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ORIGINAL PAPER

Phenotypic and Genotypic Characterization of lactic acid bacteria isolated from Turkish dry fermented sausage

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Abstract

Fourty-six strains of lactic acid bacteria (LAB) isolated from Turkish fermented sausages (sucuk) were identified according to their phenotypic and genotypic characterization subjected to phenotypic and genotypic identification. The phenotypic characterization of predominant LAB isolated from the fermented Turkish dry sausages was based on general morphology, physiological tests and API system. The genotypic characterization of LAB was based on the repetitive elements found in the genome of Streptcococcus pneumoniae (BOX-PCR). Differentiation at species, subspecies and strain level was possible for the isolates. Predominant functional LAB strains associated with the fermented Turkish dry sausages were identified as Lactobacillus plantarum, Pediococcus pentosaceus, Lactococcus lactis ssp. lactis, L. curvatus ssp. curvatus, L. brevis, L. fermentum, Weisella viridescens, L. delbrueckii ssp. delbrueckii, W. confusa, L. collinoides and Leuconostoc mesenteroides ssp. mesenteroides/ dextranicum. It can be concluded from these data that the dominant flora in sausage is Lactobacillus species and its varieties (L. plantarum).

Keywords: Turkish dry fermente sausage, Phenotypic characterization, Genotypic characterization, *Lactobacillus, Leuconostoc, Lactococcus, Pediococccus*

Introduction

Fermented sausages have the highest rates of production and consumption because of their high nutritive value, distinguished taste and good texture. In fermented sausages, taste, consistency, aroma and color depending on the reactions of bacterial, enzymatic and bio-chemical substances during the maturity are shaped [1,2]. Especially during the production period, the use of starter culture can provide both the guarantee of food safety and the desired color and aroma at the end of the short period of maturity [3].
Microbial activity is the most important factor in the development of the quality and maturity of fermented sausages. Lactic acid bacteria (LAB) and micro-organism of *Staphylococcus* have the main roles in maturity [3-6].

It is the LAB that especially has a significant role in meat preservation and fermentation processes and are accepted technologically crucial. They are able to decrease pH by lactic acid production, produce bacteriocins to prevent the growth of pathogenic and spoilage microorganisms, provide diversity by the modification of raw material to obtain new sensory properties, improve the safety, the stability and the shelf life of meat products [7].
It is well known that LAB, in particular *Lactobacillus*, represent the dominant flora depending on the suitable environment for themselves in sausage material, by reaching the higher rates after the 24-28 hours of maturity period [8,9].

Different phenotypic methods are used to identify LAB important for fermentation technology. However, these methods are not sufficient to characterize sub-species and strains in a genus. Thus, new methods have been developed depending on genotypical features and used effectively for the definition of the bacteria [10,11].

The methods used for the current study of LAB such as 16S rRNA sequencing, ribotyping, protein profilling, and pulsed-field gel electrophoresis (PFGE) are either too laborious and limited in their resolving power or require a species-specific methodology. Therefore, a method that is universally suitable for the LAB with a high resolving power both on the species and intraspecies level would be a highly valuable tool. In this regard, PCR-based genomic fingerprinting techniques are believed to have the most potential, and are easy to perform [6,12,13].

Highly conserved repetitive DNA elements, such as Repetitive Extragenic Palindromic (REP) elements, Enterobacterial Repetitive Intragenic Consensus (ERIC) elements and BOX elements seem to be widely distributed in the genomes of various bacterial groups. PCR amplification of repetitive bacterial DNA elements (rep-PCR) has been known as a simple PCR-based technique with the following characteristics: (1) low cost, (2) a high discriminatory power (3) suitability for a high-throughput of strains, and (4) a reliable tool for classifying and typing a wide range of Gram-negative and some Gram-positive bacteria [13-15].

The main purpose of this paper is to characterize the LAB isolated from Turkish fermented sausage with phenotypic methods and to find out if this information is supported by BOX-PCR, a genotypic fingerprinting analyzing method.

Materials and Methods

Samples

Fifteen sausage samples were obtained from retail markets and butchers from different cities. The samples were carried to the laboratory and kept in a refrigerator. They were used for isolation and identification of LAB.

Isolation of lactic acid bacteria

The sausage casing was removed aseptically. For microbiological analysis, a 25 g sample was prepared by homogenizing with 225 physiological saline water [0.85 NaCl % (Merck 1.06404.1000)] in a stomacher (Laboratory Blender Stomacher 400, Seward Medical, London, UK) for 1 min. Further decimal delusions were prepared from this homogenized mixture. The following incubation conditions of isolation and identification of LAB were used: De Man Ragosa Sharpe Agar (Merck) for 2-3 days at 30°C (anaerobe) for LAB [16].

Phenotypic characterization

Cell morphology of all isolates was determined using a microscope. Isolates were Gram-stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties such as carbon dioxide production from glucose, growth at different temperatures as well as the ability to grow in different concentrations of sodium chloride and pH in De Man, Ragosa, Sharpe (MRS) broth. Sugar fermentation patterns of LAB isolates were determined using the API 50 CHL test strips (bioMérieux, France)[2].

Genotypic characterization

DNA isolation and purification

Total genomic DNA of 46 strains was extracted as per the method of Khoodo and Jaufeerally-Fakim [17] with little modification.

BOX PCR Genomic Fingerprinting

DNA (75 ng) was subjected to PCR utilizing the primer BOX A1R (CTA CGG CAA GGC GAC GCT GAC G) as Versalovic *et al.* [14] described. Each 27 µl PCR reaction contained 5 µl 5× Gitschier buffer (1 M $(NH_4)_2SO_4$, 1 M Tris-HCl (pH 8.8), 1 M $MgCl_2$, 0.5 M EDTA (pH 8.8) and 14.4 M β-mercapto-ethanol add double distilled water till 200 ml), 0.6 mg/ml BSA (Sigma, A-7906), 100% DMSO (Sigma, D-8418), 0.2 mM dNTP (Sigma, D7295), 0.5 µM oligonucleotide primer, 1 units of Taq DNA polymerase (Sigma, D1806) and distilled water. PCR amplifications were performed in a DNA thermal cycler (Techne, Touchgene, UK) with an initial denaturation step (95°C, 7 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (53°C, 1 min) and extension (65°C, 8 min), and a single final extension step (65°C, 16 min). The amplified fragments were fractionated on a 1.5% w/v agarose gel during 200 min at a constant voltage of 40 V in 0.5×TAE (Tris-Acetat EDTA) at 4°C. A 10-kb reference marker (Sigma, D7058) was used to allow standardization. Following staining with ethidium bromide and visualization by using the Bio Doc Image Analysis System with Unisoft analysis package (Cambrige, UK).

Data Analysis

All BOX-PCR fingerprints patterns were transformed into a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position) and analyzed by using SPSS program (SPSS, version 12.0 for Windows). Data were used to calculate a Jaccard (1908) similarity [18].

All of the experiments in this study were repeated at least twice.

Results

Phenotypic characterization of LAB

The bacterial strains isolated in this study were subjected to various morphologic, bio-chemical and physiologic tests. The results showed that all strains were gram positive, catalase negative. The results of the morphologic, bio-chemical and physiologic study of micro-organism such as the gas production from the glucose, the development of different heat and salt rate, cell morphology are shown in Table 1.

Table 1. Phenotypic characteristics of the LAB from Turkish fermented sausages (sucuk)

Number of strains	Cell shape	Gram reaction	Catalase reaction	Gas from glucose	Growth at 10°C	Growth at 15°C	Growth at 45°C	Growth at 2.5% NaCl	Growth at 4% NaCl	Growth at 6.5% NaCl
1	Coccoid	+	-	+	+	+	-	+	+	-
2	Coccoid	+	-	-	+	+	+	+	+	+
3	Rod	+	-	-	-	+	-	+	+	-
4	Coccoid	+	-	+	+	+	-	+	+	-
5	Rod	+	-	+	-	+	-	+	+	+
6	Rod	+	-	+	-	+	-	+	+	+
7	Rod	+	-	-	-	+	-	+	+	-
8	Rod	+	-	+	-	+	-	+	+	+
9	Coccoid	+	-	-	+	+	+	+	+	+
10	Rod	+	-	+	-	+	-	+	+	+
11	Rod	+	-	-	-	+	+	+	+	±
12	Rod	+	-	-	-	+	-	+	+	-
13	Rod	+	-	+	-	+	-	+	+	+
14	Rod	+	-	+	-	+	-	+	+	-
15	Rod	+	-	-	-	+	-	+	+	-
16	Rod	+	-	+	-	+	-	+	+	-
17	Coccoid	+	-	-	+	+	-	+	+	-
18	Coccoid	+	-	-	+	+	+	+	+	+
19	Coccoid	+	-	-	+	+	+	+	+	+
20	Rod	+	-	-	-	+	-	+	+	±
21	Rod	+	-	-	-	+	+	+	+	±
22	Coccoid	+	-	-	+	+	-	+	+	-

23	Rod	+	-	+	-	+	-	+	+	+
24	Rod	+	-	-	-	+	+	+	+	±
25	Rod	+	-	-	-	+	-	+	+	-
26	Rod	+	-	-	-	+	-	+	+	±
27	Rod	+	-	-	-	+	-	+	+	-
28	Rod	+	-	+	-	+	-	+	+	±
29	Rod	+	-	-	-	+	+	+	+	±
30	Rod	+	-	-	-	+	+	+	+	±
31	Coccoid	+	-	-	+	+	+	+	+	+
32	Coccoid	+	-	-	+	+	-	+	+	-
33	Rod	+	-	-	-	+	-	+	+	-
34	Rod	+	-	-	-	+	-	+	+	-
35	Coccoid	+	-	-	+	+	-	+	+	-
36	Coccoid	+	-	-	+	+	+	+	+	+
37	Coccoid	+	-	-	+	+	+	+	+	+
38	Rod	+	-	+	+	+	-	+	+	-
39	Rod	+	-	+	-	±	+	+	+	±
40	Rod	+	-	+	-	±	+	+	+	±
41	Coccoid	+	-	-	+	+	-	+	+	-
42	Rod	+	-	-	-	+	+	+	+	±
43	Rod	+	-	-	-	+	-	+	+	±
44	Coccoid	+	-	-	+	+	-	+	+	-
45	Coccoid	+	-	-	+	+	-	+	+	-
46	Rod	+	-	+	-	+	-	+	+	-

+; positive, -; negative, ±; weak growth

At the end of API result analysis, it was determined that 10 isolates are (G11,G15,G20, G21, G24, G26, G29, G30, G42, G43 isolates) *Lactobacillus plantarum* (21.7%), 8 (G2, G9, G18, G19, G31, G36, G37, G41 isolates) *Pediococcus pentosaceus* (17.4%), 6 (G17, G22, G32, G35, G44, G45 isolates) *L. lactis* subsp. *lactis* (13.2%), 5 (G3, G7, G27, G33, G34 isolates) *L. curvatus* subsp. *curvatus* (10.9%), 5 (G6, G8, G10, G14, G16 isolates) *L. brevis* (10.9%), 3 (G38, G39, G40 isolates) *L. fermentum* (6.5%), 3 (G4, G5, G28 isolates) *Weisella viridescens* (6.5%), 2 (G12, G25 isolates) *L. delbrueckii* subsp. d*elbrueckii* (4.3%), 2 (G13, G23 isolates) *W. confuse* (4.3%), 1 (G46 isolate) *L. collinoides* (2.18%), and 1 (G1 isolate) *Leuconostoc mesenteroides* subsp. *mesenteroides/ dextranicum* (2.18%).

The distributions of lactic acid bacteria strains as to the API data were shown at Table 2.

BOX-PCR genotypic fingerprinting

The BOX A1R primer generated DNA fragments from 300 to 4000 bp for all isolates and banding patterns containing 6-15 bands (Figure 1-3).

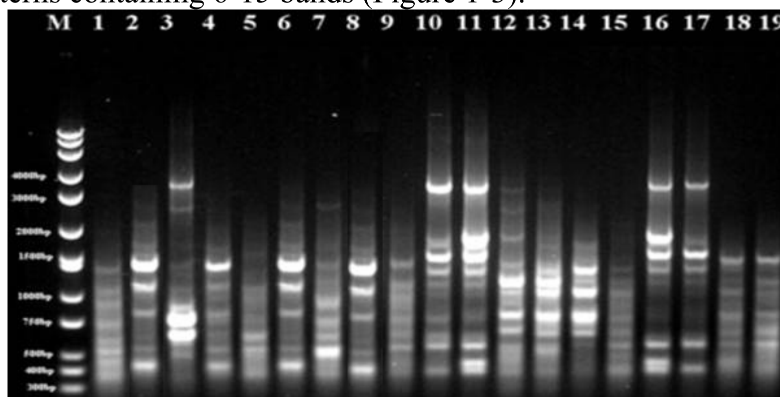

Figure 1. BOX PCR profiles generated with BOX A1R, Lines: **1:** 1, **2:** 2, **3:** 9, **4:** 18, **5:** 19, **6:** 31, **7:** 36, **8:** 37, **9:** 41, **10:** 3, **11:** 7, **12:** 27, **13:** 33, **14:** 34, **15:** 4, **16:** 5, **17:**28, **18:** 17, **19:** 22, **M:** Molecular Marker (10 kb)

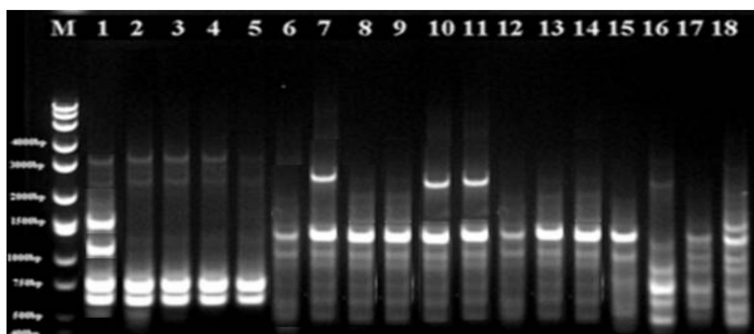

Figure 2. BOX PCR profiles generated with BOX A1R, Lines: **1:** 10, **2:** 14, **3:** 6, **4:** 8, **5:** 16, **6:** 20, **7:** 21, **8:** 30, **9:** 43, **10:** 11, **11:** 15, **12:** 26, **13:** 29, **14:** 42, **15:** 24, **16:** 38, **17:**39, **18:** 40, **M:** Molecular Marker (10 kb)

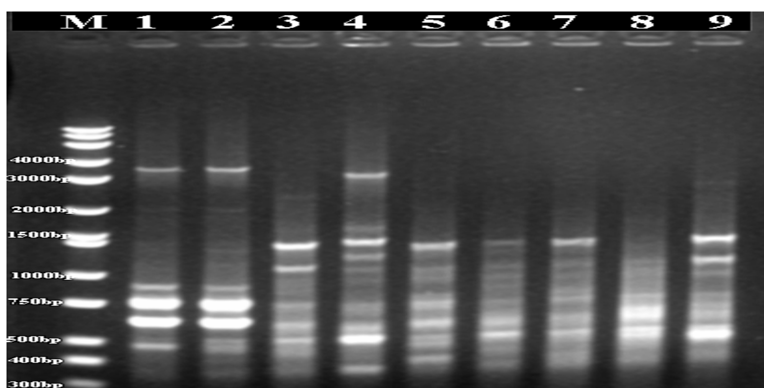

Figure 3. BOX PCR profiles generated with BOX A1R, Lines: **1:** 13, **2:** 23, **3:** 12, **4:** 25, **5:** 32, **6:** 35, **7:** 44, **8:** 45, **9:** 46, **M:** Molecular Marker (10 kb)

Table 2. Distribution of lactic acid bacteria strains as to manufacturers.

	Manufacturer							
	A	B	C	D	E	F	Total	(%)
L plantarum	1	1	3	2	2	1	10	21.
P. pentosaceus	-	1	1	3	2	1	8	17.
Lc. lactis ssp. lactis	1	1	2	1	1	-	6	13.
L curvatus ssp curvatus	2	2	1	-	-	-	5	10..
L brevis	1	-	1	1	1	1	5	10.9
L fermentum	-	2	-	-	1	-	3	6.5
Weisella viridescens	-	-	2	-	-	1	3	6.5
L. delbrueckii ssp. d*elbrueckii*	2	-	-	-	-	-	2	4.3
W. confuse	-	-	-	1	-	1	2	4.3
L. collinoides	-	1	-	-	-	-	1	2.1
Leuconostoc mesenteroides ssp. *mesenteroides/*	-	-	-	-	-	1	1	2.1
Total	7	8	10	8	7	6	46	100

After BOX PCR cluster analysis, it was observed that tested bacteria were divided into 5 main clusters (Figure 4). The most numerous was cluster I with 35 strains, followed by cluster V (5 strains), cluster III (3 strains), cluster II (2 strains) and cluster IV (1 strain).

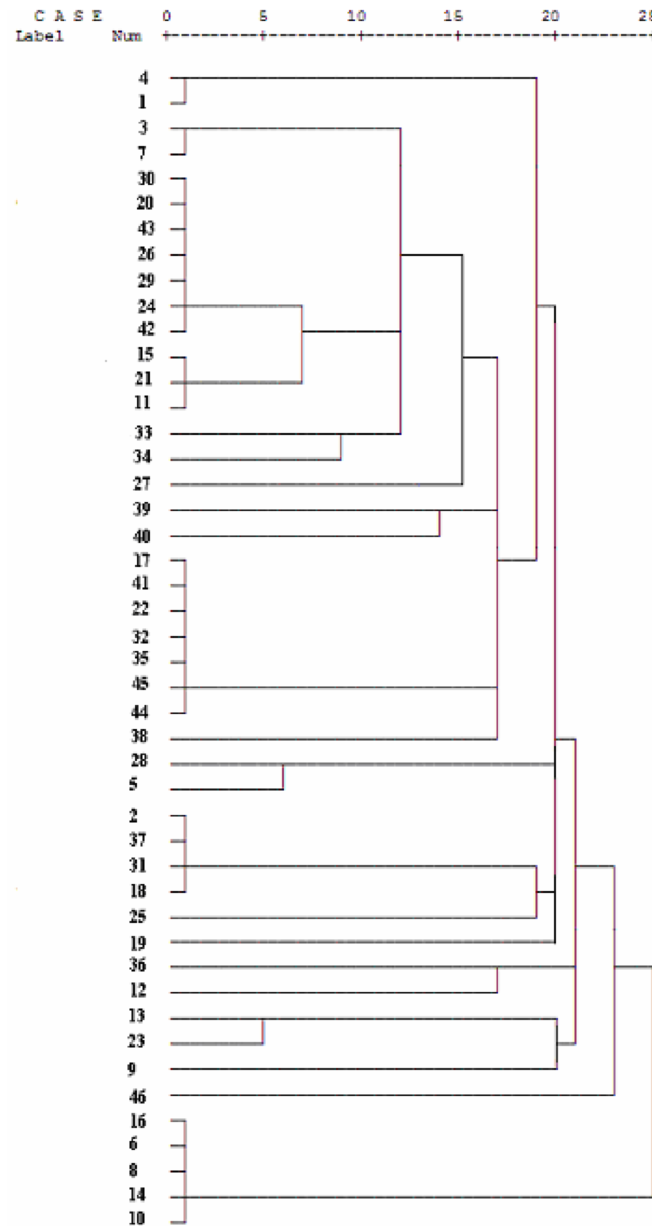

Figure 4. UPGM dendogram showing the relationship of LAB

According to the API data, it was determined that G41 isolate fixed as *Pediococcus pentosaceus*, at the end of BOX-PCR cluster analysis was a strain belonging to *Lactococcus lactis* subsp. *Lactis* and showed a similarity to G44, G45, G35, G32, G22 and G17 isolates at 98%.

According to the API results, it was also determined that the G19 strain identified as *Pediococcus pentosaceus* showed a similarity to isolates numbered G2, G37, G31 and G18 at 22% at the end of BOX-PCR cluster analysis, and this may mean that G19 strain can be a different species.

According to the cluster analysis results, it was determined that G1 and G4 isolates were similar at 98%, and the G4 isolate identified as *Weisella viridescens* according to API data was actually *Leuconostoc mesenteroides* subsp. *mesenteroides/dextranicum* with 1st isolate. It was observed that G5 and G28 isolates were similar at 76%, and they were both identified as

Weisella viridescens according to API data, but isolate G28 might be different from isolate G5 at sub-species level.

These examples were multiplied and it was still observed that the phenotypic data were to be supported with genotypic ones.

Discussion

Fermented sausages are common products throughout Europe with great diversity in production methods and organoleptic characteristics between different countries and also different regions of the same country [19]. Sausage fermentation is a well-known microbial process and ecological studies during ripening date back to the 1970s. These studies emphasize that two main populations are involved in the process. In the fermentation of sausages, the main transformations that lead to the final product involve the activity of two microbial groups: LAB and micro/staphylococci. The LAB are responsible for the acidification, while the micro/staphylococci produce lipases, eventually releasing short-chain fatty acids that are responsible for the aroma of the fermented sausage [1]. Molecular analysis of microbial changes during fermentation demostrate that by three days of maturation, these two main groups of organisms are the most abundant in the sausages [20]. Among LAB, the species most commonly determined in meat and meat products including dry sausages processed with different technologies are *L. sakei*, *L. curvatus*, *L. plantarum* and *L. sakei* being the most frequently isolated species [19,21].

In this research, the LAB isolated from Turkish fermented sausage were characterized with phenotypic and genotypic methods. We found out that *Lactobacillus plantarum* is the dominant flora as indicated by the study of Toksoy *et al*. [22], Drosinos *et al*. [23] and Kaban [24].

Pediococcus pentosaceus was determined to be the second dominant flora in this study. Ozdemir *et al*. [5], Kaban [24] estimated that *L. plantarum* and *P. pentosaceus* accepted as the dominant flora in sausage samples can be found in great numbers at high maturity heat rates (>25˚C). In this study, the fact that *L. plantarum* and *P. pentosaceus* were identified as the dominant flora indicates that the sausage samples could be subjected to heating over 25˚C during the maturity period.

Drosinos *et al*. [25] identified *Lactococcus lactis* subsp. *lactis* 4.9%, Rantsious *et al*. [1] 1%, and Kaban [24] 7.0%**.** In this study, *Lactococcus lactis* subsp. *lactis* was identified 13.2%.

Greco *et al*. [26] determined that 13.3% of these isolates was *L. curvatus* subsp. *curvatus*, Drosinos *et al*. [23] 4.3% and Kaban [24] 10.9%. Comi *et al*. [27] characterized 150 LAB with molecular methods by isolating them and identified 54 isolates (36%) as *L. curvatus* subsp. *curvatus.* In this study, *L. curvatus* subsp. *curvatus* was identified 10.9%.

The other strains isolated from sausage samples consist of *L. brevis*, *L. fermentum*, *Weisella viridescens, W. confusa, L. delbrueckii* subsp. *delbrueckii*, *L. collinoides*, *Leuconostoc mesenteroides* subsp. *mesenteroides/ dextranicum*. Similar strains are identified by Rantsious *et al*. [1], Parente *et al*. [9], Drosinos *et al*. [23] and Kaban [24], Papamanoli *et al*. [28], Klinberg *et al*. [29], Aymerich *et al*. [30],

Since the isolated LAB from fermented sausage samples were characterized with phenotypic and genotypic methods in this study, it can finally be informed that the phenotypic methods should be supported with the genotypic ones as also indicated by Gevers *et al*. [6], Conter *et al*. [10], Cocolin *et al*. [20] and De Urraza *et al*. [31].

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