Evolution of fermenting microbiota in tarhana produced under controlled technological conditions

Luca Settanni a,*, Hasan Tanguler b, Giancarlo Moschetti a, Stefano Reale c, Valeria Gargano c, Huseyn Erten b

a DEMETRA Department, University of Palermo, Viale delle Scienze 4, 90128 Palermo, Italy
b Cukurova University, Faculty of Agriculture, Department of Food Engineering, 01330 Adana, Turkey

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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 ITS 1 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 5.8 ITS rRNA gene, were mainly represented by the species Saccharomyces cerevisiae in both productions.

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 (Daglioglu, 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 (Daglioglu, 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 (Ibanoğlu and Ibanoglu, 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 Yılmazer, 1998; Erbaş et al., 2005; Sengun et al., 2009; Turanțaş and Kemahlioglu, 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...
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 (Turantas and Kemahlioglu, 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 (Ibanoglu 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-aW (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; coliflora 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 lycine 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 ≤ 0.05.

After growth, colonies of various shapes (at least 5 with identical morphology) of yeasts and Gram-positive [Gregersen (1978)] and catalase negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H\(_2\)O\(_2\) 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\(_2\)O\(_2\) 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 (Biomera, 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 RNA 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 analyses 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 (Qiagen S.p.a., Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequences were determined by the dyeoxy 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 CfoI, HaeIII, and HinfI (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 as indicator strains. Tests were carried out in triplicate.

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 eighth 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 Aw, negligible differences (P > 0.05) were found between the two tarhana fermentations.

### Table 1

<table>
<thead>
<tr>
<th>Tarhana productions</th>
<th>Chemical parameters</th>
<th>Microbial counts (log CFU g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>TA (g L⁻¹)</td>
</tr>
<tr>
<td>Dough A:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀</td>
<td>4.49 ± 0.00</td>
<td>4.54 ± 0.01</td>
</tr>
<tr>
<td>2d</td>
<td>3.73 ± 0.01</td>
<td>7.59 ± 0.10</td>
</tr>
<tr>
<td>4d</td>
<td>3.63 ± 0.00</td>
<td>10.61 ± 0.13</td>
</tr>
<tr>
<td>6d</td>
<td>3.61 ± 0.02</td>
<td>13.64 ± 0.59</td>
</tr>
<tr>
<td>8d</td>
<td>3.62 ± 0.04</td>
<td>12.88 ± 0.60</td>
</tr>
<tr>
<td>Dough B:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀</td>
<td>4.49 ± 0.00</td>
<td>4.55 ± 0.13</td>
</tr>
<tr>
<td>2d</td>
<td>4.29 ± 0.01</td>
<td>6.07 ± 0.20</td>
</tr>
<tr>
<td>4d</td>
<td>4.02 ± 0.01</td>
<td>10.23 ± 0.49</td>
</tr>
<tr>
<td>6d</td>
<td>4.07 ± 0.01</td>
<td>11.37 ± 0.19</td>
</tr>
<tr>
<td>8d</td>
<td>4.05 ± 0.01</td>
<td>10.23 ± 0.39</td>
</tr>
</tbody>
</table>

*As lactic acid.*

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 (T₀); 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
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 CO2 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. CO2 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 rDNA 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 than 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.

<table>
<thead>
<tr>
<th>Characters</th>
<th>Clusters</th>
<th>I (n = 61)</th>
<th>II (n = 8)</th>
<th>III (n = 30)</th>
<th>IV (n = 11)</th>
<th>V (n = 112)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td></td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Coccus</td>
<td>Rod</td>
</tr>
<tr>
<td>Growth: 15 °C</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>45 °C</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH 9.6</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6.5% NaCl</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CO2 from glucose</td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pentose carbohydrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. not determined.

Figure 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 CfoI, three with HaeIII and three with HinfI (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 CfoI and HinfI, while HaeIII 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 BUS.
differences in TA and pH, while produced in standard thermal conditions (25°C), the fermentation temperatures were not provided. The acidification kinetics of our experiments were confirmed by TA which, according to pH decrement, showed an acid content increment. Similar changes in TA for tarhana have been previously observed (Ibanoglu 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 Kemahlioglu, 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 3

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

<table>
<thead>
<tr>
<th>Species</th>
<th>BLAST results</th>
<th>Isolation source</th>
<th>Growth medium</th>
<th>Incubation (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITS</td>
<td>% homology</td>
<td>Acc. No.</td>
<td>16S</td>
</tr>
<tr>
<td>L. plantarum (group I)</td>
<td>457</td>
<td>99</td>
<td>JN014074</td>
<td>720</td>
</tr>
<tr>
<td>L. plantarum (group II)</td>
<td>269</td>
<td>99</td>
<td>JN014072</td>
<td>679</td>
</tr>
<tr>
<td>L. brevis (group III)</td>
<td>443</td>
<td>100</td>
<td>JN014073</td>
<td>713</td>
</tr>
<tr>
<td>L. brevis (group IV)</td>
<td>348</td>
<td>100</td>
<td>JN014075</td>
<td>672</td>
</tr>
<tr>
<td>P. acidilactici</td>
<td>295</td>
<td>99</td>
<td>JN014068</td>
<td>714</td>
</tr>
</tbody>
</table>

### Table 4

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

<table>
<thead>
<tr>
<th>Species</th>
<th>ITS (bp)</th>
<th>RFLP products (bp)</th>
<th>Isolation source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITS</td>
<td>bp</td>
<td>% homology</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>850</td>
<td>360 + 340 + 140 + 50</td>
<td>320 + 220/239 + 160/170</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>850</td>
<td>360 + 340 + 140 + 50</td>
<td>350 + 225 + 175 + 130 + 50</td>
</tr>
<tr>
<td>R. glutinis</td>
<td>650</td>
<td>300 + 210/220 + 90</td>
<td>210/215 + 130 + 50 + 60</td>
</tr>
</tbody>
</table>
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 (Birillo 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 (T0), yogurt represented the 26.4% (w/w) of the mixture and its dilution may have determined a decrease of LAB below the plate count-forward limit (Biraghi for 1005). The 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 Whiteley, 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 (Motlagh 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 *S. cerevisiae* (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. Blastomycetous investigatation 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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