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Potential bacteriocinogenic lactic acid bacteria from pastırma

Pastırmadaki potansiyel bakteriyosinojenik laktik asit bakterileri

Medine GÜLLÜCE[1a], Mükerrem KAYA[2b], Güzin KABAN[2c], Mehmet KARADAYI[1d*], Burak ALAYLAR[3e], Aybike KAMILOĞLU[4f], Ceyda IŞIK[5g], Kübra FETTAHOĞLU[6h]

[1] Department of Biology, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey.
[2] Department of Food Engineering, Faculty of Agriculture, Atatürk University, 25240 Erzurum, Turkey.
[3] Department of Molecular Biology and Genetics, Faculty of Science and Art, Agri Ibrahim Cecen University, 04100 Ağrı, Turkey.
[4] Department of Food Engineering, Bayburt University, 69000, Bayburt, Turkey.
[5] Institute of Natural and Applied Sciences, Atatürk University, 25240, Erzurum Turkey.
[6] Department of Food Processing, Meat and Products Technology, Vocational School of Doğubayazit Ahmed-i Hani, Agri Ibrahim Cecen University, 04100 Ağrı, Turkey.

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Abstract
Recent researches conducted have focused on the possibility of using bacteriocin produced by lactic acid bacteria both (LAB) as protective cultures and as natural antimicrobial agents in dry cured meat products such as pastırma. In the present study, 50 lactic acid bacteria strains, previously isolated from various traditional pastırma samples, were tested for their antimicrobial potentials. Determination of antagonistic activities against food-borne pathogenic strains (Listeria monocytogenes, Staphylococcus aureus and Bacillus cereus) were done by using agar-spot and well diffusion assays. Then, active isolates were identified by using 16S rRNA gene sequence analysis. According to the results, 11 of tested LAB were determined with significant bacteriocinogenic potential and these were assigned to as 1 Lactobacillus plantarum, 9 L. plantarum subsp. plantarum and 1 Pediococcus pentosaceus. In conclusion, it was reached that the isolated/identified bacteriocin-producing lactic acid bacteria strains from pastırma samples have a significant potential to prepare pure or crude bacteriocin preparations.

Keywords: Bacteriocin, Lactic acid bacteria, Pastırma

Öz
Son yapılan araştırmalar hem koruyucu kültürler hem de pastırma gibi kürlenmiş kurutulmuş et ürünlerinde doğal antimikrobiyal maddeler olarak laktik asit bakterileri tarafından üretilen bakteriyosinlerin kullanılabilirliği üzerine yoğunlaşmıştır. Bu çalışmada, daha önce çeşitli geleneksel pastırma örneklerinden izole edilmiş 50 laktik asit bakteri suşu, antimikrobiyal potansiyelleri açısından test edilmiştir. Gıda kaynaklı patojenik suşlara (Listeria monocytogenes, Staphylococcus aureus ve Bacillus cereus) karşı antagonistik aktivitelerin belirlenmesi agar-spot ve kuyu difüzyon deneyleri kullanılarak yapılmıştır. Daha sonra, aktif izolatlar 16S rRNA gen dizisi analizi kullanılarak tanılanmıştır. Deney bulgularına göre test edilen LAB'nin 11'i, önemli bakteriyosinojenik potansiyele sahip olarak belirlenmiş ve bunlar, 1 Lactobacillus plantarum, 9 L. plantarum subsp. plantarum ve 1 Pediococcus pentosaceus olarak tanılanmıştır. Sonuç olarak, pastırma örneklerinden izole edilmiş/tanılanmış bakteriyosin üreten laktik asit bakteri suşlarının saf veya ham bakteriyosin preparasyonları hazırlamak için önemli bir potansiyele sahip olduğu görülmüştür.

***Anahtar kelimeler**: Bakteriyosin, Laktik asit bakterileri, Pastırma*

*[d] Mehmet KARADAYI; mkaradayi@atauni.edu.tr, orcid.org/0000-0002-2743-0409
[a]orcid.org/0000-0002-5957-8259 [b]orcid.org/0000-0001-6340-828X [c]orcid.org/ 0000-0001-6720-7231
[e]orcid.org/ 0000-0001-6737-3440 [f]orcid.org/ 0000-0002-6756-0331 [g]orcid.org/ 0000-0002-7889-4636
[h]orcid.org/ 0000-0002-9464-0660

1. Introduction

Traditional food products have been an important part of food technology and widely consumed around the world for many centuries. Traditional cured meat products represent one of the most commonly used groups that share a huge financial aspect in the food industry and frequently vary from a culture to another. In Turkey, pastırma is a cured and dried meat product without heat treatment, obtained by pressing and drying after curing and washing processes in accordance with the technology of the piece meat separated from bovine carcasses according to the procedure and re-drying following fenugreek (Türk Gıda Kodeksi 2019). In the general production processes of pastırma, the raw product is processed by initial bacteriological activities, curing and drying steps (Dincer and Kivanc, 2012; Kaban 2013; Akköse and Aktaş, 2014; Çakıcı et al. 2015; Özturk 2015; Öz et al. 2017).

The initial bacteriological activities in the traditionally production is carried out by various bacterial strains, generally known as starter cultures, in the natural micro flora of meat product environment. In this manner, lactic acid bacteria (LAB) play a crucial role in the modification of raw material and the development of flavour, colour and texture (Kaban 2013; Kargozari et al. 2014). Moreover, LAB strains also have a special importance to improve food safety during the processing steps by producing inhibitory compounds including organic acids and ribosomally synthesized antagonistic polypeptides named as bacteriocins (Xiraphi et al. 2008; Pal et al. 2010; Liu et al. 2012; Biscola et al. 2013; Todorov et al. 2013; Kumariya et al 2019).

The characteristics of bacteriocins produced by LAB species are their proteinaceous nature, small sizes, resistance to stress conditions such as pH, high salt concentration and heat, antibiotic activity against pathogens and opportunistic bacteria that cause food spoilage. With these valuable properties, bacteriocins, as promising bioactive agents, have attracted many scientific interests for last few decades due to the emergence of antibiotic resistant bacteria and increasing incidence of food borne diseases (Mitić-Ćulafić et al. 2014; Müller-Auffermann et al. 2015; Schelegueda et al. 2015; Winkelströter et al. 2015).

On the other hand, many of bacteriocin producing LAB strains has not been yet explored and traditional food products have left as unique sources for isolation of these strains. Thus, the present study was designed to detect bacteriocionogenic potential of 50 lactic acid bacteria strains from various traditional pastırma samples and make molecular identification of the competent strains.

2. Materials and methods

2.1. Bacterial strains

50 lactic acid bacteria (LAB) strains used in the present study were previously isolated from pastırma (traditional Turkish dry cured meat product) samples bought from local producers. Known bacteriocinogenic strain *Lactococcus lactis* subsp. lactis GYL32 was used as the positive control (Koral and Tuncer, 2014). These LAB isolates and the standard bacteriocin producer strain were grown in de Man, Rogosa and Sharpe (MRS) broth or on MRS agar at 30 °C under unaerobic conditions. The food-borne pathogenic indicator strains were *Listeria monocytogenes* C3970, *Staphylococcus aureus* ATCC29213 and *Bacillus cereus* 11778. These strains were grown in Nutrient broth or BHI broth at 37 °C under shaking (200 rpm). All the standard bacterial strains were kept at -86 °C in lactobacilli MRS media and BHI or Nutrient media with 20% glycerol (v/v). Before being used, the strains were propagated twice in their respective media and growth suitable conditions (Omar et al. 2008; Devi and Halami, 2011; Macwana and Muriana, 2012).

2.2. Phenotypic and biochemical tests

All of the related tests including Gram staining, catalase activity, gas production from glucose and growth examinations in MRS broth with altering NaCl and temperature values were performed as previously described to check strain (Schillinger and Lücke, 1987; Harrigan 1998). Sugar fermentation pattern was determined using API 50 CHL (BioMerieux, SA, France) and the identification was performed by APIWEBTM (APIWEBTM standalone V 1.2.1 Ref 40012, Biomérieux®).

2.3. Determination of antimicrobial activities

The inhibitory potential of the LAB isolates was determined in both solid and liquid media by using the previous methods (Schillinger and Lücke, 1989; Todorov 2008; Devi and Halamii, 2011; Hurtado et al. 2011; Edalatian et al. 2012; Biscola et al. 2013). The agar spot test was used to check the antagonistic activity in solid media. In this procedure, samples from the overnight cultures

were taken and spotted on the surfaces of MRS agar plates. The plates were incubated at 30 °C for 24 h to allow spot development. Then, the spots were covered with 7 ml of the corresponding soft agar (0.75%) for each indicator inoculated at 0.25%. The cultures were incubated for 24 h under the required conditions for the respective indicators. After the incubation period, halos of growth inhibition around the spots were examined as a marker of the antagonistic activity.

The well diffusion assay was used to check the antimicrobial activity of positive strains in liquid media. For this aim, the active strains in the agar spot test were grown in MRS broth for 18 h. The supernatant from each culture was obtained by centrifugation (5000 rpm, 4 °C for 10 min) and filtered through a 0.2 µm pore membrane to get the sterilized culture filtrate (SCF). Then, 30 µl of SCF was transferred into holes drilled into MRS agar or BHI agar inoculated with indicator microorganism (1%, v/v) and the cultures were incubated under appropriate conditions for the indicators. In the end of the incubation time, observation of the clear inhibition zones around the wells was evaluated as a sign of the antimicrobial activity. Moreover, 3 µL of proteinase K solution (10 mg/mL) was added into the control wells to prove proteinaceous nature of the antimicrobial agent in the SCF. Loss of the inhibition zones indicated that the antimicrobial compound was proteinaceous in nature (Altuntas et al. 2010).

2.4. Molecular identification of bacteriocinogenic strains

DNA isolation studies of the active bacterial strains were performed with the method described by Wilson in 1997. By the *in vitro* polymerase chain reaction (PCR), unique 16S rDNA gene regions of the bacterial isolates were amplified by using a universal primer set (27F [5'-AGAGTTTGATCMTGGCTCAG-3'] and 1492R [5'-CGGTTACCTTGTTACGACTT-3']. Total volume of the reaction mixture was 30 µl and it included DMSO (1.2 µl), magnesium chloride (1.5 mM), dNTPs (0.2 mM), primers (25 pmoles of each one), DNA template (50 ng) and Taq DNA polymerase (5 U) along with reaction buffer. The reaction was started with an initial step at 95 ºC for 2 min, continued 36 cycles of 1 min at 94 ºC, 1 min at 53 ºC, 2 min at 72 ºC, and finally terminated by a final 5 min extension step at 72 ºC.

The amplicons were examined by the ethidium bromide staining agarose gel (1.5% w/v). The electrophpresis running conditions were 90 V for 120 min in 0.5× Trise-Borate-EDTA buffer. The gel was visualized by using the Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK). Then, confirmed amplicons were sequenced by Macrogen Inc®. (Netherlands). The sequence data was assigned to The Basic Local Alignment Search Tool (BLAST) of NCBI in order to determine the nucleotide sequence homology. The identification data from the molecular homology results was submitted to GenBank® and accession numbers were assigned.

3. Results and discussion

After the phenotypic and biochemical tests including Gram staining, catalase activity, gas production from glucose and growth examinations in MRS broth with altering NaCl and temperature values, all of the strains were validated as lactic acid bacteria (Table 1). Sugar fermentation pattern was also supported these results (data was not shown).

Table 1. Phenotypic and biochemical test results of the bacteriocinogenic isolates.

	Phenotypic and biochemical tests							
Isolate Code	*Gram property*	*Catalase activity*	*Gas production from glucose*	*Growth on MRS agar supplemented with NaCl*		*Growth on MRS agar at various incubation temperatures*		
				6.5%	*10%*	*8 ºC*	*15 ºC*	*45 ºC*
P122	+	-	-	+	+	+	+	+
P124	+	-	-	+	+	+	+	+
P128	+	-	-	+	+	+	+	+
P132	+	-	-	+	+	+	+	+
P145	+	-	-	+	+	+	+	+
P146a	+	-	-	+	+	+	+	+
P146b	+	-	-	+	+	+	+	++
P153	+	-	-	+	+	+	+	+
P154	+	-	-	+	+	+	+	+
P155	+	-	-	+	+	+	+	+
P161	+	-	-	+	+	+	+	+

Among these 50 LAB strains, 11 active bacteriocinogenic LAB isolates with antagonistic and antimicrobial potential against food-borne pathogens were determined by using the agar spot test and the well diffusion assay systems. These results were shown in Table 2. When compared to control, the P124 isolate showed the lowest antimicrobial activity against the food-borne pathogenic indicator strains. On the other hand, the P161 isolate was the most noticeable strain with its the strongest inhibitory activity almost equal to the control. Other 9 isolates also showed moderate antimicrobial activity.

Table 2. Bacteriocinogenic isolates with antagonistic activities against food-borne pathogenic indicator strains.

	Food-borne pathogenic indicator strains		
	L. monocytogenes	*S. aureus*	*B. cereus*
Isolate Code	C3970 Inhibition zone diameter (cm)	ATCC29213 Inhibition zone diameter (cm)	11778 Inhibition zone diameter (cm)
P122	0.2	0.3	0.1
P124	0.1	0.2	-
P128	0.1	0.3	0.2
P132	0.2	0.3	0.2
P145	0.4	0.4	0.2
P146a	0.1	0.3	0.2
P146b	0.1	0.1	0.5
P153	0.2	0.4	0.4
P154	0.1	0.5	0.2
P155	0.4	0.4	0.2
P161	0.4	0.5	0.4
***L. lactis* GYL32 (Control)**	**0.5**	**0.4**	**0.5**

Data of the 16S rDNA gene sequencing showed that the active strains grouped in *Lactobacillus* and *Pediococcus* genera. 1 *Lactobacillus plantarum*, 9 *L. plantarum* subsp. *plantarum* and 1 *Pediococcus pentosaceus*. According to the results, 1 isolate was assigned to *Lactobacillus plantarum* (P155), 9 isolates to *L. plantarum* subsp. *plantarum* (P122, P124, P128, P132, P145, P146a, P153, P154, P161) and 1 isolate to *Pediococcus pentosaceus* (P146b). Detailed data about taxonomic affiliation of active strains and GenBank® accession numbers was given in Table 3.

Table 3. Taxonomic affiliation of active strains and GenBank® accession numbers

Sample Code	**Accession Number**	**Closest relative species**	**% Identity**
P122	KR010985	*Lactobacillus plantarum subsp. plantarum*	99
P124	KR010986	*Lactobacillus plantarum subsp. plantarum*	99
P128	KR010987	*Lactobacillus plantarum subsp. plantarum*	99
P132	KR010988	*Lactobacillus plantarum subsp. plantarum*	99
P145	KR010989	*Lactobacillus plantarum subsp. plantarum*	99
P146a	KR010990	*Lactobacillus plantarum subsp. plantarum*	99
P146b	KR010991	*Pediococcus pentosaceus*	99
P153	KR010992	*Lactobacillus plantarum subsp. plantarum*	99
P154	KR010993	*Lactobacillus plantarum subsp. plantarum*	100
P155	KR010994	*Lactobacillus plantarum*	99
P161	KR010995	*Lactobacillus plantarum subsp. plantarum*	99

Pastırma is a well-known and the most popular traditional dry-cured meat product in Turkey. Up to date approximately 20 types of pastırma with different shape and quality characteristics, which are fundamentally named as şekerpare, sırt, bohça, kuşgömü, antrekot, tütünlük, dilme, arkabaş, etek, omuz, bez, mehle, kenar, kürek, kapak, döş, kavram, potuk and meme according to the region of carcass where muscle groups as raw materials for pastırma production obtained, have been described and widely consumed in the traditional Turkish cuisine (Kaban 2013; Çakıcı et al. 2015). Due to its popularity and great variety, pastırma production has always had an economic importance in the meat industry and many scientific efforts focusing on improving pastırma production processes have done for decades.

Recent research trends on pastırma production have especially focused on conservation and improvement of traditional production methods and development of natural food additives due to consumer's debates about rising usage of synthetic chemical additives in the industrial production processes and their unexpected hazardous effects on human health. In this regard, natural microflora in pastırma, mainly consists of lactic acid bacteria (LAB) and catalase positive cocci (micrococci and coagulase negative staphylococci), provides a unique source for development of natural food additive preparations (Dincer and Kivanc, 2012; Çinar 2014; Özturk 2015; Sınmaz 2013).

Especially LAB strains distinguish themselves from other bacterial strains via their GRAS (Generally Recognized as Safe) property accepted by FDA (American Food and Drug Administration) and unique capabilities for production of natural antimicrobial metabolites as well as their crucial roles in sensory and textural development of the product through acid production. These antimicrobial metabolites of LABs are generally referred as bacteriocins, which have a proteinaceous nature, small sizes, resistance to stress conditions such as pH, high salt concentration and heat, antagonistic activity against pathogens and opportunistic bacteria that cause food spoilage (Omar et al. 2008; Xiraphi et al. 2008; Pal et al. 2010; Devi and Halami, 2011; Hurtado et al. 2011; Edalatian et al. 2012; Liu et al. 2012; Macwana and Muriana, 2012; Biscola et al. 2013; Todorov et al. 2013; Costa et al. 2019).

In this context isolation of bacteriocin producing LAB strains from traditional pastırma samples and their molecular characterization seem as a fundamental route providing a futuristic, promising and useful approach to development of natural preservative agents for processing steps in pastırma production. With the present study, 11 bacteriocin-like metabolite producing LAB strains with antagonistic activity against food-borne pathogens *L. monocytogenes* C3970, *S. aureus* ATCC29213 and *B. cereus* 11778 were determined. Among these, P124 and P161 showed the lowest and highest antimicrobial activity properties, respectively. Besides, the remaining 9 isolates also had moderate antimicrobial activity ranging from P124 to P161.

Compared to antimicrobial activity results of the control strain *Lactococcus lactis* subsp. lactis GYL32, P161 isolate have significant potential for development of new bacteriocin preparations. On the other hand, other 10 isolates with moderate and low antimicrobial activity can also be improved by the further optimization studies altering several parameters (pH, temperature, NaCl concentration, carbon and nitrogen sources) for the growth and bacteriocin production (Iyapparaj et al. 2013; Malheiros et al. 2015).

The active isolates were identified as 1 *Lactobacillus plantarum*, 9 *L. plantarum* subsp. *plantarum* and 1 *Pediococcus pentosaceus*. As expected, the maximum variation was seen between *L. plantarum* P155 and *P. pentosaceus* P145 when 16S rRNA gene sequence information was compared with each other. The identity value was calculated as 91.18% for these bacteria. Percent identity matrix created by Clustal 2.1 (Table 4). *L. plantarum* P155 and *P. pentosaceus* P145 placed two outermost branches, and *L. plantarum* subsp. *plantarum* isolates grouped between them. Small differences between the phylogenetic tree and the percent identity matrix can be explained by the nature of sequence identity, the amount of characters which match exactly between two different sequences. Hereby, gaps are not counted and the measurement is relational to the shorter of the two sequences.

When the literature data is analyzed, it is not surprising to encounter *L. plantarum* because it is one of the most prevalent natural LAB strains in traditional pastırma samples (Dincer and Kivanc, 2012; Sınmaz 2013; Çinar 2014). Furthermore, it is well known that *L. plantarum* strains are the main producers for a special group of bacteriocins, which is named as plantaricins and include diverse range of ribosomally synthesized small antagonistic natural agents such as plantaricin A, plantaricin B, plantaricin C, plantaricin D, plantaricin E/F, plantaricin G, plantaricin I,

plantaricin J, plantaricin K, plantaricin N, plantaricin NC8, plantaricin S and plantaricin W (Omar et al. 2008; Hurtado et al. 2011). Similarly, parallel explanations can be also made for 9 isolated of *L. plantarum* subsp. *plantarum*. On the other hand, the surprising isolate of the present study was *P. pentosaceus*, which known as producer of pediocins, especially pediocin PA-1. This is the first report on the presence of bacteriocin like metabolite producing *P. pentosaceus* strains in pastırma. Similarly, there is not also any clear information in the literature on bacteriocinogenic *L. plantarum* and *L. plantarum* subsp. *plantarum* isolates from pastırma.

Table 4. Percent identity matrix of the representative species as inferred using Clustal 2.1

		1	2	3	4	5	6	7	8	9	10	11
1	**P146b**	100	88.91	88.49	88.95	88.95	88.95	89.37	91.18	89.32	88.91	88.87
2	**P153**		100	96.79	97.33	97.33	97.33	97.55	98.26	98.02	98.02	97.94
3	**P124**			100	99.17	99.17	99.17	98.80	98.89	98.42	97.89	96.92
4	**P128**				100	100	100	98.72	98.89	99.25	98.65	97.82
5	**P132**					100	100	98.72	98.89	99.25	98.65	97.82
6	**P145**						100	98.72	98.89	99.25	98.65	97.82
7	**P154**							100	99.76	99.32	98.87	98.05
8	**P155**								100	99.52	99.68	99.68
9	**P161**									100	99.17	98.35
10	**P122**										100	99.10
11	**P146a**											100

4. Conclusion

In conclusion, the results of the present study remark that the pastırma originated bacteriocinogenic LAB strains have a significant potential to prepare pure or crude bacteriocin preparations as antagonistic natural food additives against food-borne pathogens and opportunistic bacteria that cause food spoilage.

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