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Evolution of fermenting microbiota in tarhana produced under controlled technological conditions

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ABSTRACT

The purpose of this study was to evaluate the evolution of lactic acid bacteria (LAB) and yeasts during the fermentation of tarhana produced with some pasteurised ingredients and carried out at 30 and 40 °C. The chemical parameters were those typical for tarhana production. Coliform bacteria were not detected during fermentation, while LAB and yeasts were in the range 10^7–10^8 colony forming units (CFU) g^{-1}. Plate counts showed an optimal development of both fermenting microbial groups and the differences in cell concentrations were not significant ($P > 0.05$). LAB were isolated during fermentation and grouped on the basis of phenotypic and polymorphic characteristics. LAB isolates were identified by a combined genetic approach consisting of 16S/23S rRNA intergenic spacer region (ITS) and partial 16S rRNA gene sequencing as *Pediococcus acidilactici*, *Lactobacillus plantarum* and *Lactobacillus brevis*. Hence, the pasteurisation of the vegetable ingredients, excluded wheat flour, enhanced the hygienic conditions of tarhana without influencing the normal evolution of LAB. However, the fermentation at 40 °C favoured pediococci, while the production at 30 °C was mainly characterised by lactobacilli. Yeasts, identified by the restriction fragment length polymorphism (RFLP) of the 5.8S ITS rRNA gene, were mainly represented by the species *Saccharomyces cerevisiae* in both productions.

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1. Introduction

In the last years, a new alimentary life-style is gaining consensus with consumers being more and more attracted by ethnic food products that are typical of a given geographical area.

Tarhana is a traditional Turkish cereal based lactic acid fermented food product mainly produced at home or at home-scale level. It is also made commercially on small and large scales. The methods for tarhana production differ depending on the region (Daglioğlu, 2000). However, there are four different types of tarhana, stated by Turkish Standardization Institute: flour tarhana, goce (cracked wheat) tarhana, semolina tarhana and mixed tarhana (Anonymous, 1981). The difference between them is the usage of the wheat flour, cracked wheat and semolina separately or as combinations in the recipe. Similar products are kishk (kushk) in Middle East countries, trahanas in Greece, thanu in Hungary, talkuna in Finland and atole in Scotland (Daglioğlu, 2000; Erten and Tanguler, 2010).

Tarhana is obtained mainly from lactic acid fermentation of a mixture of wheat flour, yogurt (stirred or set yogurt) and, depending on the region of production, raw or cooked vegetables (tomato, onion, pepper etc.), spices (mint, basil, dill, tarhana herb etc.), bakers' yeast and salt. This product is generally let to ferment for a week at ambient temperature and the dominant microbiota is mainly represented by lactic acid bacteria (LAB) and yeasts. The resulting product is listed among the acidic fermented foods (İbanoğlu and İbanoğlu, 1999; Sengun et al., 2009) characterised by acidic taste and yeast aroma. At the end of the fermentation, the wet tarhana is dried in the sun or by dryer and grounded. It is widely used for soup making due to having a high nutritive value. It is also consumed as a snack when dried as thin layer or nugget after fermentation, not to be ground (Erbaş et al., 2005).

In the last years, this product has been investigated for several characteristics, including its microbiological aspects (Temiz and Yilmazer, 1998; Erbaş et al., 2005; Sengun et al., 2009; Turantaş and Kemahlioğlu, in press). The presence of yeasts, mainly belonging to the species *Saccharomyces cerevisiae*, is generally attributed to the addition of baker's yeast during ingredient mixing. Several LAB species have been found associated with tarhana

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fermentation, which may have a different origin. The thermophilic LAB are commonly provided by yogurt, but also the mesophilic LAB species (*Lactobacillus* spp., *Pediococcus* spp. and *Enterococcus* spp.) may have a dairy rather than wheat origin (Settanni and Moschetti, 2010). The survival of the major food pathogenic microorganisms has been investigated during production of tarhana and it is recommended not to consume wet fresh tarhana within the first 7 days of production (Turantaş and Kemahlioğlu, in press).

LAB represent the most important microbial group for tarhana fermentation; they play a defining role in the generation of the aromatic compounds typical for the final product (İbanoğlu et al., 1995) and strongly participate to the stability of the product during storage by inhibition of several unwanted microorganisms (Settanni and Corsetti, 2008). However, yeasts may also contribute to the aromatic profile of tarhana, since they develop several by-products compounds from cereal constituents (Valmorri et al., 2010). Besides LAB and yeast species present in the raw materials, other intrinsic factors (nutrient availability, inhibitory substances, microbial competitions), as well as the technological conditions applied may affect the organoleptic characteristics of the final product. The temperature of fermentation may be selective for the growth of certain microorganisms and, on the contrary, inhibitory for others.

The main objective of the present study was to evaluate the effect of two temperatures (30 and 40 °C) on the evolution of LAB and yeasts during the fermentation of flour type tarhana produced with pasteurised additional vegetable ingredients.

2. Materials and methods

2.1. Tarhana production

Tarhana was produced with the following ingredients according to Erbaş et al. (2005): red pepper (660 g), tomato (660 g), onion (330 g), basil (50 g), mint (50 g), dill (35 g), whole wheat flour (1765 g), baker's yeast (20 g), thick yogurt (1320 g), rock salt (110 g). All raw materials were purchased in retail markets located in Adana (Turkey). Before mixing, onions were peeled and pepper seeds were removed. After that, pepper, tomato, onion, basil, mint and dill were washed, chopped and minced. This mixture was pasteurised at 65 °C for 30 min and after cooling to 30 °C it was mixed to the other ingredients and kneaded to obtain a dough.

The dough was separated into two aliquots and placed in 10-L glass jars. The two doughs were incubated at 30 °C (dough A) and 40 °C (dough B).

Tarhana samples for chemical and microbiological analysis were collected soon after mixing and at 2-d intervals, till the eighth day of fermentation.

2.2. Chemical determinations

Tarhana samples were subjected to conventional chemical analysis: pH was determined using a pH metre (Inolab WTW, Weilheim, Germany); total acidity (TA) was measured by titrating tarhana sample up to pH 8.1 with 0.1 mol L^{-1} NaOH using digital pH meter and expressed as grams of lactic acid L^{-1} (Erbaş et al., 2005); water activity (A_W) was directly determined using the water activity instrument of Novasina LabMaster-a_W (Switzerland) with 3–5 g of tarhana sample. Chemical measurements were performed in triplicate.

2.3. Microbiological analysis and microorganism isolation

Decimal dilutions of Tarhana samples (25 g) were prepared in sterile physiological solution (225 g) by homogenisation using a classic blender. Microbial suspensions were plated and incubated as follows: total mesophilic count (TMC) on plate count agar (PCA), incubated aerobically at 30 °C for 72 h; coliforms on violet red bile agar (VRBA), incubated aerobically at 37 °C for 24 h; mesophilic rod and coccus LAB on MRS and M17 agar, respectively, incubated anaerobically at 30 °C for 48 h; thermophilic rod and coccus LAB on MRS and M17 agar, respectively, incubated anaerobically at 40 °C for 48 h; total yeasts on potato dextrose agar (PDA) agar, incubated aerobically at 25 °C for 48 h; non-*Saccharomyces* yeasts on lysine agar (LA), incubated aerobically at 25 °C for 48 h. All media were purchased from Oxoid (Basingstoke, UK). Counts were carried out in duplicate.

Analysis of variance (ANOVA), elaborated with the program SAS 2004, version 9.1.2 (Statistical Analysis System Institute Inc., Cary, NC, USA), was used to evaluate differences among tarhana productions with a significance level $P \leq 0.05$.

After growth, colonies of various shapes (at least 5 with identical morphology) of yeasts and Gram-positive [Gregersen KOH method (Gregersen, 1978)] and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 5%, v/v) bacteria (presumptive LAB) were randomly picked from count plates and transferred to the corresponding broth media. The isolates were purified by successive sub-culturing and stored in glycerol at −80 °C until further experimentations.

2.4. Phenotypic grouping of isolates

Cell morphology of LAB and yeast isolates was determined by an optical microscope. Yeasts were grouped per morphology, whereas LAB were subjected to further phenotypic assays.

Rod and coccus-shaped LAB cultures were first grouped on the basis of cell disposition, growth at 15 and 45 °C and CO_2 production from glucose. The last test was carried out in the optimal growth media (MRS for rod LAB and M17 for coccus LAB) containing all components except citrate, whose fermentation by certain LAB may determine gas formation (Parente and Cogan, 2004). M17 contained glucose in place of lactose. The assay consisted of LAB inoculation into test tubes sealed with H_2O agar (2%, w/v). The strains negative to the assay were inoculated into test tubes containing the optimal growth media prepared with a mixture of pentose carbohydrates (xylose, arabinose and ribose, 8 g L^{-1} each) in place of glucose. Coccus isolates were further sub-grouped on the basis of their growth at pH 9.6 and in presence of 6.5% NaCl.

2.5. Genotypic differentiation and identification of LAB

Cell lysis for DNA extraction from LAB cultures was performed by the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as template DNA for PCR reactions.

Strain differentiation was performed by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis in a 25-μL reaction mix using single primers M13 (Stenlid et al., 1994), AB111, and AB106 (Van den Braak et al., 2000). Amplifications were performed by means of T1 Thermocycler (Biometra, Göttingen, Germany) applying the conditions reported by Zapparoli et al. (1998) for primer M13 and those reported by Van den Braak et al., 2000 for primers AB111 and AB106. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and visualized by UV transillumination after staining with SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA). Deoxyribonucleic acid ladder 1 kb (Invitrogen, Carlsbad, CA, USA) was used as a molecular size marker. RAPD-PCR profiles were analysed with the pattern analysis software package Gel Compare Version 4.1 (Applied Maths, Kortrijk, Belgium). Genetic similarities among microorganisms were calculated according to Pearson

product moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method using arithmetic average clustering algorithm.

Genotypic identification of LAB with different RAPD-PCR profiles was carried out by a polyphasic approach consisting of 16S/23S rRNA intergenic spacer region (ITS) and partial 16S rRNA gene sequencing.

ITS amplification was performed as described by White et al. (1990), whereas 16S rRNA gene amplification followed the protocol reported by Weisburg et al. (1991). Both analysis were based on the colony PCR technique: colonies grown onto the surface of agar plates were picked up by means of a sterile tip and suspended into 100 μL Tris–HCl (100 mmol L^{-1})–EDTA (1 mmol L^{-1}) (TE). Four microlitres of each cell suspension were used for PCR reactions. DNA fragments were visualized as above described, purified by the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequences were determined by the dideoxy chain termination method with the DNA sequencing kit (Perkin–Elmer Cetus, Emeryville, CA, USA) according to the manufacturer's instructions. The sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database (Altschul et al., 1997).

2.6. Yeast identification

Yeast identification was carried out by the restriction fragment length polymorphism (RFLP) of the 5.8S ITS rRNA gene. This region was amplified following the protocol reported by Esteve-Zarzoso et al. (1999). The amplified DNA was digested with the restriction endonucleases *Cfo*I, *Hae*III, and *Hinf*I (Roche Diagnostics, Mannheim, Germany) according to the supplier's instructions. PCR products and their corresponding restriction fragments were separated in 1.5 and 3% w/v agarose gels, respectively, in 1× TAE (40 mmol L^{-1} Tris-acetate, 1 mmol L^{-1} EDTA, pH 8.2) buffer. After electrophoresis, the gels were stained with SYBR safe (Molecular probes) and acquired as above described.

2.7. Assays for bacteriocin activity

The antimicrobial activity of each LAB strain was first detected by the agar-spot deferred method (ASDM) and the strains showing positive results were subsequently tested by the well diffusion assay (WDA) (Schillinger and Lücke, 1989). Both assays were performed following the modifications of Corsetti et al. (2004) using *Lactobacillus sakei* LMG 2313, *Listeria innocua* 4202 and *Listeria monocytogenes* ATCC19114^{T} as indicator strains. Tests were carried out in triplicate.

Sensitivity of active supernatants to proteolytic enzymes was tested by treatment with proteinase K (12.5 U mg^{-1}), protease B (45 U mg^{-1}) and trypsin (10.6 U mg^{-1}) at a final concentration of 1 mg mL^{-1} in phosphate buffer (pH 7.0). All enzymes were purchased from Sigma–Aldrich (Milan, Italy). The supernatants were incubated for 2 h at 37 °C and the remaining activity was determined by WDA (Settanni et al., 2005).

3. Results

3.1. Evolution of chemical parameters

The results of pH and TA of tarhana samples collected at different times from productions A and B showed a diverse ($P < 0.05$) evolution (Table 1). Dough A was characterised by lower pH values than dough B during the whole period of fermentation and it reached a final pH of 3.62 at the eight day, while dough B showed a pH value of 4.05. These data were confirmed by TA whose increment was quicker for dough A than dough B. Dough A also showed a higher final TA value (12.88 g L^{-1}) than dough B (10.23 g L^{-1}). Concerning A_w, negligible differences ($P > 0.05$) were found between the two tarhana fermentations.

3.2. Microbiological analysis

At the beginning of fermentation, both tarhana productions (Table 1) were characterised by the same microbial concentrations, since they were obtained from the same preparation bulk. Coliforms were never detected during the whole process. TMC was 7.5 log CFU g^{-1} at time zero (T_0); it reached the maximum concentration at day 4 for both doughs (8.4 and 8.3 log CFU g^{-1} for dough A and B, respectively) and after that diminished. The other microbial groups showed a similar behaviour with highest count values at day 4. However, dough A showed a quicker increment of mesophilic and thermophilic rod and coccus LAB, as well as total yeasts than dough B; in fact, these microbial groups reached approximately 8 log CFU g^{-1} at day 2 for dough A, whereas at day 4 for dough B. Non-*Saccharomyces* yeasts were about 4 order of magnitude lower than total yeasts for both tarhana productions. Unlike other microorganisms, non-*Saccharomyces* yeasts reached the maximum concentration level at day 6 instead of day 4. However, no statistical differences ($P > 0.05$) were found between the two tarhana productions.

3.3. Isolation and grouping of LAB

A total of 224 colonies were randomly collected from tarhana samples. All cultures were subjected to microscopic inspection and

Table 1
Characteristics of tarhana during fermentation.

Tarhana productions	Chemical parameters			Microbial counts (log CFU g^{-1})							
	pH	TA (g L^{-1})[a]	A_w	PCA	VRBA	MRS 30 °C	M17 30 °C	MRS 40 °C	M17 40 °C	PDA	LA
Dough A:											
T_0	4.49 ± 0.00	4.54 ± 0.01	0.905 ± 0.003	7.5 ± 0.3	0	7.4 ± 0.2	7.7 ± 0.3	6.7 ± 0.3	7.9 ± 0.3	7.2 ± 0.3	3.3 ± 0.3
2d	3.73 ± 0.01	7.59 ± 0.10	0.905 ± 0.002	8.0 ± 0.6	0	8.0 ± 0.3	8.1 ± 0.6	7.9 ± 0.6	8.0 ± 0.2	8.0 ± 0.2	2.3 ± 0.3
4d	3.63 ± 0.00	10.61 ± 0.13	0.916 ± 0.006	8.4 ± 0.4	0	8.4 ± 0.5	8.2 ± 0.3	8.2 ± 0.0	8.1 ± 0.3	8.0 ± 0.2	3.1 ± 0.4
6d	3.61 ± 0.01	13.64 ± 0.59	0.910 ± 0.006	7.3 ± 0.4	0	7.6 ± 0.5	7.6 ± 0.4	7.8 ± 0.4	7.5 ± 0.6	7.5 ± 0.2	4.0 ± 0.5
8d	3.62 ± 0.04	12.88 ± 0.60	0.907 ± 0.013	6.9 ± 0.0	0	7.2 ± 0.7	7.0 ± 0.4	7.4 ± 0.1	7.2 ± 0.0	7.3 ± 0.5	2.5 ± 0.0
Dough B:											
T_0	4.49 ± 0.00	4.55 ± 0.13	0.905 ± 0.004	7.5 ± 0.3	0	7.4 ± 0.7	7.7 ± 0.3	6.7 ± 0.3	7.9 ± 0.5	7.2 ± 0.5	3.3 ± 0.6
2d	4.29 ± 0.01	6.07 ± 0.20	0.904 ± 0.003	7.6 ± 0.3	0	7.4 ± 0.3	7.2 ± 0.2	7.0 ± 0.6	7.1 ± 0.0	7.5 ± 0.4	3.0 ± 0.0
4d	4.02 ± 0.01	10.23 ± 0.49	0.908 ± 0.001	8.3 ± 0.4	0	8.2 ± 0.3	8.2 ± 0.3	8.3 ± 0.7	7.9 ± 0.3	8.3 ± 0.3	2.6 ± 0.6
6d	4.07 ± 0.01	11.37 ± 0.19	0.906 ± 0.006	7.8 ± 0.1	0	7.8 ± 0.2	7.8 ± 0.7	7.8 ± 0.7	7.9 ± 0.3	7.9 ± 0.4	3.9 ± 0.4
8d	4.05 ± 0.01	10.23 ± 0.39	0.905 ± 0.004	7.3 ± 0.1	0	7.9 ± 0.6	7.9 ± 0.3	7.9 ± 0.4	7.8 ± 0.7	7.8 ± 0.6	2.1 ± 0.4

[a] As lactic acid.

separated in 114 cocci and 110 rods. After Gram characterisation and catalase test, 112 cocci and 110 rods were still considered presumptive LAB cultures, as being Gram-positive and catalase-negative.

All cultures were tested for growth temperature (15 and 45 °C) and CO_2 production from glucose, whereas cocci LAB were also evaluated for growth at pH 9.6 and in presence of NaCl 6.5% (w/v). The combinations of the phenotypic characters considered allowed the separation of the 222 LAB cultures into 5 groups (Table 2), 4 for rods and 1 for cocci. The less numerous groups were group II and IV that included 8 and 11 isolates, respectively. CO_2 production from glucose was scored negative for groups I and II which were tested for growth in presence of pentose sugars, evidencing their facultative heterofermentative metabolism.

3.4. Differentiation and identification of LAB

Following the methodology of De Angelis et al. (2001), about 30% of the isolates ($n = 67$) of each phenotypic group, representing the different productions and samples, was subjected to RAPD analysis using primer M13 (results not shown). The 67 isolates analysed were divided into five main clusters, one for each of the five phenotypic groups. One isolate per cluster was further processed with primers AB111 and AB106 which confirmed that the isolates analysed constituted five different strains (Fig. 1).

Two isolates per RAPD profile were identified by ITS and partial 16S rRNA gene sequencing. The BLAST search evidenced a percentage of identity with sequences available in the NCBI database of at least 97%, which is considered the minimum level of similarity of ribosomal genes of strains belonging to the same species (Stackebrandt and Goebel, 1994). Table 3 shows the results of identification of LAB and their detection during tarhana fermentation. The species with the highest number of isolates was *Pediococcus acidilactici* (group V). *Lactobacillus plantarum* was the species identified for groups I and II, while *Lactobacillus brevis* was the closest relative species for the isolates of the groups III and IV.

LAB identification demonstrated a higher presence of *P. acidilactici* then lactobacilli in tarhana fermented at 40 °C, while an opposite trend was observed for tarhana fermented at 30 °C, whose main LAB were *L. plantarum* and *L. brevis*. The isolates allotted in the last two species detected in tarhana kept at 40 °C belonged to groups II and IV, respectively.

3.5. Isolation, grouping and identification of yeasts

Ninety isolates were randomly collected from the media used for yeast count. All cultures were subjected to microscopic inspection and separated into two morphological groups: 1) globose, ellipsoid or elongate in shape and multipolar budding; 2) ovoidal and globose.

Table 2
Phenotypic grouping of LAB isolates collected during fermentation of tarhana subjected to different thermal regimes.

Characters	Clusters				
	I ($n = 61$)	II ($n = 8$)	III ($n = 30$)	IV ($n = 11$)	V ($n = 112$)
Morphology	Rod	Rod	Rod	Rod	Coccus
Growth:					
15 °C	+	+	+	+	+
45 °C	+	−	+	−	+
pH 9.6	+	+	+	+	+
6.5% NaCl	+	+	+	+	+
CO_2 from glucose	−	−	+	+	−
Growth in presence of pentose carbohydrates	+	+	n.d.	n.d.	n.d.

n.d. not determined.

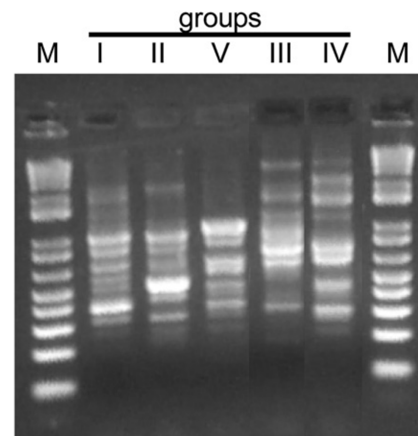

Fig. 1. RAPD-PCR profiles obtained with primer M13 representative of the five groups of LAB isolated during tarhana fermentation.

Twenty-one isolates were selected from the various samples of the two productions. Ten representative cultures were subjected to genetic identification. The first analysis consisted in molecular weight determination of the ribosomal RNA region 5.8S ITS which generated two products (Table 4). The electrophoretic separation of DNA fragments obtained after enzymatic digestion produced two different profiles with endonuclease *Cfo*I, three with *Hae*III and three with *Hinf*I (Table 4). The species *S. cerevisiae* was identified by an ITS band of 850 bp recognised for eight isolates, but the main restriction products reported in literature (Esteve-Zarzoso et al., 1999; De Llanos Frutos et al., 2004; Villa-Carvajal et al., 2006) were found for seven of them. *Rhodotorula glutinis* (ITS = 650 bp) was identified for two isolates, whose RFLP patterns were those reported in literature after digestion with *Cfo*I and *Hinf*I, while *Hae*III produced an atypical profile.

3.6. Bacteriocin-like inhibitory substances (BLIS) production

In order to better investigate the low biodiversity of tarhana LAB, the five strains were initially tested for antibacterial compound production by means of the ASDM against three indicator strains with high sensitivity to bacteriocins (Hartnett et al., 2002; Corsetti et al., 2008). In this step, the possible inhibitory effect of the organic acids and of hydrogen peroxide was not excluded, since the overlay containing the indicator strains was on direct contact with colonies of the producer strains. All strains produced a clear halo of inhibition zone against one or more indicator strains. Subsequently, the active supernatants were treated with catalase, neutralised, sterilised by filtration and tested by the WDA against the same 3 indicators. Three supernatants belonging to the two strains of *L. plantarum* and the strain of *P. acidilactici* kept the antimicrobial activity showing the following clear zones around the wells: *L. plantarum* group I 15 (±1) mm against *L. innocua* 4202, 20 (±2) mm against *L. monocytogenes* ATCC 19114, 25 (±2) mm against *L. sakei* LMG 2313; *L. plantarum* group II 14 (±1.5) mm against *L. innocua* 4202, 20 (±2.5) mm against *L. sakei* LMG 2313; *P. acidilactici* 20 (±1.5) mm against *L. sakei* LMG 2313. Differences in the sensitivity *versus* the indicator strains indicated that inhibition caused by tarhana LAB was strain-specific. Both strains of *L. brevis* were negative to WDA. The antibacterial compounds were inactivated by proteolytic enzymes, proving their proteinaceous nature, a general characteristic of bacteriocins (Tagg et al., 1976; Jack et al., 1995). These substances have not yet been characterised for amino acid and gene sequences, therefore they will be referred to as BLIS.

Table 3
Species of LAB isolated during fermentation of tarhana subjected to different thermal regimes.

Species	BLAST results						Isolation source		Growth medium		Incubation (°C)	
	ITS			16S			Dough A	Dough B	MRS	M17	30	40
	bp	% homology	Acc. No.	bp	% homology	Acc. No.						
L. plantarum (group I)	457	99	JN014074	720	99	JN014071	■		■		■	
L. plantarum (group II)	269	99	JN014072	679	99	JN089400	■	■	■		■	■
L. brevis (group III)	443	100	JN014073	713	98	JN014070	■		■		■	
L. brevis (group IV)	348	100	JN014075	672	99	JN089401	■	■	■		■	■
P. acidilactici	295	99	JN014068	714	99	JN014069	■	■	■	■	■	■

4. Discussion

The general production of tarhana includes main ingredients, wheat flour and yogurt, and other ingredients, represented by several vegetables, salt and baker's yeast. In the present work, tarhana was produced following the traditional recipe of Adana, but a technological step was added during preparation, since the vegetable mixture was pasteurised before mixing with flour, yogurt and baker's yeast. This modification of the production process may play a defining role in the hygienic characteristics of the final product.

Tarhana is traditionally produced at home, where the temperature applied for incubation cannot be kept under control easily and it may be subjected to consistent fluctuations, between night and day hours and, especially, between summer and winter months. Tarhana is also manufactured at industrial level. Hence, the technological conditions applied during production may vary greatly, affecting the overall characteristics of the final products. At this proposal, the temperature of fermentation may be selective for the growth of certain microorganisms and, on the contrary, inhibitory for others. For this reason, the work was undertaken to evaluate the impact of two different fermentation temperatures, 30 and 40 °C, on the evolution of the main microbial groups (LAB and yeasts) of tarhana.

Yogurt is a fermented milk obtained after acidic coagulation of milk proteins thanks to the action of thermophilic LAB (Oberman and Libudzisz, 1998). The experimental design of this work considered a high fermentation temperature (40 °C) that could favour the development of species of yogurt origin rather than mesophilic LAB. This innovation could result in products characterised by peculiar organoleptic features, mainly sensory, which, possibly, are appreciated by unusual tarhana consumers, especially when it is prepared in form of dried snack.

Chemical analysis of tarhana produced at 30 and 40 °C showed differences in TA and pH, while A_w measurements did not evidence consistent variations among productions. These data are in agreement with those reported by similar works performed on tarhana produced in standard thermal conditions (25–28 °C) (Erbaş et al., 2005; Turantaş and Kemahlioğlu, in press). Sengun et al. (2009) reported higher values of pH (mainly ranging from 4.0 to 4.9) for tarhana samples fermented from one to 21 days, but the fermentation temperatures were not provided. The acidification kinetics of our experimentations were confirmed by TA which, according to pH decrement, showed an acid content increment. Similar changes in TA for tarhana have been previously observed (İbanoğlu et al., 1995).

Microbiological investigation included TMC, coliforms, LAB (rods and cocci, mesophilic and thermophilic) and yeasts. TMC reached the maximum concentration at the fourth day for both doughs and diminished soon after. Comparable concentrations and a similar behaviour was observed for mesophilic rod and coccus LAB and thermophilic coccus LAB. Slightly lower levels were found for thermophilic rod LAB and yeasts. These data showed a general behaviour for tarhana (Erbaş et al., 2005; Sengun et al., 2009; Turantaş and Kemahlioğlu, in press). Our results also displayed that mesophilic and thermophilic LAB concentrations were almost superimposable to one another, but this is not a general behaviour of tarhana LAB. Sengun et al. (2009) observed that some samples were characterised by higher counts of thermophilic LAB while, on the contrary, other samples showed higher concentrations of mesophilic LAB. Furthermore, in some cases prevailed coccus LAB over rod LAB, while in some productions rod LAB became dominant. These observations underline the statement that different technologies of production may determine differences in the microbial ecology of tarhana during fermentation.

The high number of yeasts was not surprising, since comparable concentrations of LAB and yeasts are generally reported for this product (Sengun et al., 2009). Count values obtained from the two media differed between 4 and 6 orders of magnitude. The low numbers of non-*Saccharomyces* yeasts could be due to the addition of the common baker's yeast to help fermentation, which is mainly constituted by *S. cerevisiae* (Valmorri et al., 2010).

Microbial evaluations also included the determinations of coliform bacteria. This group of bacteria is generally hosted in the intestinal tract and for this reason they are considered to be indicators of food safety (Jay et al., 2009). From our investigation, coliforms were not detected during the entire fermentation period, showing that the pasteurisation was effective.

LAB isolates were subjected to several phenotypic tests, generally employed to perform bacterial grouping (Valmorri et al., 2006), which resulted into five groups. RAPD-PCR confirmed the phenotypic grouping. The identification of LAB resulted in the following

Table 4
Species of yeasts isolated during fermentation of tarhana subjected to different thermal regimes.

Species	ITS (bp)	RFLP products (bp)			Isolation source	
		*Cfo*I	*Hae*III	*Hinf*I	Dough A	Dough B
S. cerevisiae	850	360 + 340 + 140 + 50	320 + 220/230 + 160/170 + 140	380/390 + 125 + 50	■	■
S. cerevisiae	850	360 + 340+140 + 50	350 + 225+175 + 130 + 50	380/390 + 125 + 50	■	
R. glutinis	650	300 + 210/220 + 90	210/215 + 130 + 50 + 60	340/360 + 200 + 50/60	■	

three species: *L. brevis*, *L. plantarum* and *P. acidilactici*. All species are commonly reported to be associated with fermented food products (Wood, 1998). Furthermore, *L. plantarum* and *P. acidilactici* are typical of tarhana (Sengun et al., 2009). Two strains, one of *L. plantarum* and one of *L. brevis*, showed the growth at 45 °C, an atypical behaviour for strains of these species (Hammes and Vogel, 1995). However, *L. plantarum* with this characteristic has been already isolated from tarhana (Sengun et al., 2009). *P. acidilactici* was represented by a single strain, which dominated the fermentation at 40 °C. The massive presence of *P. acidilactici* in tarhana is common (Sengun et al., 2009).

The three LAB species detected may be found associated with raw materials used in food processing (Fleming and McFeeters, 1981) and *P. acidilactici* is also found at high numbers in some yogurt productions (Birollo et al., 2000; Badis et al., 2004). This observation suggested that yogurt maybe the ultimate source of *P. acidilactici* inoculums in tarhana.

The typical yogurt LAB, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, were never isolated during tarhana fermentation. This observation was not surprising. After ingredient mixing (T_0), yogurt represented the 26.4% (w/w) of the mixture and its dilution may have determined a decrease of LAB below the plate count detection limit. During fermentation, yogurt does not contain enough lactose to further support the growth of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. The total content of fermentable sugars of wheat flour barely varies from 1.55 to 1.85% (w/w) (Martinez-Anaya, 1996), with maltose, which is not utilized by any of the two species (Hammes and Vogel, 1995; Hardie and Whiley, 1995), being the most abundant. Furthermore, the presence of salt at 2.2% (w/w) makes the environment quite hostile for these species, since it is known that 2.0% NaCl does not allow the growth of *L. delbrueckii* subsp. *bulgaricus* (Wheater, 1955), while it may determine only a weak development of *S. thermophilus* (Zirnstein and Hutkins, 2000).

Pasteurisation has undoubtedly played a defining role in keeping tarhana LAB biodiversity at low levels, but un-treated flour, which has been added after thermal treatment of the other additional vegetable ingredients, may vehiculate living LAB (Corsetti et al., 2007). Thus, in order to better investigate the factors affecting the low biodiversity found among tarhana LAB, the five strains were characterised for antimicrobial compound production. Both *L. plantarum* strains and *P. acidilactici* were found to be BLIS producers. Several strains of these species have been found to produce bacteriocins (Mothlagh et al., 1992; Nissen-Meyer et al., 1993; Jimenez-Diaz et al., 1995). It is worth of note that, although the only bacteriocin approved for utilization as preservative in many foods is nisin (Federal Register, 1988), produced by *Lactococcus lactis* and commercially available as Nisaplin™ (Danisco, Copenhagen, Denmark), pediocin PA-1, produced by *P. acidilactici*, is being marketed (under the brand ALTA™ 2431, Kerry Bioscience, Carrigaline, Co. Cork, Ireland). It may be stated that, besides pasteurisation, BLIS production contributed to the low LAB biodiversity found in this study; in fact all BLIS were active against a lactobacillus indicator. The production of antimicrobial substances may confer a competitive advantage over non-bacteriocin producing strains (Franciosi et al., 2009) and may warrant a longer persistence of the producing strains themselves (Settanni et al., 2005).

The levels of concentration detected for yeasts were of the same order of magnitude than LAB. This finding is not surprising, because already reported for tarhana (Erbaş et al., 2005). The high number of yeasts may be due to the addition of baker's yeast as common ingredient for tarhana production. Blastomycetic investigation at species level resulted in the identification of *S. cerevisiae* throughout tarhana production from the beginning of fermentation till the eighth day. The addition of baker's yeast explains its presence at high numbers (Valmorri et al., 2010). Interestingly, this species was also detected on LA, a medium that is generally employed for differential counts of non-*Saccharomyces* yeasts. From our practical observations some *Saccharomyces* strains may develop colonies on this medium. *R. glutinis*, a species found associated to several fermented foods (Arroyo-López et al., 2008; Francesca et al., 2010), was revealed at subdominant levels in this work.

S. cerevisiae is the common fermenting agent of several foods and beverages, such as bread, beer, wine etc. The finding of this species in tarhana fermented at 40 °C is worth of note. This species performs optimal fermentations at mesophilic temperature, but its persistence at high temperature is not new. Survival of *S. cerevisiae* cells under conditions of extreme heat may be possible due to trehalose, a disaccharide that enables proteins to retain their native conformation at elevated temperatures and suppresses the aggregation of denatured proteins (Singer and Lindquist, 1998). This behaviour may open several further application of *S. cerevisiae* to produce foods.

5. Conclusions

The process of pasteurisation of the vegetable additional ingredients of tarhana did not influence the common LAB evolution during fermentation. In terms of LAB counts, no statistical differences were found between the two tarhana productions carried under diverse thermal regimes, but the identification of LAB during the eight days of fermentation showed a higher presence of *P. acidilactici* then lactobacilli in tarhana fermented at 40 °C, while an opposite trend was observed for tarhana fermented at 30 °C, whose main LAB were *L. plantarum* and *L. brevis*. Yeasts were mainly represented by *S. cerevisiae*.

Works are being prepared in order to better characterize the two tarhana productions for their chemical and sensory characteristics and to determine the acceptability by consumers.

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