DYNAMICS OF LACTIC ACID BACTERIA DURING PASTIRMA PRODUCTION

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ABSTRACT
Pastirma is a traditional dry-cured meat product of Turkey, produced from whole beef or muscles of water buffalo. Studies on the microbial diversity of pastirma generally focus on the final product. In this study, we aimed to determine the lactic acid bacteria (LAB) dynamics during the pastirma production process. Samples were obtained from a commercial producer at four different production stages (after curing, after first drying, before cemen coating, and final product). During the production, the pH level slightly increased from ~5.6 to ~5.8, while the water activity (aw) decreased to ~0.86 until cemen addition, after which it increased to 0.89. Total mesophilic aerobic bacteria (TMAB) and LAB counts increased during the production stages reaching 7.15 and 6.64 log cfu/g, respectively. The most dominant LAB for all stages was Lactilactobacillus sakei group with a relative abundance (RA) of 52-73% RA. Weissella species W. viridescens and W. halotolerans followed the L. sakei group. Phylogenetic analysis of 16S rRNA gene indicated that all L. sakei isolates were of subsp. carnous, however, (GTG)5 fingerprinting demonstrated a high degree of intraspecies variation. Moreover, fingerprinting analysis showed that L. sakei isolates of specific fingerprinting groups were selected towards the final production stages. The present study elucidates how the LAB diversity changes both at the species and intraspecies level during pastirma production.

Keywords: dry-cured meat products, (GTG)5 fingerprinting, lactic acid bacteria dynamics, Lactilactobacillus sakei, pastirma

INTRODUCTION
Pastirma is a dry-cured meat product produced using whole muscles of beef and water buffalo (Kaban, 2009). Pastirma is produced through a series of drying and pressing steps and the production does not involve heating or smoking (Kaban, 2013). During pastirma production, after the carcass breakdown, muscles are separated from fat and connective tissues and they are rubbed with curing salts including salt, nitrate, and nitrite (figure 1; Fettaboglu et al., 2019; Kaban, 2009). After washing to remove excess salts, the meat pieces are air-dried by hanging (first drying), pressed to ease drying by forcing water out, and dried again (second drying) (Kaban, 2009). The meat pieces are pressed once more (second pressing) and dried further (third drying) before coating with cemen (Kaban, 2009). Cemen is a paste prepared from fenugreek (Trigonella foenum-graecum) flour, red, pepper, and garlic (Fettaboglu et al., 2019). Cemen gives pastirma its characteristic flavor and contributes to microbiological stability of the product and protects further drying (Dincer & Kivanc, 2012; Ozturk, 2015). The pieces are subjected to a final drying step before consumption (figure 1).

The main bacterial microbiota of pastirma consists of lactic acid bacteria (LAB) and catalase positive cocci (Öz et al., 2017). Catalase positive cocci are important for color formation, oxidative stability, and aroma formation in pastirma (Fettaboglu et al., 2019). LAB, on the other hand, produce lactic acid from fermentable sugars decreasing pH and contribute to the safety of meat products by inhibiting pathogenic and spoilage bacteria (Cocolin et al., 2011; Kroeckel, 2013). The competitiveness of LAB also results from the metabolites they produce, such as bacteriocins and hydrogen peroxide (Kroeckel, 2013). In addition, LAB contribute to flavor formation with the mild acidic taste of lactic acid and small amounts of other metabolites, such as acetic acid, pyruvic acid, ethanol, acetoin, and CO2 (Kroeckel, 2013). The LAB species in pastirma were determined in few studies (Cinar et al., 2019; Dincer & Kivanc, 2012; Öztürk et al., 2017; Özdemir & Strikken, 1996); and these studies have been conducted on the final product rather than the production process. In this study, we aimed to determine how LAB diversity changes both at the species and at intraspecies level during the pastirma production process using samples obtained from a commercial facility. The isolates were identified molecularly by 16S rRNA sequencing and analyzed using (GTG)5 fingerprinting. Microbiological counts as well as the chemical parameters, pH and aw, were determined to correlate with LAB dynamics. To the best of our knowledge, this study represents the first to reveal the LAB dynamics during the stages of pastirma processing.

MATERIAL AND METHODS

Samples
Pastirma samples from four production stages (figure 1), after curing (stage 1), after first pressing (stage 2), before cemen coating (stage 3), and the final product (stage 4), in a single lot were kindly provided by an industrial producer located in Afyonkarahisar (Turkey) in October 2018. The samples were brought to the laboratory in cold-chain and analyzed in one day. Three samples were combined (25 g) and homogenized in 225 mL physiological salt solution (0.85% NaCl, w/v [Merck KGaA, Darmstadt, Germany]) using a Stomacher (Bagmixer 400, Interscience, Saint Nom, France) (Hazart et al., 2017). All analyses were performed in two replicates.

Determination of pH and aw
The pH value was measured using a HI 2211 pH meter (Hanna Instruments Inc., Woonsocket, RI, USA). Water activity (aw) was determined using a water activity meter (Labsswift-aw, Novasina AG, Lachen, Switzerland).

Bacterial counts
Serial dilutions of the homogenized samples were inoculated on different media for bacterial enumerations. Total mesophilic aerobic bacteria (TMAB) were plated on plate count agar (PCA) (Biolife, Milan, Italy) and incubated aerobically at 30°C for 2 days. Lactic acid bacteria (LAB) were inoculated on de Man, Rogosa and Sharpe (MRS) agar (Merck KGaA, Darmstadt, Germany) and M17 agar (Biolife) and the plates were grown for 2 days at 30°C in anaerobic conditions (Dincer & Kivanc, 2012; Öztürk et al., 2017).

Bacteria isolation
From MRS and M17 agar plates containing ~25-250 colonies, approximately square root of morphologically different colonies were picked and streaked onto
agar plates containing the same isolation media. Single colonies were streaked consecutively twice for purification. Isolates were grown in liquid media containing 20% glycerol and stored at -80°C (Dincer & Kivanc, 2012). Gram reaction and catalase test were performed on the isolates as described previously (Chester, 1979; Powers, 1995).

**DNA extraction and polymerase chain reaction (PCR)**

DNA extraction was performed using a salting-out method as described previously (Martin-Platero et al., 2007). The isolates were grouped by (GTG)5 fingerprinting using the PCR conditions described previously (Versalovic et al., 1994). PCR reaction mixture was prepared as described previously (Seri & Metin, 2021). PCR reactions were conducted using T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were run in a 1.5% (w/v) agarose gels containing Red Safe nucleic acid staining solution (Intron Biotechnology Inc., Korea) in 1 x TAE buffer (Bio-Rad) using Wide Mini-Sub Cell GT electrophoresis system (Bio-Rad) and visualized by Gel Doc EZ Imager (Bio-Rad).

Selected isolates according to the grouping patterns were subjected to 16S rRNA PCR using the universal primers 27F (5’-AGAGTTTGATCCTGCGCTCAG) and 1492R (5’-GTTACCTTGTGACTCAG) (Lane, 1991). The PCR mixture was prepared as described for (GTG)5 PCR reaction except that 2 µL of both forward and reverse primer was used. PCR was carried out as described previously (Seri & Metin, 2021). PCR reaction products were analyzed on 0.8% (w/v) agarose gels, purified using a GeneJet PCR purification kit (Thermo Fisher Scientific) according to manufacturer’s instructions and sequenced with the primers used for PCR.

**Statistical analyses**

Data were analyzed using JMP 14.1.2 software (SAS Institute Inc., Cary, NC, USA). A comparison among different stages was performed using a one-way analysis of variance (ANOVA) at a confidence level of 95% (p < 0.05).

**Table 1 Change of pH, a<sub>0</sub>, and bacterial counts during pastirma manufacture**

<table>
<thead>
<tr>
<th>Stage no</th>
<th>Stage</th>
<th>pH</th>
<th>a&lt;sub&gt;0&lt;/sub&gt;</th>
<th>LAB</th>
<th>M17</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>After curing</td>
<td>5.63±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.923±0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.88±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.23±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>After first pressing</td>
<td>5.73±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.859±0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.29±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.67±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Before çemen coating</td>
<td>5.81±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.862±0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.13±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.83±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Final product</td>
<td>5.80±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.889±0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.15±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.64±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

Legend: <sup>a</sup> Different superscripts represents values that differ significantly within a column at P<0.05

During pastirma production, a<sub>0</sub>, significantly decreased from 0.923 (±0.003) at stage 1 in the salted meat to 0.859 (±0.003) at stage 2 after first pressing (p<0.05) resulting in about 7% reduction, after which, it remained relatively constant until çemen coating. Osmotic pressure created by salt and pressing operation accelerated the water loss and drying resulting in a significant decrease in a<sub>0</sub>. Çemen addition slightly increased the a<sub>0</sub> of the final product to 0.889 (±0.002) (p<0.05), likely because of diffusion of water in çemen into the meat. Similar a<sub>0</sub> decreases were reported during the pastirma production process (Inat, 2008; Kaban, 2009; Ozturk, 2015); however, the stages where the most significant reduction takes place differ among the studies. Kaban (2009) reported a significant decrease after the end of first drying (−0.96) to the final product (−0.87). According to their results, a<sub>0</sub> decrease continued at a similar rate during the first and the second drying periods until the final product was obtained. However, our results indicated that the most significant reduction occurred during the first drying and the first pressing stages. In the study conducted by Ozturk (2015), a decrease of a<sub>0</sub> from 0.97 to 0.90 occurred during salting to the first drying stage, while pressing and the second drying stage had little effect. The differences observed between the studies might be due to the differences in the process conditions such as temperature and relative humidity during drying, or the pressing force applied. A<sub>0</sub> is an important parameter determining the shelf life of the product. Reduction of a<sub>0</sub> during the drying steps makes pastirma an intermediate-moisture food, increasing its microbial stability (Leistner, 1985).

**Phylogenetic analyses of 16S rRNA gene**

Phylogenetic analyses were conducted using MEGA X (Kumar et al., 2018). The model describing the substitution pattern the best was determined to be Kimura 2-parameter with Gamma distribution, which resulted in the lowest Bayesian information criterion score.

**RESULTS AND DISCUSSION**

**Chemical properties of pastirma during the production process**

Change of pH and a<sub>0</sub>, was monitored during the production process because of their importance in microbiota development. Results indicated an increase of pH from 5.63 (±0.02) at stage 1 (after curing) to 5.81 (±0.01) at stage 3 (before çemen coating) (p<0.05), after which no significant change was observed (Tab 1). Previous studies on the pastirma production process indicated a similar trend of pH increase especially after the first drying step (Doğruer et al., 2003; Kaban, 2009; Ozturk, 2015). For instance, in the study conducted by Kaban (2009), pH value first decreased during the curing stage (~ pH 5.5) compared to the fresh meat, and then increased significantly during the later stages until the final product (~ pH 5.9) is obtained. Similar to our study, çemen addition had little effect in pH change. Although there are reports of a relatively constant pH level during the production of certain dry-cured meat products around the world (Shu et al., 2017; Vargas-Ramella et al., 2020), most studies have indicated (Benlacheheb et al., 2019; Lorenzo, 2014; Lorenzo et al., 2008; Pateiro et al., 2015; Virgili et al., 2007). For example, in Spanish Celta dry-cured loin, the pH value increases from 5.6 to 5.8 during dry-ripening (Pateiro et al., 2015). A similar pH increase from ~5.7 to ~5.9 was observed in Italian dry-cured ham during ageing (Virgili et al., 2007). The increase of pH during the ripening period is suggested to be due to proteolytic activity taking place in the muscle (Virgili et al., 2007). The origin of proteolysis in meat products has been suggested to be mainly due to meat-originated proteolytic enzymes; however, microbial enzymes have also been reported to be involved (Duri et al., 2004; Petrova et al., 2015; Scannell et al., 2004). pH increases have also been reported in other dry-cured products such as Spanish dry-cured lacon and dry-cured loin cevica (Lorenzo et al., 2014; Lorenzo et al., 2008).

**Bacterial counts of pastirma during the production process**

During the production process, TMAB and LAB counts changed in a similar manner: they increased during the production process until the final step, çemen coating, which did not change the counts significantly (p<0.05). The lowest TMAB count was 4.88 (±0.01) log cfu/g at stage 1, after curing, and it increased to 7.15 (±0.05) in the final product. Similar to our results, previous studies showed an increasing trend of TMAB loads with similar pH values during the pastirma production process (Doğruer et al., 1997; Guner et al., 2008; Gürbüz et al., 2003). LAB enumerated on MRS agar exhibited an increasing trend similar to TMAB, having the lowest load at stage 1 (4.23±0.01 log cfu/g), which increased to 6.83 (±0.05) before çemen addition. The decrease of LAB counts to 6.64 (±0.01) in the final product after çemen addition was statistically insignificant (p>0.05). LAB counts in different studies demonstrated a similar rising trend during the production stages although the values vary with final product counts changing from −4.5 to 7.25 log cfu/g (Doğruer et al., 1997; Guner et al., 2008; Gürbüz et al., 2003; Kaban, 2009). This variation was also indicated in studies that conducted LAB counts in different pastirma samples from the market to reveal an extensive variation between −3 – 8 log cfu/g (Akou & Kaya, 2001; Öz et al., 2017; Özdemir et al., 1998). Differences observed in the LAB loads of different pastirma samples are probably because of the variation of the properties of raw material, the production conditions used in different facilities as well as the storage period in the market. In addition, specifically in our study, microbial counts might have been affected by the cold chain transportation process of samples from the production facility in Afyonkarahisar to our laboratory in Istanbul. Although the loads differ in different studies, the rising trend during the production stages seem to be common.

Other dry-cured products also reported an increase in LAB counts after salting. For example, in dry-cured lacinon production, LAB counts increased during the post-salting stage and in the weeks of the ripening period (Vilar et al., 2000). In another dry-cured product el-gueddie, one log increase from 10<sup>2</sup> to 10<sup>3</sup> was observed in LAB load during the ripening stage compared to the after salting stage (Benlacheheb et al., 2019). M17 counts also had an increasing trend during the production process. The counts were similar with no statistical significance during salting and cold-pressing.
All 82 MRS isolates (named AB#) and only 7 of 80 M17 agar isolates (named AC#) were Gram positive and catalase negative putative LAB. These isolates were then typed by (GTG)5 fingerprinting analysis (Figure 2). Selected isolates from each fingerprinting group were subjected to 16S rRNA gene sequencing for member in different phylogenetic groups in the pastirma samples. These isolates harbor the strain diversity during the production process.

Different stages of the pastirma production process recruited L. sakei isolates of different (GTG)5 fingerprinting clades (figure 2). For example, while isolates of clade II-a and VI were isolated mostly from the first two stages, clades I, II-b and II-c were generally obtained from the last two stages. This finding indicates a selection of strains with certain genetic background during specific stages of the process. This selection process might be related to the pH or aw variations during production. It could also be related to the competitive ability of the strain because total bacterial counts increase from ~5 log cfu/g during the first two stages to ~7 log cfu/g during the last two stages (Tab 1). The strain with a better competing ability would be expected to appear during later stages of production. Similar to our finding, in the production of the Italian fermented sausage Ventriconia, three different biotypes of L. sakei were observed and during maturation, a specific biotype was selected and outcompeted others (Tremonte et al., 2017). How specific strains are selected during pastirma production process is an interesting question and requires further experiments, such as determination of pH, aw, and salt tolerance, as well as fitness of each strain, which could be performed in future studies.

In all pastirma production stages analyzed, L. sakeiL. graminis was the most dominant group with its relative abundance (RA) changing between 52-73% during different production stages (Figure 4). Weissella species follow this group with W. viridescens (21-28% RA) and W. halotolerans (4-21% RA). During the stages 3 and 4, other Weissella species, W. helenica/W. saugenius and W. thanlandensis were also observed (Figure 4). Weissella species are heterofermentative and produce ethanol, CO2, and acetic acid from gluconic (Kroeckel, 2013). The species W. viridescens, W. halotolerans, and W. helenica have been described as meat clade and isolated in various fermented meat products (Albano et al., 2009; Fusco et al., 2015; Kesen et al., 2012; Samelis et al., 1994). But they are generally present as a minority group among LAB (Fessard & Remize, 2017). In this study, the species W. helenica and W. saugenius could not be discriminated using 16S rRNA sequences. LI et al. (2020) reported that the phs gene coding for phenylalanine-rRNA synthetase alpha subunit, was very successful in discriminating Weissella species. Phsβ was also described a successful marker for other LAB species (Naser et al., 2005; Sánchez-Juanes et al., 2020). Therefore, this marker can be used in future studies if the isolates are further analyzed. In the final product, Leu. citreum (11% RA) appeared, possibly originating from cemen. Other species observed included C. diversors in the first and L. curvatus in the third stage.

In fermented meat products where the LAB dynamics was followed, different trajectories were reported. For example, in Ventriconia, RA of L. sakei progressively increased during maturation until only this species was detected (Tremonte et al., 2017). In harbin dry sausage production, on the other hand, the RA of L. sakei increased up to ~60% after six days and remained approximately around this level later (Hu et al., 2021). To the best of our knowledge, how the diversity of lactic acid bacteria changes during pastirma production has not been investigated before. We present here that in pastirma, L. sakeiL. graminis remained the predominant species group with an RA of 52-73% throughout the production process. However, the strain diversity of this species changed during different production stages. Analysis of microbial dynamics of samples from different producers in future studies and comparison of the results with the current study will be more comprehensive in understanding pastirma microbial ecology. The effects of seasonal variations on the microbiota of pastirma can also be explored. For example, in spontaneously fermented Italian sausages, L. sakei pangenome changed to a greater extent during the production indicating a strain diversity among production batches from different seasons (Franciosa et al., 2021).

Figure 1 Pastirma production process (Kahan 2009; Tekiņen and Dogran 2000). Samples obtained from different processing stages were indicated.
Figure 2. (GTG)5 fingerprinting analysis of pastirma LAB isolates. Selected isolates from each clade were identified by 16S rRNA sequencing. Isolate name, identification result and isolation stage were indicated next to the fingerprinting pattern of each isolate. The clades harboring different species or intraspecies groups were indicated as I-IX.
Figure 3 The phylogenetic analysis of LAB isolates and closely related type strains using 16S rRNA gene sequence. The analysis involved 830 nucleotides of 16S rRNA gene.
Figure 4 Relative abundance of LAB species in each production stage of pastirma

CONCLUSION

In conclusion, in this study, we have determined certain physicochemical parameters, bacterial dynamics, and LAB diversity during pastirma production process using samples from a commercial producer. During the production, while the pH increased from ~5.6 at the curing stage to 5.8 in the final product, a decrease from 0.92 to ~0.86 before çemen coating, after which it increased to ~0.89 in the final product. Both TMAB and LAB counts increased towards the final stages, where 7.15 and 6.64 log cfu/g were attained in the final product, respectively. The most common LAB in pastirma production was L. sakei, population. The different (GTG)5 clades observed for the L. sakei group were specific to different production stages, which indicated selection of strains with certain genetic background towards the final stages of pastirma.

Understanding the strain-level dynamics is important for comprehensive analyses of the production process. How specific strains are selected and the attributes they provided to the final product are exciting topics to be analyzed in future studies.

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