فصلنامه علمی زیست‌شناسی میکروارگانیسم‌ها
سال یازدهم، شماره ۴۴، زمستان ۱۴۰۱ ، صفحه ۱۴۴-۱۲۹
تاریخ دریافت: ۱۴۰۱/۰۴/۱۶ – تاریخ پذیرش: ۱۴۰۱/۰۷/۱۲

فعالیت ضدمیکروبی سویه‌های *پدیوکوکوس اسیدی لاکتیسی* PTCC 1954 و *لوکونوستوک مزانتروئیدس* PTCC 1953 جداسازی‌شده از سوسیس‌های ارگانیک گوشت

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چکیده

مقدمه: محصولات گوشتی ارگانیک به‌دلیل وجود باکتری‌های اسیدلاکتیک مانند لاکتوباسیلوس‌ها، لوکونوستوک‌ها، لاکتوکوس‌ها و پدیوکوکوس‌ها بسیار شناخته‌شده هستند. هدف از این تحقیق جداسازی و شناسایی سویه‌های جدید باکتری‌های اسیدلاکتیک از سوسیس‌های ارگانیک گوشت و ارزیابی فعالیت ضدمیکروبی آنها است.

مواد و روش‌ها: در این تحقیق جداسازی باکتری‌های اسیدلاکتیک از بسته‌بندی‌های تهیه‌شده در شرایط خلأ سوسیس‌های شرکت سولیکو (تهران، ایران) انجام گرفت. شناسایی براساس ویژگی‌های مورفولوژی، بیوشیمیایی و تجزیه و تحلیل *16S rRNA* انجام شد. ارزیابی اولیه فعالیت ضدمیکروبی سویه‌ها علیه *میکروکوکوس لوئتوس* PTCC 1408 انجام گرفت. سینتیک رشد سویه MN-B و فعالیت ضدمیکروبی آن علیه *لاکتوباسیلوس کازئی* ATCC 32392

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10.22108/bjm.2022.134313.1473

در محیط MRS بررسی شد. فعالیت ضدمیکروبی علیه برخی از پاتوژن‌های منتقل‌شونده از راه غذا انجام شد. باکتریوسین تولیدشده توسط جدایه MN-B توسط روش جذبی - دفعی و به کار بردن دیالیز به شکل جزئی خالص‌سازی شد.

نتایج: در این تحقیق دو سویه از جنس پدیوکوکوس و سه سویه از جنس لوکونوستوک از سوسیس‌های بسته‌بندی‌شده در شرایط خلأ، جداسازی و با علائم اختصاری MN-A3، MN-A2-1، MN-A2، MN-B و MN-A نام‌گذاری شدند. نتایج شناسایی نشان دادند سویه‌های MN-B و MN-A متعلق به گونه *پدیوکوکس اسیدی‌لاکتیسی* و سه سویه MN-A2-1، MN-A2 و MN-A3 متعلق به گونه *لکونوستوک مزانتروئیدس* هستند. از میان سویه‌ها، سویه MN-B بالاترین فعالیت ضدمیکروبی را علیه *میکروکوکوس لوئتوس* PTCC 1408 نشان داد. وزن مولکولی باکتریوسین جداسازی‌شده از این سویه به شکل جزئی ۵ کیلودالتون است.

بحث و نتیجه‌گیری: سویه MN-B، *پدیوکوکس اسیدی‌لاکتیسی* PTCC 1954 فعالیت ضدمیکروبی مناسبی علیه *سالمونلا انتریکا* زیرگونه انتریکا سروتیپ *تیفی موریوم* PTCC 1709، *استافیلوکوکوس ارئوس* PTCC 1113، *لیستریا منوسیتوژنز* PTCC 1302 و *لیستریا اینووانی* PTCC 1303 نشان داد. با توجه به اثر ضدمیکروبی مناسب، سویه جدید جداسازی‌شده می‌تواند کاندید مناسبی به‌عنوان نگه‌دارنده در مواد غذایی باشد.

واژه‌های کلیدی: فعالیت ضدمیکروبی، جداسازی، شناسایی، سوسیس‌های ارگانیک گوشت

Biological Journal of Microorganism
Year 11, Vol.11, No.44, Winter 2023
Received: 2022-07-07 - Accepted:Page: 2022-10-04
P:129-144
(research article)

Antimicrobial Activity of Pediococcus Acidilactici PTCC 1954 and Leuconostoc Mesenteroides PTCC 1953 strains Isolated from Organic Meat Sausages

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Abstract

Introduction: Organic meat products are well known for their probiotic values due to the presence of probiotic lactic acid bacteria such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Pediococcus* strains. The present study aimed to isolate and characterize new strains of lactic acid bacteria (LABs) from organic meat sausages and evaluate their antimicrobial activities.

Materials and Methods: In this study, LAB strains were isolated from vacuum-packed organic meat sausages bought from Solico Group Co Ltd. (Tehran, Iran). All isolates were characterized by morphological, biochemical, and 16S rRNA analysis. The primary antibacterial activity of the isolates was evaluated against *Micrococcus luteus* PTCC 1408. The growth kinetics and antimicrobial activity of the strain MN-B against *Lactobacillus casei* ATCC 39392 were determined in an MRS broth medium. In addition, a bacteriocin produced by the strains MN-B revealed antimicrobial activity against selected foodborne pathogens. Bacteriocin produced by the MN-B strain was partially purified using the adsorption-desorption method followed by dialyzes.

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10.22108/BJM.2022.134313.1473

Results: In this study, two *Pediococcus* spp. and three *Leuconostoc* spp. with antimicrobial activity were isolated from vacuum-packed organic meat sausages and designated as MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3. The results showed that the isolate MN-A and MN-B belonged to the strain *Pediococcus acidilactici* and the MN-A2, MN-A2-1, and MN-A3 isolates were identified as *Leuconostoc mesenteroides* strains. Amongst the isolates, the strain MN-B showed the highest antimicrobial activity against the *Micrococcus luteus* PTCC 1408. The molecular weight of bacteriocin was about 5.0 KDa.

Discussion and Conclusion: *Pediococcus acidilactici* (strain MN-B) showed significant antimicrobial activity against *Salmonella enterica* subsp. enterica serotype Typhimurium PTCC 1709, *Staphylococcus aureus* PTCC 1113, *Listeria monocytogenes* PTCC1302, and *Listeria inovani* PTCC 1303. Therefore, this strain can be considered a potential candidate in different sectors such as the food industry as a preservative.

Key words: Antimicrobial Activity, Isolation, Characterization, Organic Meat Sausages

Introduction

Meat and meat products contain high nutritional value and are a good source of bioactive compounds such as vitamins, minerals, peptides, and fatty acids with a good effect on human health. There are many valuable compounds including essential amino acids, micronutrients, and bioactive compounds (carnitine, conjugated linoleic acids (CLA), antioxidant, and creatine) in raw red meat. In addition, the value of meat can be improved during processing, and the use of fermentation and ripening leads to preserving meat to avoid the loss of valuable compounds as well as biochemical changes, generation of bioactive peptides, and development of potentially probiotic bacterial strains (1-3). Lactic acid bacteria (LAB) are used for the production of fermented sausages. Their application allows controlling the process and improving the quality and safety features of sausages. These lactic acid bacteria are normal flora or can be added manually as a starter culture for the fermentation process (4, 5).

Fermentation can improve the shelf life and improve the flavor of many foods. Some variety of lactic acid bacteria (LAB) strains such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Pediococcus* are involved in food fermentation and have crucial health-improving effects. Currently, LAB is considered "generally recognized as safe" microorganisms and are generally used in the food industry. LAB can increase food taste and nutritional value by generating aromatic compounds and converting isoflavone glucosides into aglycones. Therefore, screening probiotic LAB from fermented foods has gained increasing attention in recent years (3, 6, 7).

Also, lactic acid bacteria have antimicrobial activities against pathogenic microorganisms by producing metabolites and other mechanisms including competitively inhibiting pathogen binding, increasing the host immune system, and producing compounds such as organic acids, bacteriocins, and hydrogen peroxide (8).

It should be noted that not all of these bacteria could be applied to the control of food-borne pathogens in the food industry as they might produce unfavorable flavors in food. *Pediococcus* spp. is the most important LAB that is considered probiotics. They are Gram-positive, non-motile, non-spore-forming, catalase-negative, facultative anaerobic cocci arranged in tetrads. There are various strains of *Pediococcus* such as *P. pentosaceous* and *P. acidilactici* that produce a protein known as pediocin, which is considered an effective antimicrobial bacteriocin (9).

Pediocin has significant properties such as thermostability and activity at a wide

range of pH, making it an attractive class of bio preservatives, especially in the food industry. The genus *Leuconostoc* currently includes 13 species. They are widely distributed in different ecological niches such as chilled meat, honeydew, sauerkraut, fermented coffee, kimchi, boza, fermented milk, sugar beet solution, brown algae kimchi, palm wine, cane juice, sous-vide rutabaga, and wine. The species in the genus *Leuconostoc* produce bacteriocins, and most of these bacteriocins were found to be part of class IIa bacteriocins, showing specific anti-*Listeria* activity. Several strains belonging to *Leuconostoc* spp. were categorized as promising probiotic candidates. However, certain *Leuconostoc* spp. can cause spoilage in specific types of food matrices, while limited reports have shown the pathogenic potential of these strains. *Leuconostoc* strains can ferment lactose and citrate to lactic acid, acetate, CO_2, ethanol, acetaldehyde, diacetyl, acetoin, and 2, 3-butanediol, all of which are metabolites that can contribute to the flavor and texture characteristics of dairy products (10, 11).

Although there are some reports regarding the isolation and characterizations of antimicrobial activity of *Pediococcus* spp. and *Leuconostoc* spp. from different sources, to the best of our knowledge, there is little information regarding isolation, characterization, and assessing the antimicrobial activity of *Pediococcus* and *Leuconostoc* strains from organic meat sausage. The aim of the present study is to isolate, characterize, and evaluate the antimicrobial activity of native *Pediococcus* spp. and *Leuconostoc* spp. from organic meat sausage.

Materials and Methods

Samples, Isolation of Bacterial Strains, Media, and Growth Conditions: Isolation of LAB strains was performed from vacuum-packed organic meat sausages bought from Solico Group Co Ltd. (Tehran, Iran). Twenty packs of fresh organic meat sausages were bought from local markets. All packs were kept on ice bags, transferred into the laboratory, and refrigerated at 4 °C. For the isolation of the bacteria, the method provided by Vivekananda Mandal et al. 2011 was used with some modifications (12).

Each pack was opened with a sterile scissor and cut into small pieces using a sterile knife, and 1 gram of the obtained pieces was homogenized in normal saline solution. One milliliter of the homogenized solution in normal saline was added to 9 ml of Trypticase soy broth (TSB) medium to obtain a 1:10 initial dilution. Then, 100µl of each dilution was streaked on the MRS agar plate. Incubation was performed at 28 °C for 24h. MRS agar plates containing between 50 to 100 colonies were overlaid with MRS soft agar (0.8%) seeded and streaked with *Micrococcus luteus* PTCC 1408 as an indicator bacterial strain. Incubation was performed at 28 °C for 24h for observing the clear zone of growth inhibitions. Colonies showing reasonable zone of growth inhibition were picked up and transferred to MRS agar plates for obtaining pure culture.

Phenotypic, Biochemical, and Molecular Characterization of the Isolates: Phenotypic characterization including Gram staining, colony size, and morphology, biochemical tests such as carbohydrate fermentation, catalase activity, growth at 15°C and 45°C, growth at 4% and 6.5% NaCl, and motility were carried out according to Bergey's Manual of Systematic Bacteriology for the identification and characterization of the LAB isolates (13).

For the taxonomic study of *isolates*, the 16S rRNA analysis was performed. The genomic DNA was extracted from single colonies of the bacterial strains, using a Qiagen DNeasy blood and tissue kit (QIAGEN N.V, Germany) (following the

manufacturer's instructions). For all five isolates, the bacterial 16S rRNA was amplified using the forward 27f: 5'-GAGTTTGATCCTGGCTCAG -3' and reverse 1505r: 5'-GATACGGCTACCTTGTTACGA -3' primers. The PCR program was performed in a Techne FTC51S5D Thermocycler (Staffordshire, UK) for 35 cycles under the following temperature profile: 95°C for 3min (1 cycle); 93 °C for 45 s, 58 °C for 1min, and 72 °C for 90 s (35 cycles); and 72 °C for 10 min at the end of the final cycle. The PCR product was purified using a GF-1 PCR Cleanup kit (Vivantis technologies, Malaysia). The purified products were sequenced using the below primers:

27f: 5'-GAGTTTGATCCTGGCTCAG -3', 16r339: 5'-ACTGCTGCCTCCCGTAGGAG -3'

16f358: 5'-CTCCTACGGGAGGCAGCAG-3', 704f: 5'- GTAGCGGTGAAATGCGTAGA- 3'

1505r: 5'-GATACGGCTACCTTGTTACGA -3'

The Sanger's dideoxy chain-termination sequencing method was used with an ABI 3730XL DNA analyzer (Thermo Fisher Scientific Inc. USA). The similarities between 16S rRNA sequences were compared in the National Center for Biotechnology Information (NCBI), Ribosomal Database. The phylogenetic tree based on 16S rRNA gene sequence was created against different related strains according to the partial sequence analysis. The evolutionary distances were calculated using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 28 nucleotide sequences. All locations with less than 95% site coverage were eliminated (i.e. fewer than 5% alignment gaps), and missing data and ambiguous bases were allowed at any position (partial deletion option). Phylogenic dendrograms were generated through the neighbor-joining method with a bootstrap test (1,000replicates) using MEGA X (14, 15).

Primary Antibacterial Activity Determination by the LAB Isolates: The antibacterial activity of the isolated lactic acid bacteria was determined using the spot-on lawn assay method suggested by Denkova (16) with some modifications. The lactic acid bacteria isolates were grown in MRS broth overnight. Then, cell-free supernatants (CFS) were collected by centrifugation (10000 g at 4 °C for 10min), and filter sterilized using a 0.2µ Whatman membrane filter, Merck Darmstadt, Germany**.** Then, MRS agar plates were overlaid with 5 ml melted soft agar (0.7%) inoculated with 100 µl of an overnight culture of *M. luteus* PTCC 1408 (~ 10^8 CFU/ml) as the indicator strain. Then, 20 µl of each crude filtered CFS (pH 4.7) of *Lactobacillus* strains was spotted on the medium inoculated with the test strain. The incubation was performed at 37°C for 24h. The clear zones surrounding each spot indicate the antibacterial activity of compounds produced by LAB strains against *Micrococcus luteus* PTCC 1408.

Determination of the Antibacterial Activity of the Produced Bacteriocin by LAB Isolates against some Selected Food-borne Pathogens: For the determination of the antibacterial activity of the bacteriocin produced by the selected *Lactobacillus* isolates, *the spot-on lawn and agar disk diffusion methods* were used against some selected food-borne pathogens. The selected food-borne pathogens including Salmonella *enterica* subsp. enterica serotype Typhimurium PTCC 1709, *Staphylococcus aureus* PTCC 1113, *Listeria monocytogenes* PTCC1302, and *Listeria inovani* PTCC 1303 were obtained from the Persian Type Culture Collection (PTCC, Tehran, Iran). For the spot-on lawn method, *Lactobacillus* strains were

grown in MRS broth (Merck Co. Darmstadt, Germany) at pH 6.8 and maintained aerobically at 35 °C for 48 h. The bacterial cells were removed from the growth medium by centrifugation (10000X g for 30 min at 4 °C) and passed through the Whatman membrane filter (0.2 µm diam. 47 mm). Then, pre-poured MRS agar plates were overlaid with 5ml melted soft agar (0.7%) inoculated with 100 µl of overnight cultures (~ 10^8 CFU/ml) of the selected food-borne pathogens. In the next step, 20 µl of each crude filtered *Lactobacillus* isolates was spotted on the MRS media. For the agar disk diffusion, the method described by Balouiri et al. (2016) was used (17). Agar plates containing MRS medium were inoculated with 100µl of test strains and streaked using a glass spreader. Next, blank filter paper discs (about 6 mm in diameter) were placed on the agar surface. Then, 20 µl of each crude filtered *Lactobacillus* isolates containing were added to filter paper discs.

The incubation was performed at 37 °C for 24h. The clear zones surrounding each spot indicate the antibacterial activity regarding the bacteriocin production by LAB strains against selected foodborne pathogens. The activity of cell-free supernatant was described in arbitrary units per ml (AU/ml). A unit activity of the bacteriocin was defined as an arbitrary unit (18); 1 AU is a unit area of inhibition zone per unit volume, in this case, mm2/ml (19). The bacteriocin activity was determined using the following formula: Bacteriocin activity (mm2/ml) = Lz-Ls/V which the parameters are LZ = clear zone area (mm2), Ls = well area (mm2), and V = volume of the sample.

Determination of the antimicrobial activity of bacteriocin production by the MN-B isolate: The growth and antimicrobial activity of bacteriocin produced by the selected *P. acidilactici* sp. (MN-B) were evaluated against *Lactobacillus casei* ATCC 39392 using an MRS broth medium. Then, 250 mL of MRS broth at pH 6.8 was inoculated with (1% v/v) cell suspension of *P. acidilactici* sp. (MN-B) and incubated at 37 °C without agitation in the presence of nitrogen for 48 h. Bacterial cell optical density, antimicrobial activity against *Lactobacillus casei* ATCC 39392 strain, and pH were measured every 2 and 4 hours during the fermentation process.

Determination of Minimum Inhibitory Concentration: For the determination of minimum inhibitory concentration, a serial dilution method for the crude bacteriocin, and selected antibiotics were used in Mueller Hinton broth (Sigma-Aldrich) medium, according to CLSI M7-A8 and M100-S28-2018 guidelines. Serial two-fold dilution (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µg/ml) of the cell-free extract of bacteriocin, and antibiotics (64, 32, 16, 8, 4, 2 and 1µg/ml) with bacterial concentration (10^8 CFU/ml 0.5 McFarland's standard) were used to determine MIC in MH broth. The control was only broth with the MN-B strain that was incubated for 24 h at 37 °C. The MIC endpoint is the lowest concentration of antimicrobial components where no visible growth is seen in the tubes. The turbidity of the tubes was distinguished, both before and after incubation to approve the MIC value.

Partial Purification of Bacteriocin Produced by the MN-B Strain: Bacteriocin produced by the MN-B isolate was purified using the adsorption-desorption method with some modifications (20).

For purification, the MN-B isolate was inoculated in 1-liter MRS broth (pH 6.5) and incubated at 37 °C for 24 h in a rotary shaker at 150 rpm. Samples of culture were taken at 12, 16, 18, 22, and 26h of incubation, respectively. Each sample was heat-killed and cooled at room temperature. Then, the pH culture was adjusted to 5.5-5.6 by 10 (N) NaOH and incubated at 37 °C

for 4h in a rotary shaker at 150 rpm, in order to adsorb the bacteriocins to the producer cells. In the next step, cells were centrifuged at 15,000 rpm for 6 min at 4 °C, then washed twice with sterile 5mM sodium phosphate buffer pH 5.5 and again centrifuged at 15,000 rpm for 6 min at 4 °C. Then, pellets were resuspended in 100 mM NaCl buffer (pH 1.5), the pH of which was adjusted with 5% phosphoric acid and stirred for 2 h at 4 °C. The cell-free supernatant (CFS) was collected by centrifugation and dialyzed in a dialysis bag (Mol. Cut off 1.0 kDa, Sigma, USA) and used as partially purified bacteriocins. In each step of purification, the number of proteins was quantified by the Bradford method using bovine serum albumin (BSA) as standard (18).

SDS–PAGE Analysis: Partially purified bacteriocin produced by the isolate MN-B was concentrated ten-fold and separated by the Lammeli method using 15% separating gel and 5% stacking gel with acrylamide and bis-acrylamide ratio 30:1 under denaturing conditions (21). Then, 30µl of partially purified bacteriocin was mixed in a 4:1 ratio with denaturing sample buffer and loaded in a well.

Electrophoresis was performed at 20 mA for the first 20 min and at 25 mA for the next 1 h. Then, the gel was removed and stained with Coomassie brilliant blue R-250. For confirmation of the protein bands as bacteriocins in the SDS-PAGE analysis, an activity gel study was performed. The gel was washed in sterile double distilled water containing 0.1% Tween 80 for 24 and used for antimicrobial activity assessment. Then, the gel was placed on a pre-poured MRS agar plate and overlaid with 10 ml melted MRS soft agar (0.6%, w/v) seeded with *Lactobacillus casei* ATCC 39392 as the sensitive indicator strain. The plate was incubated at 37 °C for 24 h and a zone of growth inhibition was observed (12, 20, 21).

Statistical Analysis: In this study, all data were expressed as mean ± standard deviation (SD) of triplicate independent experiments. All statistical evaluations were performed with the SPSS/PC software, version 20.0. The T-student test was used to evaluate the significance of the obtained data. *P*-value $\leq$0.05 considered significant.

Results

In the present study, nine *Lactobacillus* strains were isolated from twenty vacuum-packed organic meat sausages. Based on macroscopic and microscopic characteristics, all the nine isolates were Gram-positive long and slender bacilli or bent and short often-coryneform coccobacilli. In the primary screening, five strains showed antibacterial activity against *M. luteus* PTCC 1408. These isolates were designated as MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3, respectively. Zones of inhibition against *M. luteus* PTCC 1408 grown on Mueller-Hinton agar medium for the isolated strains MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3 are shown in Figure 1. The highest antibacterial activities belonged to the MN-B, MN-A, and MN-A2 isolates, respectively.

Biochemical characteristics of the five isolates along with the reference strains were provided in Table 1. According to the obtained morphological and biochemical properties, the isolates were related to the species of *Pediococcus acidilactici* (MN-A and MN-B) and *Leuconostoc mesenteroides (*MN-A2, MN-A2-1, and MN-A3), respectively.

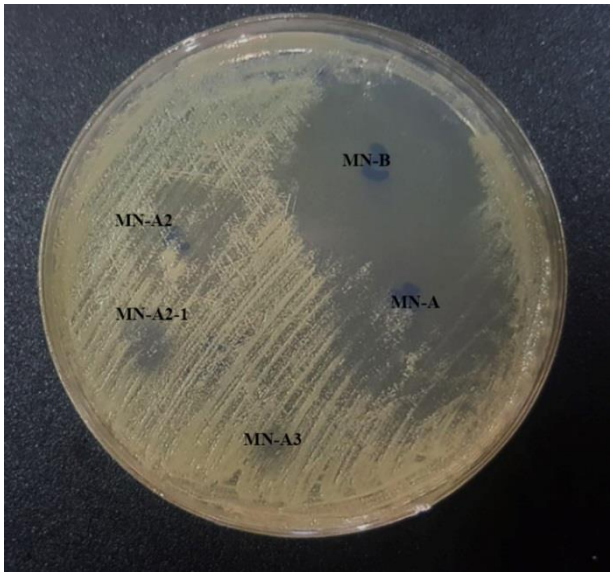

Fig. 1- Antibacterial activity of MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3 *Lactobacillus* strains isolated from organic meat sausages using spot-on lawn assay on MRS agar against *Micrococcus luteus* PTCC 1408.

Table 1- Phenotypic and Biochemical Characteristics of *Pediococcus* spp. and *Leuconostoc* spp. isolated from Organic Meat Sausages and Reference Strains

Characteristics	MN-A2	MN-A	MN-A3	MN-A2-1	MN-B	*Pediococcus acidilactici* PTCC 1602	*Leuconostoc mesenteroides* sub sp. *mesenteroides* PTCC 1059
Catalase	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-
Growth at							
pH 4.5	+	+	+	+	+	+	+
pH 7.0	+	+	+	+	+	+	+
pH 8.0	+	+	+	+	+	+	+
pH 9.0	-	-	-	-	-	-	-
35°C	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+
48°C	+	+	+	+	+	+	+
Acid from							
Lactose	d	+	d	d	+	+	+
Cellobiose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+
Ribose	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-
L-Arabinose	+	+	+	+	+	+	+
Maltose	+	-	+	+	-	-	+
Xylose	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-

Symbols and abbreviations: +, 90% or more of strains are positive; −, 90% or more are negative; d, 11–89% of strains are positive

Agarose gel electrophoresis of PCR product based on 16S rRNA gene amplification analysis of the isolates provided in Figure 2. For all five isolates, a sequence of about 1500 nucleotides was obtained.

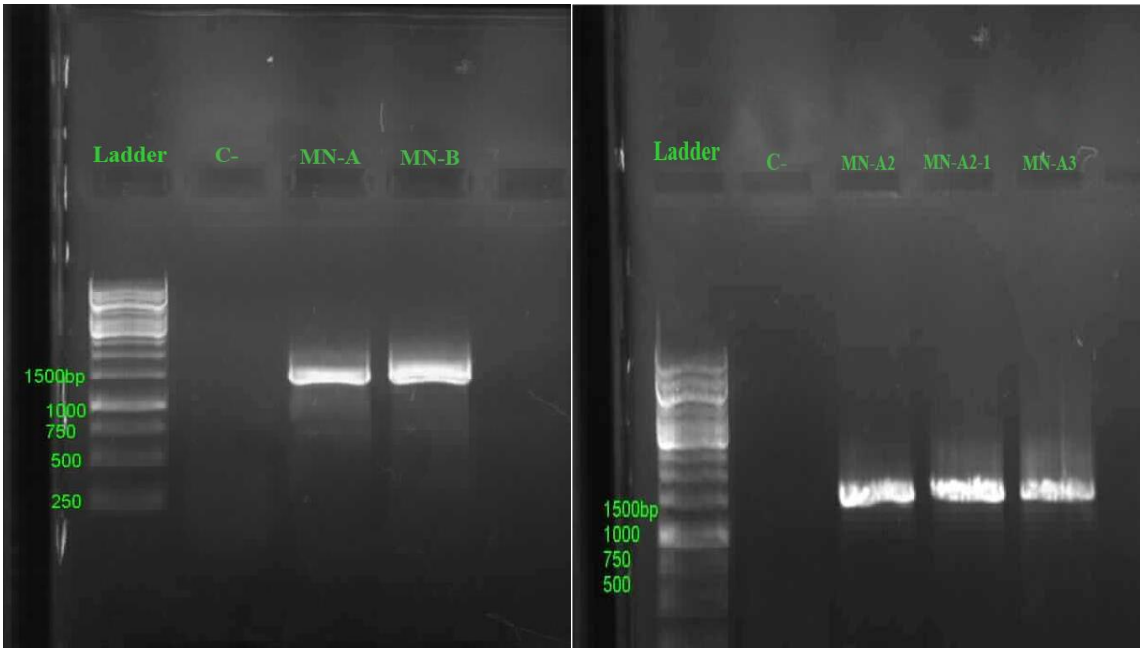

Fig. 2- Agarose gel electrophoresis of amplified 16S rRNA gene PCR products of the isolates MN-A and MN-B (left), MN-A2, MN-A2-1, and MN-A3 (right). Each well of the samples in lanes regarding the LAB isolates contains 5µl of DNA samples and 0.3µ of loading buffer.

The similarity determination partial sequencing and blast analysis for the five isolates was performed according to the National Center for Bioinformatic Information (NCBI) database. Phylogenic dendrograms of the isolates by the neighbor-joining (NJ) method and bootstrap analysis were provided in Figures 3a and 3b, respectively. Based on the obtained results, MN-A and MN-B strains showed the most similarity to the *Pediococcus acidilactici* strain as shown in Figure 3a. The MN-A2, MN-A2-1, and MN-A3 strains showed the most similarity to *Leuconostoc mesenteroides* sub sp. *Mesenteroides* and *dextranicum*, respectively (Figure 3b). Hence, the 16S rRNA characterization proposed that they are new *Pediococcus* and *Leuconostoc* species isolated from organic meat sausages. The Persian Type Culture Collection (PTCC, Tehran, Iran) determined the public accession numbers of the isolates. MN-A and MN-B isolates were determined as the same strains of the species *P. acidilactici*, and the public accession number was considered only for the MN-B strain as *P. acidilactici* PTCC 1954. Strains MN-A2, MN-A2-1, and MN-A3 were the same strains for the species of *L. mesenteroides and* the public accession number was considered only for the MN-A2-1 strain as *L. mesenteroides* PTCC 1953. The nucleotide sequences of the MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3 isolates were deposited in the GenBank database with the accession numbers: MW425291.1, MW425295.1, MW425292.1, MW425294.1, and MW425293.1, respectively.

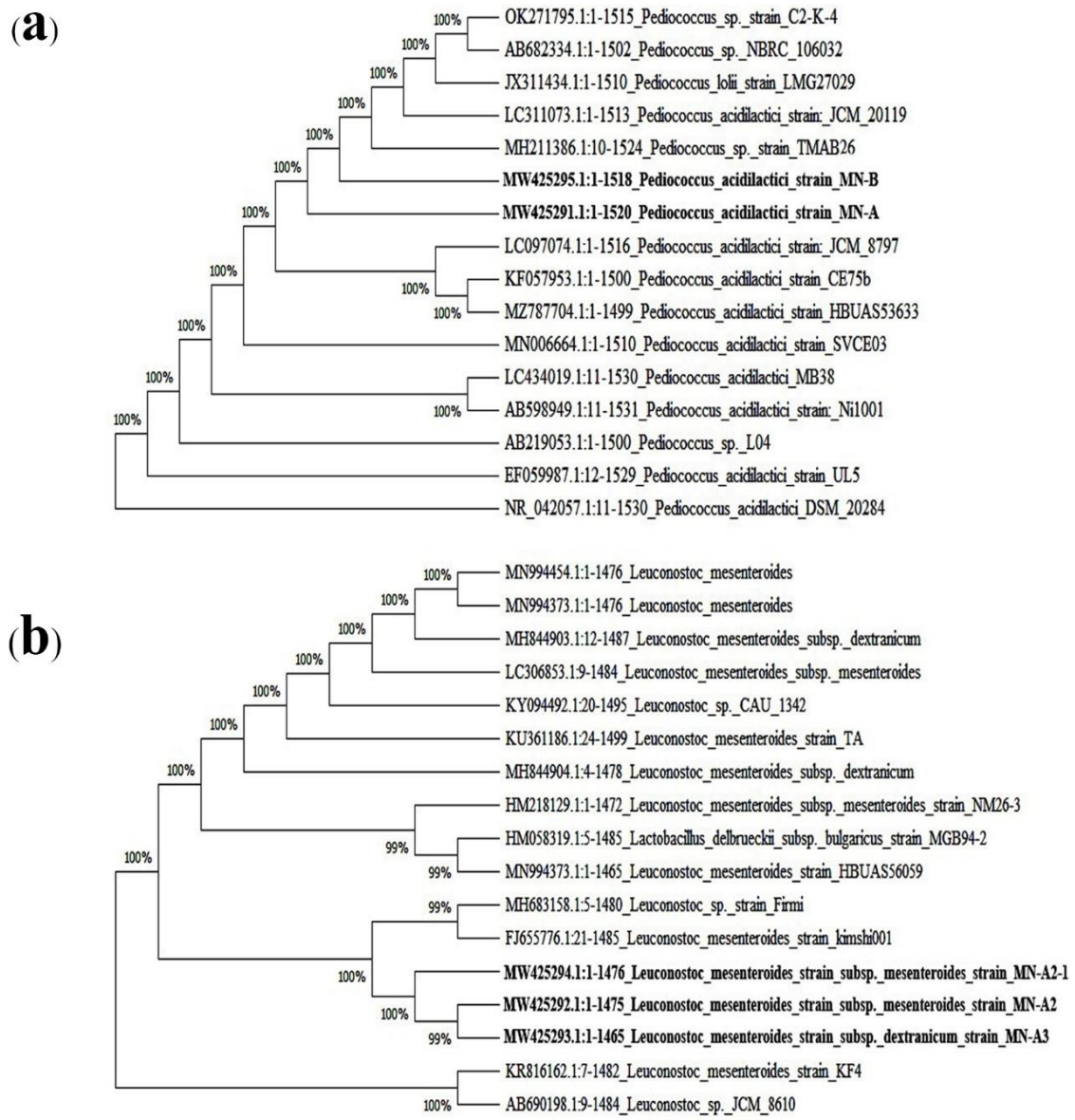

Fig. 3- Neighbor-joining trees based on partial 16S rRNA gene sequence analysis for the isolates. (a) for the *Pediococcus* isolates MN-A and MA-B in comparison to the most related *Pediococcus* strains, and (b) for the *Leuconostoc mesenteroides* isolates MN-A2, MN-A2-1, and MN-A3 in comparison to the most related taxa. Bootstrap values (expressed as percentages of 1000 replications) of 95% or more are shown at branch nodes.

Antibacterial Activity Determination of the Produced Bacteriocin by the Isolates against Foodborne Pathogens: Five isolates showed antimicrobial activity *against M. luteus* PTCC 1408 in *the spot-on lawn assay method. Also, the antimicrobial activity of the isolates was assessed against some selected foodborne pathogens including Salmonella enterica* subsp. enterica serotype *typhimurium* PTCC 1709, *Staphylococcus aureus* PTCC 1113, *Listeria monocytogenes* PTCC1302, and *Listeria inovani* PTCC 1303 using disk diffusion and spot-on lawn assay methods as shown in Figure 4. *Amongst the isolates, the strain MN-B (P. acidilactici) showed the highest antimicrobial activity against foodborne pathogens. Hence, this strain was selected for further studies.*

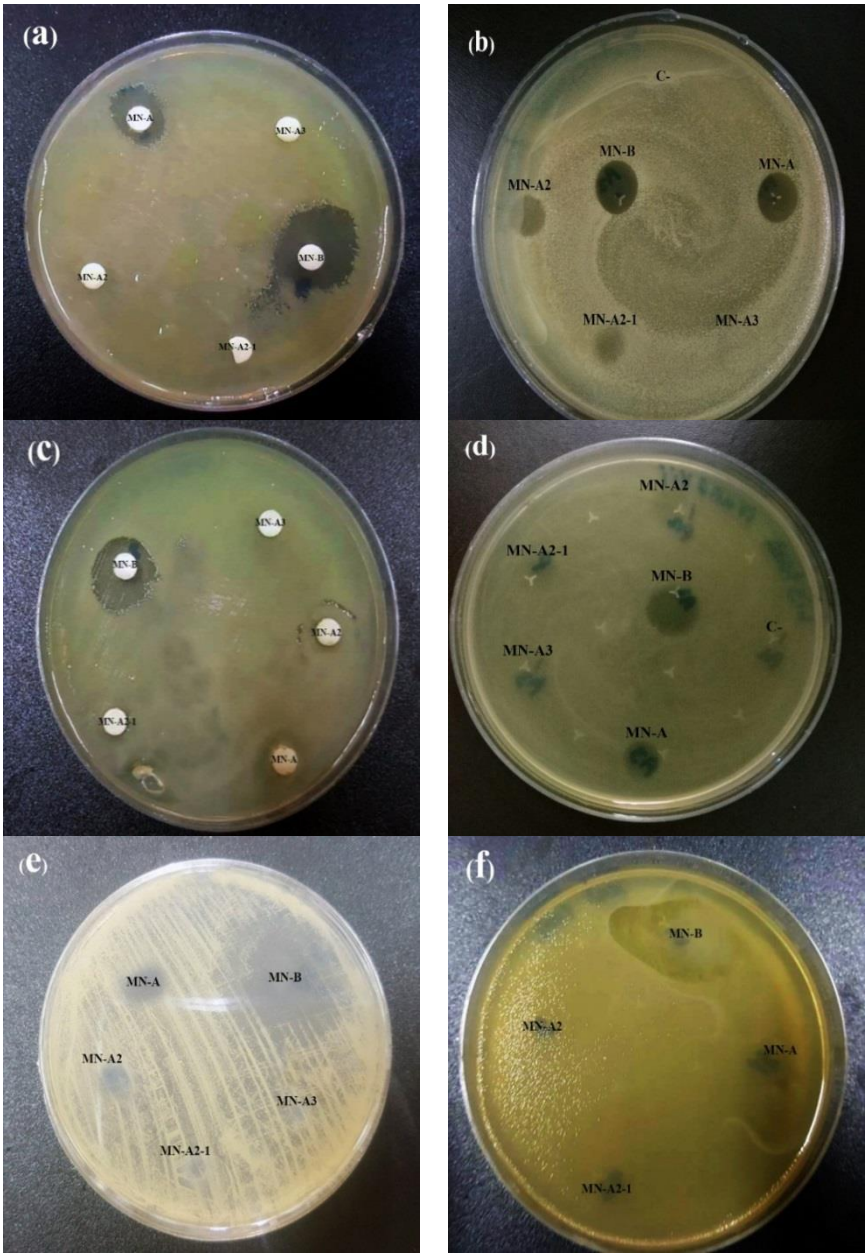

Fig. 4- The antibacterial activity of MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3 *Lactobacillus* strains isolated from organic meat sausages against some selected food-borne pathogens using disk diffusion and spot-on lawn assay methods. (a) *Listeria inovani* PTCC 1303 *(*disk diffusion), (b) *Listeria inovani* PTCC 1303 (Spot-on lawn), (c) *Listeria monocytogenes* PTCC1302 (disk diffusion), (d) *Listeria monocytogenes* PTCC1302 (Spot-on lawn), (e) *Salmonella enterica* PTCC 1709 (Spot-on lawn), and (f) *Staphylococcus aureus* PTCC 1113 (Spot-on lawn).

Determination of the Antibacterial Activity of the MN-B isolate: Figure 5 shows the growth parameters of *P. acidilactici* sp. (MN-B) strain in MRS broth at 37 °C and its antimicrobial activity against *Lactobacillus casei* ATCC 39392, based on sampling and assessing agar gel diffusion assay. As shown, the antibacterial activity is proportional to bacterial cell growth, and the dependence of antimicrobial activity on bacterial growth shows that it could be a primary metabolite. The antimicrobial activity starts at the early exponential growth phase and continues to the middle of the stationary phase at about 660 (AU/ml) at 18 h and pH 3.5.

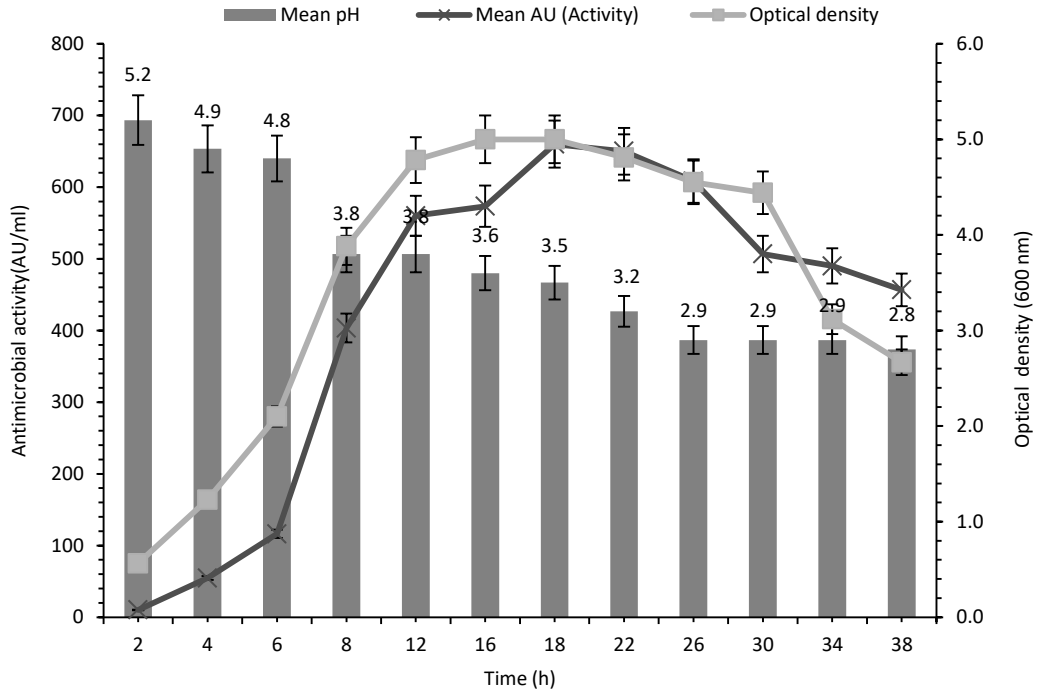

Fig. 5- Growth parameters and the antimicrobial activity of the isolate *P. acidilactici* sp. (MN-B) against *Lactobacillus casei* ATCC 39392 in MRS broth. All values are Mean ±SD of triple determination.

The minimal inhibitory concentrations of the crude bacteriocin produced by *Pediococcus acidilactici* strain MN-B against four-selected foodborne pathogens in comparison to the reference antibiotics were determined according to Table 2. Minimum inhibitory concentrations of bacteriocin against *L. monocytogenes* PTCC 1302 (p<0.05), *S. aureus* PTCC 1113(p<0.05), *L. inovani* PTCC1303 (p<0.05), and *S. enterica* PTCC 1709 (p<0.05) were 6.25, 25, 6.25, and 12.5 µg/l, respectively. *The antimicrobial compound produced by the isolate MN-B showed the highest inhibition against L. inovani and L. monocytogenes with MIC values of 6.25* (µg/mL) with (p<0.05) and (p<0.05), respectively.

Table 2- Minimum Inhibitory Concentration (MIC) Determination of Bacteriocin Produced by *Pediococcus Acidilactici* Strain MN-B and Reference Antibiotics (Control Group) against Four Selected Foodborne Pathogens ($\mu g/mL^{-1}$)

	Bacteriocin	Gentamycin	*P*-value	Tetracycline	*P*-value	Chloramphenicol	*P*-value
L.monocytogenes PTCC1302	6.25	2	0.002	4	0.002	2	0.008
S. aureus PTCC 1113	25	4	0.008	4	0.007	8	0.005
L.inovani PTCC1303	6.25	2	0.003	2	0.004	4	0.006
S.enterica PTCC 1709	12.5	4	0.005	4	0.009	8	0.007

P- Value ≤0.05 is significant

Purification of bacteriocin produced by the MN-B isolate: The result of partial purification of the bacteriocin produced by the MN-B strain using the adsorption-desorption method is provided in Figure 6. The purified fractions in the different time courses of incubation times at 12, 16, 18, 22, and 26h were used in the SDS-PAGE analysis. The obtained molecular mass of the bacteriocin was about 5.0 KDa.

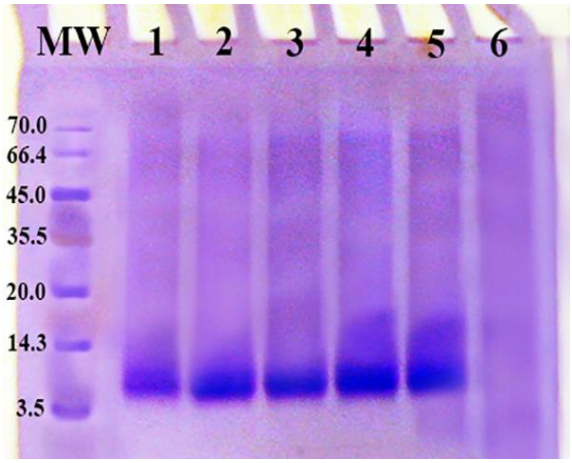

Fig. 6- Determination of molecular weight of bacteriocin produced by the *P. acidilactici* sp. (MN-B) strain using SDS-PAGE analysis. Lane MW is a molecular size marker (kDa). Lane 1-5 shows purified bacteriocin samples at 12, 16, 18, 22, and 26h of incubations, respectively. Lane 6 is negative control. The molecular size of the bacteriocin is about 5.0KDa.

Discussion and Conclusion

We isolated nine *Lactobacillus* strains from twenty vacuum-packed organic meat sausages. The isolates were Gram-positive long and slender bacilli or coccobacilli. The antimicrobial activity of the isolates was assessed against *M. luteus* PTCC 1408. The strains MN-B, MN-A, and MN-A2 showed the highest antibacterial activity, respectively. The colony morphology of the two isolates (MN-A and MN-B) was white, circular, convex, almost the entire margin, smooth, and shiny with the sizes of 2-3 mm on MRS agar. The cells were nonmotile, spherical, occasionally ovoid, and non-spore-forming. By analyzing morphological characteristics, these strains resembled the *Pediococcus spp.* The morphology of the *Pediococcus* strains is similar to that of *Tetragenococcus* species, and members of these two genera are the only lactic acid bacteria (LAB) dividing in two vertical directions resulting in the formation of pairs and tetrads, but never of typical chains. The coccoid cells are round and infrequently ovoid, in contrast to other coccus-shaped lactic acid bacteria such as *Leuconostoc*, *Lactococcus* and *Enterococcus* species. The cell size may differ from 0.6–1.0 μm in diameter, depending on the strain and species, but remains uniform within a culture.

The colony morphology of the MN-A2, MN-A2-1, and MN-A3 isolates were creamy, circular, entire margin, convex, smooth, shiny, and opaque with the sizes of 1-1.5 mm on MRS, respectively. Cells are non-spore-forming, straight, slender rods 2.5–6.5 μm long and <1.0 μm wide, usually occurring singly or as pairs but sometimes in older cultures as short chains. The features of MN-A2, MN-A2-1, and MN-A3 strains resembled the *Paralactobacillus* genus.

Based on 16S rRNA analysis, *Paralactobacillus* forms a separate branch of lactic acid bacteria and is most related to the genera *Lactobacillus* and *Pediococcus*. The highest sequence similarities are found with the *Lactobacillus casei*/*Pediococcus* cluster ranging from 90.1 to 91.7% and up to 93.1% with *Lactobacillus pantheris*. The most related species for the isolates MN-A and MN-B according to the obtained blast in NCBI were *Pediococcus acidilactici strain JCM 87979 (16S rRNA partial sequence), Pediococcus acidilactici strain JCM 20119(16S rRNA partial sequence), and Pediococcus lolli* strain LMG 27029 *(16S rRNA partial sequence) with 100% similarity as shown in Figure 3a. For the* MN-A2, MN-A2-1, and MN-A3 strains, the most similar strains were *Leuconostoc mesenteroides* strain *KF4 (16S rRNA partial sequence), Leuconostoc* sp. JCM *(16S rRNA partial sequence)*, 8610, and *Leuconostoc* sp. strain Firmi *(16S rRNA partial sequence)* with 100% similarity as shown in Figure 3b.

The MN-A and MN-B isolate both were the same strains of *Pediococcus acidilactici* species. So, the MN-B strain was deposited in PTCC with the public accession number of *P. acidilactici* PTCC 1954. The MN-A2,

MN-A2-1, and MN-A3 isolates were the same strains for *Leuconostoc mesenteroides.* So, the MN-A2-1 strain *was* deposited in PTCC with the public accession number of *P. acidilactici* PTCC 1953.

Based on the obtained results, the strain MN-B (*P. acidilactici*) showed the highest antimicrobial activity against foodborne pathogens in disk diffusion and spot-on lawn assay methods. Then, the antimicrobial activity of MN-B isolate was evaluated in the submerged culture condition using MRS broth. The production of antimicrobial compounds starts at the beginning of the exponential phase and increases upon the increase of cell density. Decreasing bacteriocin activity was observed in the middle of the stationary phase at 22h of incubation. Therefore, it can be concluded that the strain MN-B produces a primary metabolite such as bacteriocin as an antimicrobial compound. Many lactic acid bacteria such as *Lactobacillus sakei* produce bacteriocin in a cell density-dependent manner (21). For example, nisin and pediocin are typically primary metabolites produced in the exponential phase of growth (22, 23). The results obtained in this study were inconsistent with other studies. Our results showed that the antimicrobial activity decreased after 22h of incubation time. It can be concluded that proteolytic enzymes in the late stationary phase could degrade possible bacteriocin antimicrobial compounds in the culture medium. Also, the bacteriocin produced by *P. acidilactici* strain MN-B showed lower minimum inhibitory concentration against *L. monocytogenes* PTCC 1302 ($p<0.05$) and *L. inovani* PTCC1303 ($p<0.05$) in comparison to the other two test strains. The antibacterial activity of bacteriocin against *all selected* foodborne pathogens in comparison to the reference antibiotics was statistically significant ($p\leq 0.05$).

In this study, partial purification of the bacteriocin produced by the MN-B strain using the adsorption-desorption method showed 3 times more bacteriocin recovery from the strains, equivalent to 1100 AU/ml, as compared to the initial heat-killed culture aliquot activity of 200 AU/ml against *Lactobacillus casei* ATCC 39392. The bacteriocin produced by the MN-B strain showed maximum adsorption onto the cells at pH between 5.2 and 5.5 and pH and desorption at pH between 1.5 and 1.8. Many bacteriocin *Lactobacillus* producer strains such as *Pediococcus*, *Lactobacillus*, and *Leuconostoc* can absorb bacteriocins into their cells. In addition, it should be noted that the components in the culture medium such as non-ionic detergents can affect desorption in the purification process. The molecular weight of bacteriocin was about 5.0 KDa by SDS-PAGE analysis in each sample obtained at different times of the culture as shown in Figure 5.

A novel antimicrobial producing *Pediococcus acidilactici* LAB 5 was isolated from a vacuum-packed fermented meat product by Mandal et al. (2011) (12). This compound showed antimicrobial activity against some species of *Enterococcus*, *Leuconostoc*, *Staphylococcus*, and *Listeria*, many of which are associated with food spoilage. The antimicrobial compound was affected by proteolytic enzymes, but resistant to heat, and it was identified as a bacteriocin (Pediocin NV5) with a molecular weight of 10.3 kDa (24).

A bacteriocin-producing *Leuconostoc citreum* ST110LT was isolated from organic farm soil by C. Woo, et al. (2012). This bacteriocin effectively inhibited the growth of exponentially growing *L. monocytogenes* ATCC 15313 and other *Listeria* spp (11).

In a study by Charan Dey et al. (2019), three different strains of *Pediococcus* sp.

viz. *Pediococcus* sp. LAB 33 (HQ185406), *Pediococcus* sp. LAB 41 (HQ185407), and *Pediococcus* sp. LAB 51 (HQ184064) were isolated and identified from traditionally fermented foods like salami, sausage of chicken, mutton, and pork without the addition of any natural preservatives obtained from local markets in India. Based on 16S rRNA sequencing and subsequent BLAST, all three strains belonged to the *Pediococcus acidilactici* species. Bacteriocin produced by these strains showed antimicrobial activity against *E. faecalis* MB1, *L. mesenteroides* Ly, *L. monocytogenes* MTCC 657, and *P. aeruginosa* MTCC 741 based on the spot-on lawn method, and at a minimum concentration of ~100 AU/ml. The bacteriocin from these three strains was partially purified by the adsorption-desorption method and tested for autoclave heat, pH, detergent, and enzyme stability. The bacteriocins produced by the strains were resistant to autoclave heat, detergent, and a wide range of pH (23).

A new dairy strain of *Pediococcus pentosaceous* NCDC 273 was used for the production of bacteriocin (24). The produced bacteriocin was identical to pediocin PA-1 at nucleotide sequence level with the optimum activity at initial pH of 6.0 and 7.0 in basal MRS medium. The results showed that the pediocin PA-1 produced by *P. pentosaceous* NCDC 273 in the MRS medium has optimum activity at 20 h and 24 h with initial pH 6.0 and 7.0 at 20 g/l with glucose or lactose, respectively. Purification of bacteriocin using ammonium sulfate precipitation and cation-exchange chromatography showed a yield of 134.4% with more than 320-fold purification with the specific activity of 19 $\times 10^5$ AU/mg, and 4.6 kDa molecular weight (24).

A pediocin produced by *P. acidilactici* MM33 was purified by cationic exchange chromatography and a reverse phase step. Biochemical and mass spectrometry analysis of the purified protein showed homology with pediocin PA-1 and bactericidal activity against *Listeria monocytogenes*, with a minimal inhibitory concentration (MIC) of 200 AU/ml.

The strain *P. acidilactici* MM33 was isolated from human stool, and the indicator strain *Lactobacillus sakei* ATCC 1552 was very sensitive to the antimicrobial molecule secreted by this bacterium (25).

A *P. acidilactici* strain was isolated from fermented milk products (curd) by Nakvr et al. (2014). Bacteriocin produced by this strain showed broad-spectrum antimicrobial activity against many food pathogens (26). The bacteriocin was isolated by cell adsorption-desorption and followed purification by using Sephadex G-25 column, with an approximate molecular weight of 5.5 kDa. The produced bacteriocin was sensitive to proteolytic enzymes and stable over temperature ranges of (70 to 90 °C) and pH (7.0 to 7.0).

In conclusion, in this study, five lactic acid bacteria with antibacterial activity were isolated from vacuum-packed organic meat sausages. Amongst the isolates, the strain MN-B showed the highest antibacterial activity against selected food-borne pathogens. This isolate was identified as *Pediococcus acidilactici* based on morphological, biochemical, and 16S rRNA analysis, and deposited in the Persian Type Culture Collection with the accession number of *Pediococcus acidilactici* PTCC 1654. Showing significant antibacterial activity, this strain can be considered a potential candidate in different sectors such as food industries as a preservative. In addition, further studies regarding the properties of produced bacteriocin by *Pediococcus acidilactici* strain MN-B are recommended.

Acknowledgments

The laboratory facilities for microbial experiments were provided by Lahijan Branch, Islamic Azad University, Lahijan, Iran. The authors would like to thank Dr. Farzaneh Aziz Mohseni the Director of the Persian Type Culture Collection (PTCC, Tehran, Iran) for her valuable assistance in the identification of the isolates and assigning public accession numbers for the strains.

Author Contribution

This study was performed as a part of a Ph.D. Thesis addressed to Mina Nasiri Moslem. MNM, MFG, NAM, and NR contributed almost equally to this study. MFG, NAM, and NR designed the study, MNM did the experiments and performed research; MFG, NAM, and NR analyzed all data and wrote the paper.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors, and the authors financially supported this work.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The GenBank accession numbers for the 16S rRNA gene sequence of the MN-A, MN-B, MN-A2, MN-A2-1, and MN-A3 isolates are MW425291.1, MW425295.1, MW425292.1, MW425294.1, and MW425293.1, respectively.

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