OKU Fen Bilimleri Enstitüsü Dergisi
8(3): 1453-1463, 2025

OKU Journal of The Institute of Science and Technology, 8(3): 1453-1463, 2025

Osmaniye Korkut Ata Üniversitesi Fen Bilimleri Enstitüsü Dergisi

Osmaniye Korkut Ata University Journal of The Institute of Science and Technology

Isolation and Identification of Bacteriocin Producers from Bayburt Local Fermented Dairy and Characterization of Bacteriocins

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Research Article

Article History:
Received: 09.08.2024
Accepted: 18.03.2025
Published online:16.06.2025

Keywords:
Antimicrobial
Bacteriocin
Bayburt
Fermented dairy
Lactic acid bacteria (LAB)

ABSTRACT

Lactic acid bacteria (LAB) have antimicrobial activity, in addition to other antimicrobial agents, mainly due to the bacteriocins that they produce. Using bacteriocins in the production of foods has considerable contributions to improving food safety and extending shelf life. One of the essential characteristics of bacteriocins is that they have structural variations according to the variety of producers and, accordingly, they have different antimicrobial activity spectrums. Therefore, new bacteriocins still need to be investigated and characterized. In this study, bacteriocin-producing LAB were isolated from the local fermented dairy of Bayburt and these bacteria were identified. The antimicrobial spectrum of bacteriocin-producing strains was then determined and their sensitivity to different temperatures and enzymes was characterized. Bacteriocin-producing LAB were identified as *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Enterococuus faecium*, respectively. These strains were found to be antibacterial against 7, 6 and 4 different indicators, respectively. All bacteriocins produced by LAB were found to lose their bacteriocin-derived activity when treated with proteolytic enzymes and to be in protein structure. They were found to be stable after 100 °C for 10 minutes, 80 °C for 10 minutes and 80 °C for 15 minutes heat treatment, respectively, they retained their antimicrobial activity.

Bayburt Yöresel Fermente Süt Ürünlerinden Bakteriyosin Üreticilerinin İzolasyonu, Tanımlanması ve Bakteriyosinlerin Karakterizasyonu

Araştırma Makalesi

Makale Tarihçesi:
Geliş tarihi: 09.08.2024
Kabul tarihi: 18.03.2025
Online Yayınlanma:16.06.2025

Anahtar Kelimeler:
Antimikrobiyal
Bakteriyosin
Bayburt
Fermente süt ürünü
Laktik asit bakterileri (LAB)

ÖZ

Laktik asit bakterileri (LAB), diğer antimikrobiyallerin dışında özellikle ürettikleri bakteriyosinler nedeniyle de antimikrobiyal aktiviteye sahiptir. Bakteriyosinlerin gıda üretiminde kullanılması, gıda güvenliğinin iyileştirilmesi ve raf ömrünün uzatılmasına önemli katkılar sağlamaktadır. Bakteriyosinlerin temel özelliklerinden biri, üreticilerin çeşitliliğine göre yapısal varyasyonlara sahip olmaları ve buna bağlı olarak farklı antimikrobiyal aktivite spektrumlarına sahip olmalarıdır. Bu nedenle, yeni bakteriyosinlerin araştırılması ve karakterize edilmesi gerekmektedir. Bu çalışmada, Bayburt'un yerel fermente süt ürünlerinden bakteriyosin üreten LAB izole edilmiş ve bu bakteriler tanımlanmıştır. Daha sonra bakteriyosin üreten suşların antimikrobiyal spektrumu belirlenmiş ve farklı sıcaklık ve enzimlere karşı duyarlılıkları karakterize edilmiştir. Bakteriyosin üreten LAB sırasıyla *Lactobacillus plantarum*, *Pediococcus pentosaceus* ve *Enterococuus faecium* olarak tanımlanmıştır. Bu suşların sırasıyla 7, 6 ve 4 farklı indikatöre karşı antibakteriyel olduğu bulunmuştur. LAB tarafından üretilen bütün bakteriyosinlerin proteolitik enzimlerle muamele edildiklerinde bakteriyosinlerden kaynaklı aktivitelerini kaybettikleri ve protein yapısında

oldukları belirlenmiştir. Bunların sırasıyla, 100 °C 10 dakika, 80 °C 10 dakika ve 80 °C 15 dakika ısıl işlem sonrasında stabil olduğu, yani antimikrobiyal aktivitelerini koruduğu tespit edilmiştir.

To Cite: Kaya HI. Isolation and Identification of Bacteriocin Producers from Bayburt Local Fermented Dairy and Characterization of Bacteriocins. Osmaniye Korkut Ata Üniversitesi Fen Bilimleri Enstitüsü Dergisi 2025; 8(3): 1453-1463.

1. Introduction

The use of antimicrobials in the food industry is essential in terms of both reducing microbial load and extending shelf life. These substances prevent the risk of microbial spoilage or disease and enable the production of safer foods. The use of chemical preservatives at an industrial scale in food production is widespread. However, increasing consumer sensitivity to chemical food additives prioritizes studies on the detection of natural antimicrobial substances. Lactic acid bacteria (LAB), the main members of the microflora of many fermented foods, also have important potential for food preservation with the bacteriocins they produce. Due to the rapidly increasing microbial resistance to bacteriocins allowed in food production and the insufficient antimicrobial spectrum of these bacteriocins, identification and characterization of new different bacteriocins has become imperative. All living beings produce antibacterial proteins, many of which are called antibacterial peptides due to their small molecular mass (Chikindas et al., 2018). Bacteriocins are antibacterial metabolites in peptides or proteins synthesized ribosomally by various bacteria and secreted extracellularly into the environment. They generally inhibit closely related species (Zou et al., 2018). Gram-positive, and Gram-negative bacteria and archaea have also been found to produce bacteriocins (Juturu and Wu, 2018). Bacteriocins act by inhibiting or stopping the growth of prokaryotes and are also effective against some antibiotic-resistant pathogens. (Zou et al., 2018). Many different bacteria produce bacteriocins; they have been reported to be effective against bacteria that are pathogenic to humans and animals, especially vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA), and do not show toxicity (Ahmad et al., 2017, Zou et al., 2018). Bacteriocins also promote the growth of beneficial bacteria (Balciunas et al., 2013). The first bacteriocin identified was colicin, an antibacterial protein produced by *Escherichia coli* in a study conducted by Gratia in 1925 (Cavera et al., 2015, Juturu and Wu, 2018). In recent years, the search for alternative bacteriocins to other commercially used bacteriocins has been ongoing. Detailed biochemical and genetic characterization of pediocin and lacticin bacteriocins produced from *Pediococcus acidilactici* and *Lactococcus lactis* strains have been completed and they are two bacteriocins ready for industrial use (Papagianni and Anastasiadou, 2009; Bierbaum and Sahl, 2009). However, the isolation of different bacteriocin-producing strains from fermented foods rich in LAB and the characterization of the bacteriocins produced need to be continued and new bacteriocins need to be discovered.

The use of bacteriocins for food preservation began with their authorization by the US Food and Drug Administration (FDA). Today, nisin is used in the preservation of many foods, especially milk and dairy, canned foods and baby food. However, it has become clear that some fundamental problems limit its use as a preservative in foods. The most important of these problems are; high production costs due to

low production rates by producer strains and rapid resistance to pathogenic and spoilage-causing microorganisms in the microflora of foods. The isolation of LAB with different characteristics from local fermented foods with rich diversity and the determination of their antimicrobial properties is one of the most effective ways to solve this problem. To date, many bacteriocins produced by LAB have been identified. These studies have shown that the antimicrobial activities of bacteriocins have strain specificity and have led to the thesis of using bacteriocins specific to pathogens found in food systems (Hill et al., 2011). Therefore, the identification of new bacteriocins is one of the main targets to stop new pathogens that have developed in recent years. In this study, isolation and identification of bacteriocin-producing LAB from Bayburt local fermented milk products and characterisation of bacteriocins produced by LAB were carried out in the light of suggestions that may constitute a solution to some of the problems listed above.

2. Material and Methods

2.1. Microorganisms and growth conditions

Indicator bacteria (Table 1) used in the study were obtained from Pamukkale University Food Engineering Culture Collection (PUFECC). All strains were stored at -80 °C in glycerol with a final concentration of 30%. Indicators were grown in Brain Hearth Infusion (BHI, Merck, Darmstadt, Germany) medium incubated at 30 and 37 °C for 18 hours. Among LAB strains, lactobacilli were grown in de-Man Rogosa and Sharpe (MRS, Merck, Darmstadt, Germany) and lactococci were grown in M17 Glucose (M17G, 0.5% glucose, Merck, Darmstadt, Germany) medium supplemented with 0.5% glucose at 30 °C.

Table 1. Indicator bacteria used in the study

Code	Indıcator bacteria
PUS1	*Micrococcus luteus* DSM1790
PSC16	*Listeria monocytogenes* ATCC7644
PSC19	*Staphlococcus aureus* ATCC25923
PSC22	*Escherichia coli* ATCC25922
PSC25	*Enterobacter cloacae* ATCC23355
PSC31	*Enterococcus faecalis* ATCC19433
PSC37	*Pseudomonas aeruginosa* ATCC19433

2.2. Isolation of antibacterial LAB strains

A total of 30 cheese samples of 11 different varieties and 4 different village butter samples were used for the isolation of antibacterial-effective LABs. M17G agar and MRS agar media were used for the isolation of lactococci, streptococci and lactobacilli, respectively. MRS and M17G agar media were inoculated with appropriate dilutions and incubated at 30 °C for 48 hours. After this step, soft agar medium inoculated with each indicator bacteria (*Micrococcus luteus* DSM1790-PUS1 and *Escherichia coli* ATCC25922-PSC22) was slowly poured onto the Petri dishes containing approximately 30

colonies. The petri dishes were then incubated at 37 °C for 24 hours. At the end of incubation, the colonies forming a clear zone around the colonies were removed with a sterile core and grown in MRS and M17G liquid medium for 48 hours at 30 °C and stored at -80 °C in glycerol with a final concentration of 30%.

2.3. Identification of LAB with MALDI-TOF-MS

For the identification of LAB, the direct transfer method using MALDI-TOF-MS was preferred (MALDI TOF/TOF-MS Biotyper System, Bruker Autoflex Speed, Billerica, MA, USA). The identification analysis was carried out by Bolu Abant İzeet Baysal University, BETUM (Scientific, Industrial and Technological Application and Research Center). Identification was performed from fresh colonies grown on a solid medium (12-18 hours). A single colony selected from the solid medium was smeared as a thin layer on the target spot and then allowed to dry at room temperature. Then 1 µL of matrix solution (HCCA Matrix Solution, Sigma Aldrich, St. Louis, Missouri, USA) was added and allowed to dry at room temperature. The spots prepared in this way was loaded into the device and the reading was performed (Nacef et al., 2017; Karasu-Yalcin et al., 2021).

2.4. Determination of the antibacterial spectrum of LAB isolates

The antibacterial activity of 3 LAB isolates, which were isolated from different cheeses and village butter and whose highest antibacterial activity was confirmed, against 7 different indicators was determined by well diffusion method (Tagg and McGiven 1971, Kaya and Simsek 2019). LAB isolates grown in the medium for 18 hours at 30 °C were subjected to centrifugation (Hettich Universal 30 RF, Kirchlengern, Germany) at 6000 g for 15 min. The cell-free culture supernatant (contain active substances and bacteriocins) was transferred to new tubes, taking care not to mix the precipitated solid phase, adjusted to pH 6 using 6N NaOH and passed through a 0,45 µm pore diameter membrane filter (Merck, Millipore, Burlington, Massachusetts, USA). Suspensions of indicator bacteria grown separately in BHI liquid medium were inoculated into 7 mL of soft BHI agar medium containing 0,7% agar and poured homogeneously as a second layer on plates containing Nutrirent agar (Merck, Darmstadt, Germany). Following the solidification of the agar, wells with a diameter of 5 mm were made using a sterile glass Pasteur pipette, and the filtered bacterial cell-free culture supernatant were filled into the wells at the rate of 100 µL. At the end of the incubation periods, the inhibition zone formation around the wells was examined and the results were recorded.

2.5. Determination of the sensitivity of antibacterial metabolites produced by LAB isolates to enzymes

Advanced identification of bacteriocins produced in bacteriocin-producing LAB strains was based on their behavior against different enzymes. The effect of enzymes on bacteriocin activity was characterized using the cell-free culture supernatant of these media. Trypsin (pH 7.0 Merck, Germany), α-chemotrypsin (pH 7.0 Sigma Chem. Co., St. Louis, Missouri, USA), proteinase K (pH 7.0 Sigma

Chem. Co., St. Louis, Missouri, USA), pepsin (pH 3.0 Sigma Chem. Co., St. Louis, Missouri, USA) and catalase (pH 7.0 Sigma Chem. Co., St. Louis, Missouri, USA) enzymes were added to neutralized culture cell-free culture supernatants at a final enzyme concentration of 1 mg mL-1 and incubated at 30 °C for 2 hours. Enzyme activities were terminated by heat treatment at 100 °C for 5 minutes. The effect of the enzymes was determined by testing samples of these mediums against susceptible indicator bacteria. Cell-free culture supernatant s without enzyme treatment were used as controls. The effect of enzymes on bacteriocin activity was determined by well diffusion method (Tagg and McGiven 1971; Kaya and Simsek, 2019). Bacteriocin activity was determined as described in section 2.4.

2.6. Determination of the sensitivity of antibacterial metabolites produced by LAB isolates to temperature

Advanced identification of bacteriocins produced in bacteriocin-producing LAB strains was based on their behavior against different temperature treatments. The effect of temperature on bacteriocin activity was determined by subjecting the medium to temperature treatment in a water bath (Memmert, Schwabach, Germany) at 80, 90, 100 °C for 5, 10, 15 minutes and in an autoclave (Hirayama HV-50L, Minato City, Tokyo, Japan) at 121 °C for 15 minutes and then testing the samples taken from this medium against susceptible indicator bacteria. Untreated cell-free culture supernatants were used as controls. The effect of temperature on bacteriocin activity was determined by the well diffusion method (Tagg and McGiven, 1971; Kaya and Simsek, 2019). Bacteriocin activity was determined as described in section 2.4.

3. Results

3.1. Isolation and identification of LABs

Milk and dairy constitute the most important source of bacteriocin-producing LAB. Many bacteriocin-producing LAB have been isolated from milk and fermented dairy from the past to the present. Our country has a very rich variety of milk and dairy. For this reason, cheese varieties and butter produced traditionally in Bayburt province were preferred for the isolation of bacteriocin-producing LAB in this study. LAB was isolated from 34 different fermented dairy. These consisted of 8 different farm cheeses, 4 different Civil cheeses, 4 different Göğermiş cheese, 4 different Kerti Lor cheese, 3 different Tulum cheese, 2 different fresh lor cheese, Kavram lor cheese, Kol cheese, Acı cheese, Sinop cheese, Kuymak cheese and 4 different farm butter.

As a result of the isolations, 2 isolates with antimicrobial activity against *E. coli* and 1 isolate with antimicrobial activity against *M. luteus* were obtained in MRS and M17G media (Table 2, Figure 1). These isolates were obtained from Civil and Tulum cheeses.

Table 2. Isolates with antibacterial activity

Dairy-Code	Isolation Code	Indicator
Civil Cheese P14	P14	PUS1
Tulum Cheese P22	P22-1	PSC22
Tulum Cheese P22	P22-2	PSC22

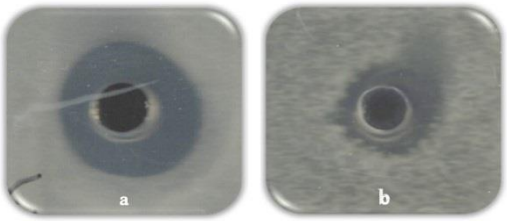

Figure 1. Antibacterial effect of cell-free culture supernatants of P14 (a) ve P22-1 (b) isolates against indicator bacterial strains (*M. luteus*, and *E. coli*)

LAB counts of cheese and butter samples were also performed on the medium used for the isolation of LAB. As a result of the counting performed for this purpose, the lowest number of lactococci and streptococci in M17G medium was 4.35, the highest was 7.69 and the average was 6.28 log CFU/g, while the lowest number of lactobacilli in MRS medium was 4.19, the highest was 7.36 and the average was 5.59 log CFU/g.

The MALDI BioTyper software, version 3.1 (Bruker Daltonics, Bremen, Germany) was used to process the raw spectra and to compare the spectra in order to classify the LAB strains. Strain P14 isolated from Civil cheese was identified as *Lactobacillus plantarum* (1.722 Score Value, NCBI Identifier 1590), while strains P22-1 and P22-2 isolated from Tulum cheese were identified as *Pediococcus pentosaceus* (1,989 Score Value, NCBI Identifier 1255) and *Enterococuus faecium* (2,002 Score Value, NCBI Identifier 1352).

3.2. Antibacterial spectrum of LAB strains

The antibacterial activity spectrum of 3 different isolates isolated from various fermented dairy against 7 different indicator bacterial species is given in Table 3.

Table 3. Antibacterial spectrum of the LAB strains

	Indicator bacteria diameter zone (mm)						
Isolate code	***M. luteus***	***L. monocytogenes***	***S. aureus***	***E. coli***	***E. cloacae***	***E. faecalis***	***P. aeruginosa***
L. plantarum	8.5	15	12.5	15	15	10.5	10.25
P. pentosaceus	-	10	10	11.5	10.5	9.5	9
E. faecium	-	8.5	-	9	9.25	-	8

Zone diameters: <1mm (no effect), 1-5 mm (low effect), 5-10 mm (medium effect),> 15 mm (high effect)

The cell-free culture supernatant of *L. plantarum* isolate from Civil cheese was found to be highly antibacterial against *L. monocytogenes, E. coli* and *E. cloacae* and moderately antibacterial against *M. luteus, S. aureus, E. faecalis* and *P. aeruginosa* strains. *P. pentosaceus* isolate isolated from Tulum cheese was found to be moderately antibacterial against six different indicators except *M. luteus* strain. *E. faecium* isolate, another isolate isolated from Tulum cheese, was found to be moderately antibacterial against *L. monocytogenes, E. coli, E. cloacae* and *P. aeruginosa*.

3.3. Effect of enzymes on cell-free culture supernatants *of LAB strains*

The activity changes of the antimicrobial metabolite produced by LAB strains isolated from Bayburt local dairy after treatment with different enzymes are shown in Table 4. Accordingly, the antimicrobial activity of the cell-free culture supernatants of all strains was found to decrease when treated with one or more of the proteolytic enzymes. On the contrary, the antimicrobial activity of the cell-free culture supernatant of all strains was maintained after treatment with the catalase enzyme. As a result of treatment with protease enzymes, activity losses occurred in cell-free culture supernatant and even disappeared completely. In particular, the loss of antimicrobial activity upon treatment with proteinase K enzyme indicates that this antimicrobial metabolite contains a protein-containing structure. Similarly, pepsin, trypsin and α-chemotryptipsin caused a decrease in antimicrobial activity in some cell-free culture supernatants, confirming the protein nature of the antimicrobial metabolite.

Table 4. Antibacterial activity of LAB strains after enzyme treatments.

Application→ / **Isolates ↓**	**Control**	**α Chemotrypsin**	**Pepsin**	**Proteinase K**	**Trypsin**	**Catalase**
L. plantarum	12.65	10.50	5.50	10.50	6.00	12.50
P. pentosaceus	17.15	12.50	13.50	12.50	14.50	17.25
E. faecium	8.65	7.50	7.50	8.50	7.50	8.50

Zone diameters: <1mm (no effect), 1-5 mm (low effect), 5-10 mm (medium effect),> 15 mm (high effect)

3.4. *Effect of temperatures on* cell-free culture supernatant*s of LAB strains*

The antimicrobial activity changes of the metabolite in the cell-free culture supernatant of LAB strains isolated from Bayburt local dairy at 80, 100 and 121°C temperatures and different time combinations are given in Table 5. As seen from this table, the antimicrobial metabolite in the cell-free culture supernatant of *L. plantarum* was stable at 80 and 100°C, and activity was lost at 121°C. The decrease in antimicrobial activity was also valid for the culture cell-free culture supernatants of *P. pentosaceus* and *E. faecium* strains. The antimicrobial activity of the cell-free culture supernatants of *P. pentosaceus* and *E. faecium* strains was lost at the end of all temperatures applied at 100 and 121°C.

Table 5. Antibacterial activity of LAB strains after heat treatment

		80˚C			100˚C			121˚C
Application→	**Control**	**5 min**	**10 min**	**15 min**	**5 min**	**10 min**	**15 min**	**15 min**
Isolates ↓								
L. plantarum	12.8	10.55	9.9	8.85	8	6	0	0
P. pentosaceus	10	8	6	0	0	0	0	0
E. faecium	19	15	13.5	11	0	0	0	0

Zone diameters: <1mm (no effect), 1-5 mm (low effect), 5-10 mm (medium effect),> 15 mm (high effect)

4. Discussion

In this study, isolation and identification of bacteriocin-producing LAB from Bayburt local fermented dairy was carried out for the first time. In this context, the activity spectrum of these bacteria was first determined and then the stability of bacteriocins against various temperature and enzyme treatments was investigated.

It has been reported in many studies that LAB isolated from different sources have various levels of antibacterial activity due to the various metabolites (lactic acid, H_2O_2, diacetyl and bacteriocin) they produce (De Vuyst and Leroy, 2007; Kaya and Simsek, 2019; Wang et al. 2024). However, the antimicrobial activity of LAB has generally been attributed to two main modes, including the effect of organic acid and bacteriocin. (Hassan et al., 2012; Chen et al. 2022). Due to the bacteriocins produced by LAB, research has focused on this subject, many studies have been carried out by researchers and thousands of bacteriocins and bacteriocin producers have been reported so far. On the other hand, the antibacterial spectrum varies depending on the diversity of bacteriocins produced by lactic strains. This is because bacteriocins show strain-specific properties depending on the mechanism of action (Dobson et al., 2012; Darbandi et al. 2022). In particular, the fact that three different isolates were effective against gram-negative indicator bacteria at medium and above levels shows that this study has achieved the desired results. It was determined that bacteriocins were similar to other bacteriocins reported in the literature with their behavior against temperature and enzyme applications. In various studies, it has been reported that bacteriocins are sensitive to proteolytic enzymes in bacteriocin characterization steps and decrease in antimicrobial activity, especially when treated with proteolytic enzymes (Lü et al., 2014; Woraprayote et al., 2015; Hu et al., 2017; Zangeneh et al., 2020). When compared with bacteriocin characterisation studies in the literature, metabolites in cell-free culture supernatants contained peptide or protein structures and thus supported that they were bacteriocins.

5. Conclusion

In this study, identification of bacteriocin-producing LAB isolated from Bayburt local fermented milk products, antimicrobial spectrum and characterization of bacteriocins were carried out for the first time. The use of bacteriocins or bacteriocin producers in foods has been recognized as one of the alternative natural preservation methods to ensure consumer and product safety. Especially since these strains are

isolated from fermented food products, they can be used in the preparation of various fermented products that are very important in terms of nutrition. The data obtained will both contribute to the literature and serve as a source for future studies. The identified LAB strains were preserved in the culture collection. The genes associated with bacteriocins have been protected.

Acknowledgement

This study was carried out with the support provided by Bayburt University, Scientific Research Projects Unit, project number 2020/01-69001-10.

Authors contribution

The design, execution, and interpretation of experimental studies, as well as the writing of the article, were done by the corresponding author.

Declaration of ethical code

The authors of this article declare that the materials and methods used in this study do not require ethics committee approval and/or legal-special permission.

Conflicts of interest

The authors declare that there is no conflict of interest.

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