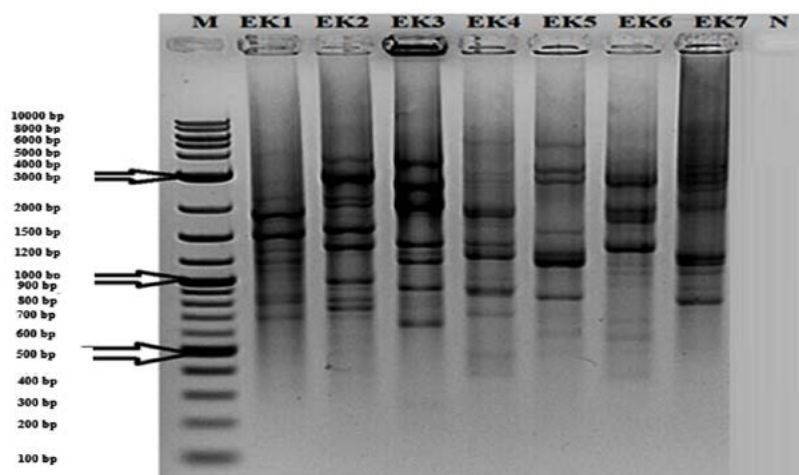


Fig. 1. (GTG)₅ profile generated with the (GTG)₅ primer. Lanes: 1) *Aerococcus urinaeequi*; 2) *Streptococcus salivarius* subsp. *salivarius*; 3) *Macrococcus caseolyticus* subsp. *hominis*; 4) *Leuconostoc mesenteroides* subsp. *dextranicum*; 5) *Lactococcus garvieae* subsp. *garvieae*; 6) *Staphylococcus saprophyticus* subsp. *saprophyticus*; 7) *Lactobacillus sakei* subsp. *sakei*; N) Negative Control; M) Molecular Marker (10 kb).

isolates EK1, EK3 and EK6 were grown optimally at between 15-37°C, EK4 and EK7 were at between 25-37°C, EK2 were at between 35-37°C and EK5 were at between 15-40°C. The tolerance of pH changes was also measured. EK2, EK4, EK6 and EK7 could show tolerance between pH 5-7, while EK1, EK3 and EK5 could survive at between pH 7-9, pH 5-9 and pH 7-9, respectively. Salt requirements of isolates were also different. EK1, EK2, EK4 and EK7 grown at between 2-4% NaCl while EK3, EK5 and EK6 could grow at between 4-10%, 4-6% and 4-8% NaCl, respectively.

Karani et al.²⁷ revealed that *M. caseolyticus* strain developed in the presence of salt as high as 8%. Diaz et al.¹⁵ stated that halophilic or halo-tolerance microorganisms tolerated high salt concentrations and this was making them very important in remediation. Considering this information, it was concluded that *M. caseolyticus*, which was not normally LAB, could be widely used in biotechnological processes because of maintaining vital functions even in high salt concentrations. Ramirez-Chavarin et al.²⁸ reported that thermotolerant lactic acid bacteria

Table 2. Comparison of 16S rRNA gene sequences of the test isolates with those in GenBank

Isolate	Species	Similarity (%)	Accession number
EK1	<i>Aerococcus urinaeequi</i>	99.86	MN045012
EK2	<i>Streptococcus salivarius</i> subsp. <i>salivarius</i>	99.86	MN045013
EK3	<i>Macrococcus caseolyticus</i> subsp. <i>hominis</i>	99.32	MN045011
EK4	<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	99.72	MN045173
EK5	<i>Lactococcus garvieae</i> subsp. <i>garvieae</i>	99.17	MN094108
EK6	<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i>	99.93	MN045172
EK7	<i>Lactobacillus sakei</i> subsp. <i>sakei</i>	99.93	MN045009

Table 3. Morphological and biochemical properties of of LAB

Isolate	Morphology	Gram reaction	Motility	Oxidase	Catalase	Gas production
EK1	smooth/coccus	+	-	-	-	Homofermentative
EK2	smooth/coccus	+	-	-	-	Homofermentative
EK3	smooth/coccus	+	-	+	+	Homofermentative
EK4	smooth/coccus	+	-	-	-	Heterofermentative
EK5	smooth/coccus	+	-	-	-	Homofermentative
EK6	smooth/coccus	+	-	-	+	Homofermentative
EK7	smooth/bacilli	+	-	-	-	Homofermentative

Table 4. Temperature, pH and salt requirements of of LAB

Isolate	Temp (°C)	pH	Salt concen (%)
EK1	15-37	7-9	2-4
EK2	35-37	5-7	2-4
EK3	15-37	5-9	4-10
EK4	25-37	5-7	2-4
EK5	15-40	7-9	4-6
EK6	15-37	5-7	4-8
EK7	25-37	5-7	2-4

became dominant flora in foods cooked like sausage and act as bioprotective agents. Varsha and Nampoothiri²⁹ found in their study that the bacteria *L. garvieae* showed growth at 42°C. The optimum pH range of *S. saprophyticus* was 5-6.8³⁰ and *Ln. mesenteroides*' optimum pH was 5.5³¹. Optimum pH range was 8.5-9 for *A. urinaeequi*³², 6-7 for *S. salivarius*³³, 6 for *L. sakei*³⁴ and 9.6 for *L. garvieae*³⁵. Karani et al.²⁷ found that the optimum pH range for *M. caseolyticus* was 6-11. Dhakar and Pandey³⁶ emphasized that bacteria, archaea

and eukaryotic organisms that had the potential to develop in a wide pH range could be used in industrially important bioprocesses.

Determination of bacteriocin presence

The potentials of isolates to produce bacteriocin were analyzed using specific PCR. Mesentericin and sacacin genes were determined in two isolates, EK4 (*Ln. mesenteroides* subsp. *dextranicum*) and EK7 (*L. sakei* subsp. *sakei*) (data not shown), respectively.

Todorov and Dicks³⁷ were reported presence of mesentericin in *Ln. mesenteroides* subsp. *dextranicum* ST99 from Boza samples. de Paula et al.³⁸ were isolated *Ln. mesenteroides* SJRP55 from cheese samples and purified bacteriocins which were identical to mesentericin Y105 and mesentericin B105. Hechard et al.³⁹

purified and characterized mesentericin Y105 from *L. mesenteroides*.

Sawa et al.⁴⁰ isolated *L. sakei* D98 from rice malt and purified three novel bacteriocin belonging to class IIa and class II d. Moretro et al.⁴¹ optimized sacacin P production from *L. sakei* CCUG 42687 in a completely defined medium. Jiang et al.⁴² defined a novel sacacin from *L. sakei* LSJ61.

Antimicrobial effect of LAB on pathogenic strains

When the effects of the test isolates against different pathogenic microorganisms were investigated by agar disc diffusion method (Table 5), it was observed that, the isolates EK4 (*Ln. mesenteroides* subsp. *dextranicum*) had antimicrobial effect on *E. faecalis* ATCC 29212 and *S. dysenteriae* ATCC 13313; another isolate, EK7 (*L. sakei* subsp. *sakei*) was effective on *E. faecalis*

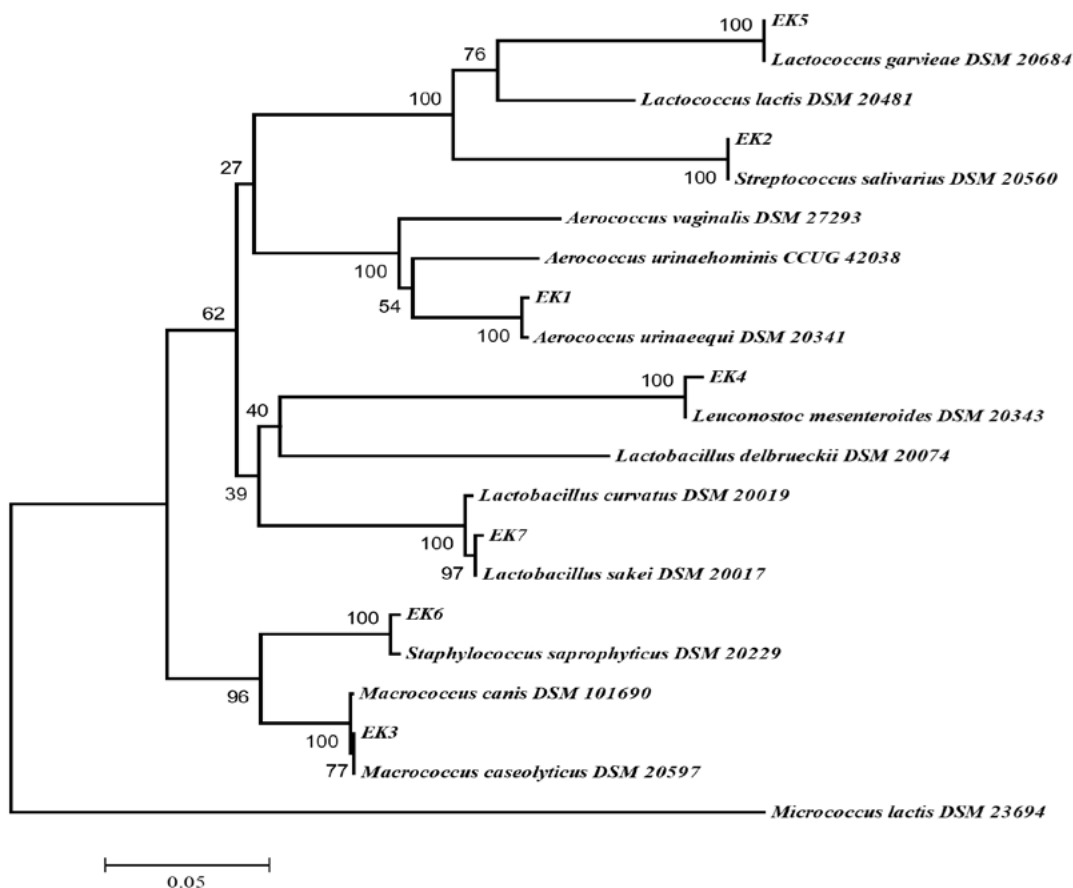


Fig. 2. Phylogenetic tree (based on neighbor-joining method) of lactic acid bacteria created with the data from 16S rRNA gene analysis results (Bootstrap value was 1000 repeats and no values below 50% were shown on nodes. Scale bar represented 0.5% deviation. *Micrococcus lactis* was used as outer group).

Table 5. Antimicrobial effect of test isolates against pathogenic microorganisms

Isolate	Species	E. faecalis	E.coli O157:H7	S. dysenteriae	P. aeruginosa	K. pneumoniae	E. cloacae	S. aureus
EK1	<i>Aerococcus urinaeequi</i>	-	-	-	-	-	-	-
EK2	<i>Streptococcus salivarius</i> subsp. <i>salivarius</i>	-	-	-	-	-	-	-
EK3	<i>Macrococcus caseolyticus</i> subsp. <i>hominis</i>	-	-	-	-	-	-	-
EK4	<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	+	-	+	-	-	-	-
EK5	<i>Lactococcus garvieae</i> subsp. <i>garvieae</i>	-	-	-	-	-	-	-
EK6	<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i>	-	-	-	-	-	-	-
EK7	<i>Lactobacillus sakei</i> subsp. <i>sakei</i>	+	+	-	-	-	-	-

ATCC 29212 and *E. coli* O157: H7 ATCC 35150.

Mesentericins from *Ln. mesenteroides* SJRP55 showed antimicrobial effect against to *E. faecalis* ATCC 19443, *Enterococcus* sp. and *E. faecium*³⁸. Mesentericin from *Ln. mesenteroides* subsp. *dextranicum* ST99 was also active on *E. faecalis*. Bacteriocin containing supernatants of isolates from fresh fruit and vegetables had antimicrobial effect against different LAB and non-LAB⁴³.

Sacacin-like bacteriocins from *L. sakei* D98 had antimicrobial activity on *E. faecalis* JCM 5803⁴⁰. A novel bacteriocin from *L. sakei* LSJ61 showed antimicrobial effect on *E. coli* ECX4⁴². Another novel bacteriocin from *L. sakei* C2, isolated from traditional Chinese fermented cabbage had a broad antimicrobial spectrum and showed inhibitory effect on *S. flexneri* CMCC 51606 and *E. coli* ATCC 25922⁴⁴. Bacteriocin from *L. sakei* ST154Ch was active on *E. faecalis*, *E. faecium* and *E. coli*, while bacteriocins from *L. sakei* ST22Ch and *L. sakei* ST153Ch had antimicrobial effect on *E. faecium* and *E. coli*⁴⁵.

CONCLUSION

Reliable foods would be crucial for human growth and development. In order for food to be reliable, it is necessary to avoid process applications and to use natural additives. Bacteriocins, one of these natural additives, play an important role in food preservation by inhibiting both pathogenic and spoilage microorganisms. In this study, traditional sausage samples demonstrated a diverse of microorganisms and introduced bacteriocin production ability. It could be concluded that fermented foods had a good potential for bacteriocin producing microorganisms and these bacteriocins would be a decent source for food hygiene problems. In addition, (GTG)₅-PCR, which is one of the rep-PCR methods, exhibited the distinction between food-borne test strains at species and subspecies level.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

EA collected the sausage samples, EA and SA carried out the study, BG, HO and MAO participated in design to draft, GA and AA wrote the manuscript. All the authors read and approved the final manuscript

FUNDING

None.

ETHICS STATEMENT

All text, data, figures/tables or other illustrations presented in the manuscript are completely original and does not contain or include material taken from other copyrighted sources. This article does not contain any studies about human or animal objects.

DATA AVAILABILITY

All datasets obtained or studied during this study are incorporated in the manuscript.

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