

Genotypic Identification of Lactic Acid Bacteria in Pastirma Produced with Different Curing Processing

Kübra ÇINAR ^{1,a} Kübra FETTAHOĞLU ^{2,b} Güzin KABAN ^{2,c}

¹ Bayburt University, Faculty of Engineering, Department of Food Engineering, TR-69000 Bayburt - TURKEY

² Atatürk University, Faculty of Agriculture, Department of Food Engineering, TR-25100 Erzurum - TURKEY

^a ORCID: 0000-0002-3715-8739; ^b ORCID: 0000-0002-9464-0660; ^c ORCID: 0000-0001-6720-7231

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Abstract

The lactic acid bacteria isolated from pastirma, produced under controlled conditions using two different curing temperatures (4°C or 10°C) and two different curing agents (150 mg/kg sodium nitrite or 300 mg/kg potassium nitrate), were subjected to genotypic (16S rRNA sequencing) identification. According to the identification results, 68 of 87 isolates (78.16%) was identified as *Pediococcus pentosaceus*. This species was followed by *P. acidilactici* (14.94%), *Lactobacillus sakei* (4.60%) and *L. plantarum* (2.30%), respectively. *P. pentosaceus* was dominant species in all curing applications (4°C/nitrate or nitrite or 10°C/nitrate or nitrite). Another species determined in all groups was *P. acidilactici*. While *L. plantarum* was only isolated from samples produced with nitrate (4°C or 10°C), *L. sakei* was isolated from samples produced with nitrite (4°C or 10°C). The effect of the curing agent on the biodiversity of lactic acid bacteria in pastirma was more effective than the curing temperature.

Keywords: Pastirma, Nitrate, Nitrite, *Pediococcus*, *Lactobacillus*, 16S rRNA

Farklı Kürleme İşlemleri İle Üretilen Pastirmada Laktik Asit Bakterilerinin Genotipik İdentifikasyonu

Öz

İki farklı kürleme sıcaklığı (4°C veya 10°C) ve iki farklı kürleme ajanı (150 mg/kg sodyum nitrit veya 300 mg/kg potasyum nitrat) kullanılarak kontrollü şartlar altında üretilen pastirmadan izole edilen laktik asit bakterileri, genotipik (16S rRNA) identifikasyona tabi tutulmuştur. İdentifikasyon sonuçlarına göre, 87 izolattın 68'i (%78.16) *Pediococcus pentosaceus* olarak tanımlanmıştır. Bu türü sırasıyla *P. acidilactici* (%14.94), *Lactobacillus sakei* (%4.60) ve *L. plantarum* (%2.30) takip etmiştir. Tüm kürleme uygulamalarında (4°C/nitrat veya nitrit veya 10°C/nitrat veya nitrit), *P. pentosaceus* dominant türdür. Tüm gruplarda belirlenen diğer bir tür ise *P. acidilactici*'dir. *L. plantarum* yalnız nitrat (4°C veya 10°C) ile üretilen örneklerden identifiye edilirken, *L. sakei* yalnızca nitrit (4°C veya 10°C) kullanılarak üretilen örneklerde tanımlanmıştır. Pastirmada laktik asit bakterilerinin biyoçeşitliliği üzerine kürleme ajanının etkisi, kürleme sıcaklığından daha etkili olmuştur.

Anahtar sözcükler: Pastirma, Nitrat, Nitrit, *Pediococcus*, *Lactobacillus*, 16S rRNA

INTRODUCTION

Pastirma, a traditional Turkish dry-cured meat product, is made from beef or water buffalo meat. Its production stages consist of curing, drying, pressing and çemen covering. The production of pastirma takes about one month and the heating or smoking stages do not include in the process ^[1]. Curing process is one of the most important stages in the production of pastirma. Product-type and process conditions are considered in the selection of curing

agent in meat products ^[2]. European Parliament and Council Directive 2014/601/EC (section 08.3.1) allows use of 150 mg nitrite (ingoing amount)/kg and 150 mg nitrate (ingoing amount)/kg in non-heat-treated meat products ^[3]. In the same fashion, Turkish Food Codex Regulation on Food Additives (2013/28693) also allows the use of 150 mg/kg nitrite in non-heat-treated meat products while it forbids the use of nitrate in pastirma ^[4]. However, nitrate is the most commonly used curing agent in pastirma ^[1,2,5-9]. These curing agents are important additives for cured



İletişim (Correspondence)



+90 442 2312425 Fax: +90 442 2315878



gkaban@atauni.edu.tr

meat products due to formation of color and flavor, antioxidant and antimicrobial properties^[9,10]. On the other hand, nitrate must be converted to nitrite for observation of the expected effects from nitrate in the processes with nitrate^[11].

In pastirma microbiota, Gr (+), catalase-positive cocci (coagulase-negative *Staphylococcus* and *Kocuria*) and lactic acid bacteria constitute two important microorganism groups^[12]. The number of lactic acid bacteria in the final product may vary depending on the process conditions of the enterprise where production is made because pastirma is produced by traditional methods. It is possible to face very low numbers during production performed under controlled conditions from raw material with quite a low number of initial microorganisms. On the other hand, it is also possible to encounter high numbers of lactic acid bacteria in pastirma although it is rare. Although the number of lactic acid bacteria varied between 10^4 - 10^8 cfu/g in some studies^[13,14], counts varying between 10^3 - 10^6 cfu/g^[15], 10^2 - 10^7 cfu/g^[16], 10^3 - 10^7 cfu/g^[17] were reported in other studies.

Various species belonging to *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Weisella* genera were determined in studies for the isolation and identification of the lactic acid bacteria in pastirma^[17-19]. There is a study on the biodiversity of lactic acid bacteria in pastirma under different curing processing, in which lactic acid bacteria were identified phenotypically using API CHL^[20]. The aim of this study was to genotypically identify the isolates of lactic acid bacteria from pastirma produced with different curing agents and curing temperatures (4°C or 10°C).

MATERIAL and METHODS

Isolates

Eighty seven lactic acid bacteria isolates, obtained from pastirma produced under controlled conditions using two different curing temperatures (4°C or 10°C) and two different curing agents (150 mg/kg sodium nitrite or 300 mg/kg potassium nitrate)^[20] were subjected to genotypic (16S rRNA sequencing) identification.

Genotypic Identification

A High Pure PCR template preparation kit (Roche, Indianapolis, IN) according to the manufacturer's protocol was used to isolation of genomic DNA. 16S rDNA coding region sequence was selected and amplified by PCR (TC-4000 Techne). Faststart High Fidelity PCR System dNTPack kit (Roche) was used in PCR and 27F (5'-AGAGTTTGATCM TGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGA CTT-3') universal primers were also used to amplify the 16S rRNA gene. The amplification program was initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension

at 72°C for 1.5 min; and a final extension step at 72°C for 7 min. Cycle sequencing reaction products were purified with a Sephadex column. 16S rDNA sequence analysis of PCR products was carried out by the Macrogen company (Netherlands). The sequence results obtained were aligned with the NCBI database using the BLAST program (<http://blast.ncbi.nlm.nih.gov>).

RESULTS

A total of 87 lactic acid bacteria isolates belonging to four different species and two different genera were identified. As a result of identification, 68 isolates were identified as *Pediococcus pentosaceus*, 13 isolates as *Pediococcus acidilactici*, 4 isolates as *Lactobacillus sakei* and 2 isolates as *Lactobacillus plantarum* (Table 1). When they were evaluated on the basis of species, it was determined that 93.10% of the isolates consisted of two species belonging to *Pediococcus* genera, and 6.90% of the isolates consisted of two species belonging to *Lactobacillus*. According to the results, *P. pentosaceus* (78.16%) was found as dominant species in pastirma, and this species was followed by *P. acidilactici* (14.94%). The isolation ratios of *L. sakei* (4.60%) and *L. plantarum* (2.30%) species were quite low.

Twenty four isolates were identified in pastirma samples in which nitrite as the curing agent and the curing temperature of 4°C were used; 22 isolates were identified in pastirma samples in which nitrite as the curing agent and the curing temperature of 10°C were used. Twenty isolates were identified in pastirma samples in which nitrate as the curing agent and the curing temperature of 4°C were used; a total of 21 isolates were identified in pastirma samples in which nitrate was used at 10°C (Table 2). Twenty isolates in the combination of nitrite and curing temperature of 4°C and 16 isolates in the combination of nitrite and curing temperature of 10°C were identified as *P. pentosaceus*. In the groups in which nitrate was used as the curing agent, 18 isolates at 4°C curing temperature and 14 isolates at 10°C curing temperature were identified as *P. pentosaceus* (Table 2).

Four different species (*P. acidilactici*, *P. pentosaceus*, *L. plantarum*, *L. sakei*) both at 4°C curing temperature and at 10°C curing temperature were identified independently of the curing agent. The isolates were identified as *P. acidilactici*, *P. pentosaceus* and *L. sakei* in nitrite-curing process, while *P. acidilactici*, *P. pentosaceus* and *L. plantarum* were identified in the presence of nitrate. According to these results, *L. plantarum* was only identified in case of using nitrate, and *L. sakei* was only identified in the presence of nitrite.

DISCUSSION

In the studies conducted on commercially available pastirma samples, quite different numbers of lactic acid bacteria

Table 1. Genotypic identification results of lactic acid bacteria isolated from pastirma produced under different curing temperature and curing agents

Isolate No	Code	Species	Number of Base	Identity (%)	Isolate No	Code	Species	Number of Base	Identity (%)	Isolate No	Code	Species	Number of Base	Identity (%)
1	K4	<i>P. pentosaceus</i>	1133	99	30	K52	<i>P. pentosaceus</i>	1100	100	59	K98	<i>P. acidilactici</i>	1011	100
2	K5	<i>P. pentosaceus</i>	1388	100	31	K53	<i>P. acidilactici</i>	1014	97	60	K99	<i>P. acidilactici</i>	1268	10
3	K6	<i>P. pentosaceus</i>	1258	100	32	K54	<i>P. pentosaceus</i>	1114	99	61	K100	<i>P. acidilactici</i>	1155	100
4	K7	<i>P. pentosaceus</i>	1169	99	33	K55	<i>P. pentosaceus</i>	1025	100	62	K101	<i>P. pentosaceus</i>	1054	98
5	K8	<i>P. pentosaceus</i>	1141	99	34	K56	<i>P. pentosaceus</i>	1016	100	63	K102	<i>P. pentosaceus</i>	1077	100
6	K9	<i>P. pentosaceus</i>	1136	100	35	K57	<i>P. acidilactici</i>	1035	99	64	K103	<i>L. sakei</i>	1074	98
7	K10	<i>P. pentosaceus</i>	1123	100	36	K58	<i>P. acidilactici</i>	876	99	65	K104	<i>P. pentosaceus</i>	1110	99
8	K13	<i>P. pentosaceus</i>	1048	100	37	K59	<i>P. acidilactici</i>	1120	100	66	K105	<i>P. pentosaceus</i>	1153	99
9	K14	<i>P. pentosaceus</i>	1267	100	38	K60	<i>P. acidilactici</i>	1075	100	67	K106	<i>P. pentosaceus</i>	1062	100
10	K15	<i>P. pentosaceus</i>	1052	100	39	K61	<i>P. pentosaceus</i>	1158	100	68	K107	<i>P. pentosaceus</i>	1045	100
11	K21	<i>P. pentosaceus</i>	1284	100	40	K62	<i>P. acidilactici</i>	1019	100	69	K108	<i>L. sakei</i>	1098	99
12	K22	<i>P. pentosaceus</i>	1169	100	41	K63	<i>P. acidilactici</i>	1113	98	70	K109	<i>L. sakei</i>	1127	99
13	K23	<i>P. pentosaceus</i>	1297	100	42	K64	<i>P. acidilactici</i>	1254	100	71	K110	<i>P. pentosaceus</i>	1240	100
14	K24	<i>P. pentosaceus</i>	1086	100	43	K66B	<i>P. pentosaceus</i>	1266	100	72	K111	<i>L. sakei</i>	994	100
15	K31	<i>P. pentosaceus</i>	1143	10	44	K66S	<i>P. pentosaceus</i>	1144	99	73	K112	<i>P. pentosaceus</i>	919	100
16	K32	<i>P. pentosaceus</i>	1109	97	45	K67	<i>P. pentosaceus</i>	1045	100	74	K113	<i>P. pentosaceus</i>	1139	100
17	K33	<i>P. pentosaceus</i>	989	99	46	K72	<i>L. plantarum</i>	1076	99	75	K114	<i>P. pentosaceus</i>	1109	99
18	K34	<i>P. pentosaceus</i>	1048	99	47	K73	<i>L. plantarum</i>	934	99	76	K115	<i>P. pentosaceus</i>	1069	98
19	K35	<i>P. pentosaceus</i>	1126	99	48	K74	<i>P. pentosaceus</i>	1046	100	77	K116	<i>P. pentosaceus</i>	1041	100
20	K36	<i>P. pentosaceus</i>	1113	99	49	K75	<i>P. pentosaceus</i>	1217	100	78	K117	<i>P. pentosaceus</i>	1029	99
21	K37	<i>P. pentosaceus</i>	1288	100	50	K76	<i>P. pentosaceus</i>	1034	100	79	K118	<i>P. pentosaceus</i>	1190	99
22	K38	<i>P. pentosaceus</i>	1061	100	51	K79	<i>P. pentosaceus</i>	1072	99	80	K119	<i>P. acidilactici</i>	1188	99
23	K39	<i>P. pentosaceus</i>	1214	100	52	K81	<i>P. pentosaceus</i>	1109	100	81	K120	<i>P. pentosaceus</i>	1155	99
24	K40	<i>P. pentosaceus</i>	1089	100	53	K82	<i>P. pentosaceus</i>	1041	100	82	K121	<i>P. pentosaceus</i>	1106	99
25	K41	<i>P. pentosaceus</i>	1185	100	54	K83	<i>P. acidilactici</i>	1132	99	83	K122	<i>P. pentosaceus</i>	1143	100
26	K42	<i>P. pentosaceus</i>	1087	99	55	K85	<i>P. pentosaceus</i>	1204	100	84	K124	<i>P. pentosaceus</i>	1177	99
27	K44	<i>P. pentosaceus</i>	1022	99	56	K86	<i>P. pentosaceus</i>	1123	99	85	K127	<i>P. pentosaceus</i>	1023	100
28	K45	<i>P. pentosaceus</i>	1004	100	57	K87	<i>P. pentosaceus</i>	1235	100	86	K128	<i>P. pentosaceus</i>	1259	100
29	K51	<i>P. pentosaceus</i>	1145	100	58	K97	<i>P. pentosaceus</i>	1041	100	87	K129	<i>P. pentosaceus</i>	1090	100

Table 2. Diversity and prevalence of lactic acid bacteria isolated and genetically identified from pastirma produced under different curing temperature and curing agents

Isolates	Curing Temperature								Total Number of Isolates (%)
	4°C				10°C				
	Nitrate		Nitrite		Nitrate		Nitrite		
	Isolates	%	Isolates	%	Isolates	%	Isolates	%	
<i>P. pentosaceus</i>	18	90	20	83.33	14	66.67	16	72.72	68 (78.16)
<i>P. acidilactici</i>	1	5.0	3	12.50	6	28.57	3	13.64	13 (14.94)
<i>L. sakei</i>	-	-	1	4.17	-	-	3	13.64	4 (4.60)
<i>L. plantarum</i>	1	5.0	-	-	1	4.76	-	-	2 (2.30)
Total	20	100	24	100	21	100	22	100	87 (100)

were determined [13-15,17]. On the other hand, in a study investigating the effect of different levels of sodium and potassium nitrate on the quality properties of pastirma, it was found that the use and levels of sodium or potassium nitrate did not cause any differences in the microbiological properties of the product [21]. *Pediococcus* spp. which are dominantly available in experimental pastirma were also found in samples taken from the market. Sinmaz et al. [17] identified 5.7% of the lactic acid bacteria isolated from pastirma as *P. pentosaceus* and 4.7% of them as *P. acidilactici*. Dinçer and Kivanç [19] isolated *P. acidilactici* as well as *L. plantarum*, *L. sakei* and *Enterococcus faecium* from pastirma. However, *Pediococcus* species were not found in pastirma samples in the study carried out by Özdemir and Siriken [18]. In another study on lactic acid bacteria isolated from pastirma produced with different curing condition, 72.41% of the isolates were phenotypically identified as *P. pentosaceus* [20]. Similar result (78.16%) was also observed in the present study where genotypic identification was used.

Pediococci are microorganisms of the homofermentative character and they are important microorganisms for food microbiology in terms of being tolerant to salt and also developing in a wide temperature range [22,23]. It is stated that the optimum growth temperature of *pediococci* varies between 25 and 40°C [24]. In addition, it is also indicated that some species show a wide tolerance against pH and salt as well as temperature [25]. In this present study, it is thought that *pediococci* show tolerance to salt at the curing and drying stages, and could survive at later stages.

In this present study, *L. plantarum* and *L. sakei* were also identified. These species belonging to *Lactobacillus* genera are facultative heterofermentative and produce a high proportion of lactic acid by following Embden-Meyerhoff-Parnas (EMP) called glycolysis [26]. Özdemir and Siriken [18] identified 5 isolates from 40 isolates which were isolated from pastirma by them as *L. plantarum* and 8 isolates as *L. curvatus*. Dinçer and Kivanç [19] identified *L. plantarum* as the dominant species as a result of the biochemical tests on 92 isolates obtained from pastirma samples by

using API50 CH. Çınar [20] reported that *L. sakei* was not determined in pastirma sample, while *L. plantarum* (2.30%), *L. curvatus* (2.30%), *L. brevis* (2.30%) and *L. collinoides* (1.15%) were isolated from pastirma samples. In the present study, *Lactobacillus* isolates were identified as *L. sakei* and *L. plantarum*. Sinmaz et al. [17] identified *L. plantarum* only in one sample and *L. paraplantarum* in another sample as a result of 16S rDNA sequence analysis on 14 pastirma samples. In the same study, *L. curvatus* was identified in two samples and it was stated that *L. sakei* was the dominant species.

As a conclusion, *P. pentosaceus* was the dominant species in pastirma under different curing conditions. In addition, the effect of the curing agent on the biodiversity of lactic acid bacteria in pastirma was more effective than the curing temperature. The species of *P. acidilactici*, *P. pentosaceus*, *L. sakei* and *L. plantarum* were detected both at 4°C and at 10°C. However, *L. plantarum* was only identified from pastirma cured with nitrate, and *L. sakei* was only identified from the samples cured with nitrite.

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