

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 çemen coating, and final product). During the production, the pH level slightly increased from ~5.6 to 5.8, while the water activity (a_w) decreased to ~0.86 until çemen 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 *Latilactobacillus 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. *carneus*, 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, *Latilactobacillus 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; Fettahoğlu 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 çemen (Kaban, 2009). Çemen is a paste prepared from fenugreek (*Trigonella foenum-graecum*) flour, red-pepper, and garlic (Fettahoğlu et al., 2019). Çemen 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 (Fettahoğlu 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 (Çinar et al., 2019; Dincer & Kivanc, 2012; Öz et al., 2017; Özdemir & Siriken, 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 a_w , 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 çemen 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) (Hazar et al., 2017). All analyses were performed in two replicates.

Determination of pH and a_w

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

Bacterial counts

Serial dilutions of the homogenated 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; Öz 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 restreaked 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 (Martín-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 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'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-GGTACCTGTTCAGACTT) (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 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).

(GTG)5 fingerprinting analysis

(GTG)5 fingerprinting patterns were analyzed using temporary Bionumerics (ver 8, Applied Maths, Sint-Martens-Latem, Belgium) evaluation licence that we have received permission to publish. A cluster analysis was performed using Ochiai similarity coefficient with 1% optimization and 1% band matching tolerance. Dendograms were generated using unweighted pair grouping by mathematical averaging (UPGMA).

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.

Evolutionary analyses were conducted using maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980) with Gamma distribution (5 categories, parameter = 0.1275).

RESULTS AND DISCUSSION

Chemical properties of pastirma during the production process

Change of pH and a_w 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 (Sha et al., 2017; Vargasa-Ramella et al., 2020), most studies indicate similar pH value increases (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 (Durá 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 lacón and dry-cured foal cecina (Lorenzo, 2014; Lorenzo et al., 2008).

Table 1 Change of pH, a_w and bacterial counts during pastirma manufacture

Stage no	Stage	pH	a _w	Bacterial counts (log cfu/g)		
				TMAB	LAB	M17
1	After curing	5.63±0.02 ^C	0.923±0.003 ^A	4.88±0.01 ^C	4.23±0.05 ^C	4.92±0.02 ^B
2	After first pressing	5.73±0.01 ^B	0.859±0.002 ^C	5.29±0.06 ^B	5.67±0.02 ^B	4.63±0.02 ^B
3	Before çemen coating	5.81±0.005 ^A	0.862±0.001 ^C	7.13±0.02 ^A	6.83±0.05 ^A	7.25±0.16 ^A
4	Final product	5.80±0.015 ^A	0.889±0.002 ^B	7.15±0.05 ^A	6.64±0.01 ^A	6.65±0.12 ^A

Legend: ^{A,B,C} Different superscripts represents values that differ significantly within a column at P<0.05

During pastirma production, a_w 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_w. Çemen addition slightly increased the a_w 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_w 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_w 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_w 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_w is an important parameter determining the shelf life of the product. Reduction of a_w during the drying steps makes pastirma an intermediate-moisture food, increasing its microbial stability (Leistner, 1985).

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 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±0.05 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 (Aksu & 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 lacón production, LAB counts increased during the post-salting stage and in the first weeks of the ripening period (Lorenzo et al., 2010; Vilar et al., 2000). In another dry-cured product el-gueddid, one log increase from 10⁶ to 10⁷ 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

stages, after which they increased to 7.25 (± 0.16) before çemen coating ($p < 0.05$) and remained similar in the final product ($p > 0.05$).

LAB diversity during the production process

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 molecular identification. A phylogenetic analysis was conducted using the 16S rRNA gene sequences of the isolates together with the type strains of the species recognized in BLAST search (figure 3). As a result, five genera and nine species were identified. While the mostly encountered species was *Latilactobacillus sakei*, renamed after the new taxonomy (Zheng *et al.*, 2020), this species was followed by *Weissella viridescens* and *Weissella halotolerans* (figure 3). The other species encountered were *Latilactobacillus graminis*, *Latilactobacillus curvatus*, *Carnobacterium divergens*, *Leuconostoc citreum*, *Weissella helenica/sagaensis* (could not be discriminated using 16S rRNA sequence), and *Weissella thailandensis*.

There are only a limited number of studies on the identification of LAB species of pastirma. In one of these studies, the most common LAB species in pastirma samples from different manufacturers was *L. sakei* which was followed by *Weissella cibaria* and *Weissella confusa* (Öz *et al.*, 2017). Another study, using biochemical characterization tests, similarly identified *L. sakei* as the dominant species (Özdemir & Siriken, 1996). The other two studies found different results; in one of them, *Pediococcus pentosaceus* was the main species dominating the samples in all different curing conditions tested (Çinar *et al.*, 2019), while the other study identified *Lactiplantibacillus plantarum* as the main species of pastirma samples collected from different cities of Turkey (Dincer & Kivanc, 2012). Microbial load of meat and the processing environment as well as processing conditions are effective on the microbiota. Generally, in fermented meat products, the mostly encountered LAB species is *L. sakei* followed by *L. curvatus*, and less frequently *L. plantarum*, while *Weissella*, *Leuconostoc*, *Pediococcus* and *Lactococcus* were reported as minority species (Aquilanti *et al.*, 2016; Fontana *et al.*, 2012; Franciosa *et al.*, 2021; Lebert *et al.*, 2014). The wide presence of *L. sakei* in meat products has been explained by its low temperature and salt tolerance as well as the versatile genomic repertoire to adapt to and grow on meat, outcompeting other microorganisms (Chaillou *et al.*, 2005; Zagorec & Champomier-Vergès, 2017).

In the current taxonomy, *L. sakei* group comprises four related species, *L. sakei*, *L. graminis*, *L. curvatus*, and *L. fuchuensis* (Zheng *et al.*, 2020). In addition, *L. sakei* is divided into two subspecies *L. sakei* subsp. *sakei* and *L. sakei* subsp. *carnosus* (Koort *et al.*, 2004; Zheng *et al.*, 2020). However, genome-wide analyses indicated large amount of diversity in this species group and the existence of different lineages (Eisenbach *et al.*, 2018, 2019; Kim *et al.*, 2021; Terán *et al.*, 2018). The intraspecies variation is not only at the genetic level, but also expresses itself at the phenotype (Montanari *et al.*, 2018), which is important for specific strains to be selected in certain conditions such as during growth on meat. In 16S rRNA phylogeny, all *L. sakei* isolates of pastirma were clustered together with *L. sakei* subsp. *carnosus* rather than *L. sakei* subsp. *sakei* (figure 3). However, (GTG)5 fingerprinting analysis indicated a highly diverse *L. sakei* population separated into three main clades, I, II, and VI, and subclades (figure 2). *L. graminis* isolates were also located in clades II and VI together with *L. sakei* isolates and could not be separated from *L. sakei* by (GTG)5 fingerprinting (figure 2).

Similar to the present study, previous studies conducting RAPD analysis reported a high degree of heterogeneity among *L. sakei* isolates (Bonomo *et al.*, 2008; Cocolin *et al.*, 2009; Comi *et al.*, 2005; Urso *et al.*, 2006). A similar finding was observed using (GTG)5 fingerprinting analyses in fermented Italian sausages (Amadoro *et al.*, 2015; Franciosa *et al.*, 2021). The presence of a diverse population of *L. sakei* was also reported in genome-wide comparison studies. For example, a study conducted with 9 *L. sakei* genomes showed that the core genome, shared by all genomes, consisted of approximately half the pan genome, which is described as the total set of non-redundant genes in the dataset (Guimarães *et al.*, 2015), indicating large amount of variability among strains (Eisenbach *et al.*, 2019). In another study performing phylogenetic analysis on the core-genomes of 63 *L. sakei* group strains showed the presence of different lineages in both subspecies, *sakei* and *carnosus* (Kim *et al.*, 2021). These findings indicate that *L. sakei* taxonomy might be revised in the future with possible new subspecies'. Understanding the conditions that select certain strains and the attributes that different strains give to the product is of utmost importance in the production of specific products.

The presence of different lineages in *L. curvatus* based on genome data were also reported in different studies (Eisenbach *et al.*, 2018; Kim *et al.*, 2021; Terán *et al.*, 2018). Terán *et al.* (2018) defined two lineages in *L. curvatus*; one comprising the type strain DSM 20019 and the other containing the strain FLEC03. (GTG)5 fingerprinting well-separated the *L. curvatus* (clade VII) from *L. sakei* and *L. graminis* (figure 2). In addition, in 16S rRNA phylogeny, *L. curvatus* AB63 was aligned with *L. curvatus* FLEC03 (figure 3), rather than the type strain DSM 20019, indicating that AB63 belongs to the *L. curvatus* lineage harboring the strain FLEC03.

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 a_w 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 be selected during later stages of production. Similar to our finding, in the production of the Italian fermented sausage Ventricina, 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, a_w , and salt tolerance, as well as fitness of each strain, which could be performed in future studies.

In all pastirma production stages analyzed, *L. sakei/L. 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. sagaensis* and *W. thailandensis* were also observed (Figure 4). *Weissella* species are heterofermentative and produce ethanol, CO₂, and acetic acid from glucose (Kroeckel, 2013). The species *W. viridescens*, *W. halotolerans*, and *W. helenica* have been described as meat-associated and isolated in various fermented meat products (Albano *et al.*, 2009; Fusco *et al.*, 2015; Kesmen *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. sagaensis* could not be discriminated using 16S rRNA sequences. Li *et al.* (2020) reported that the *pheS* gene coding for phenylalanyl-tRNA synthetase alpha subunit, was very successful in discriminating *Weissella* species. *PheS* 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 çemen. Other species observed included *C. divergens* 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 Ventricina, 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 $\sim 60\%$ 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. sakei/L. 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 according to the season indicating a strain-level diversity among production batches from different seasons (Franciosa *et al.*, 2021).

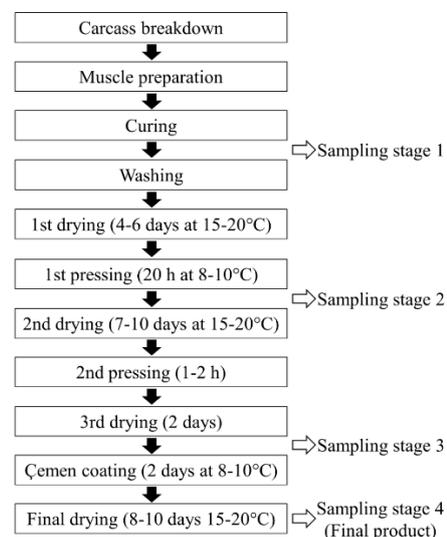


Figure 1 Pastirma production process (Kaban 2009; Tekinşen and Doğruer 2000). Samples obtained from different processing stages were indicated.

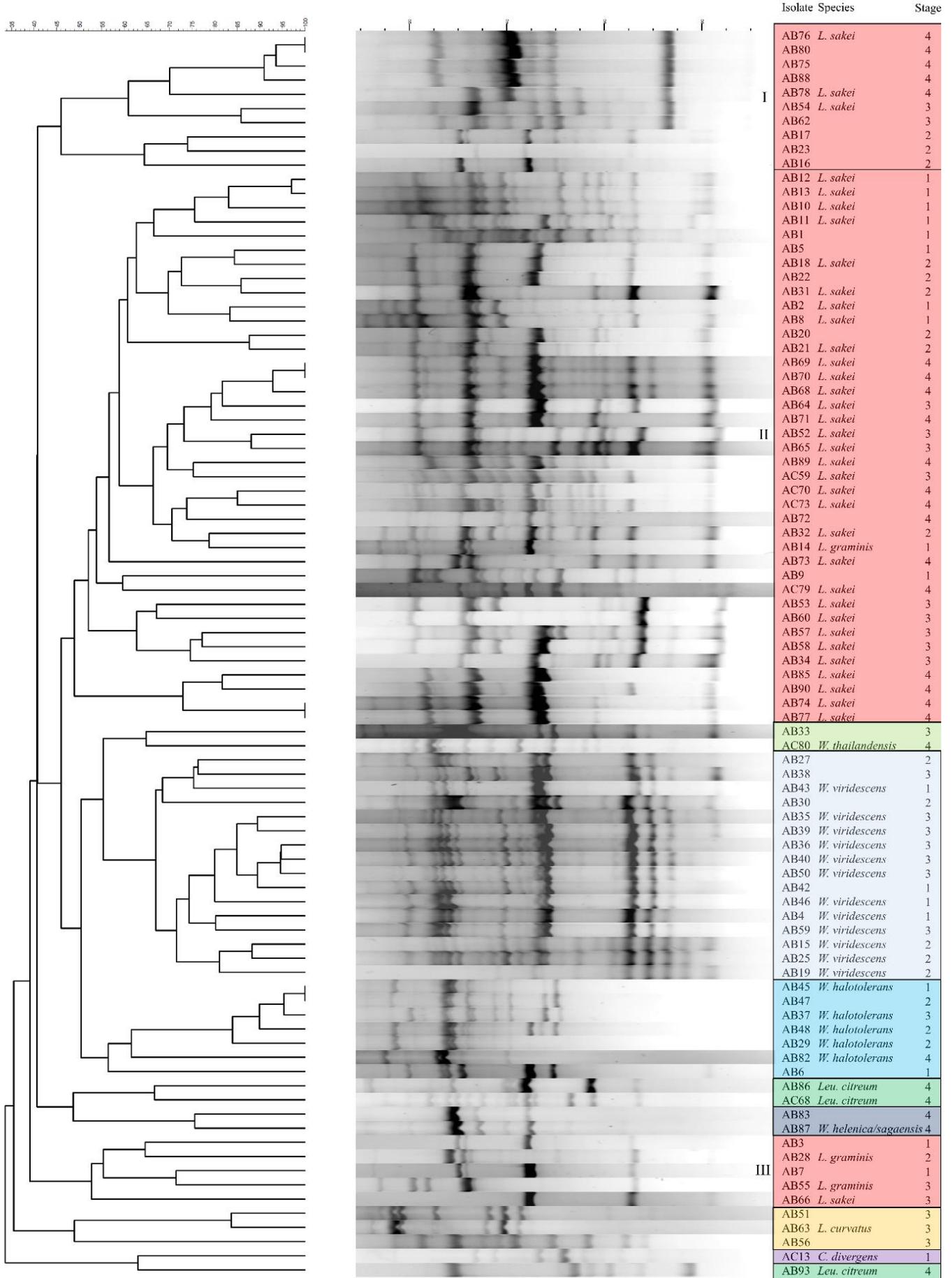


Figure 2 (GTG)5 fingerprinting analysis of pastirma LAB isolates. Selected isolates from each clade were identified by 16S rRNA sequencing. Isolate name, identification result and isolation stage were indicated next to the fingerprinting pattern of each isolate. The clades harboring different species or intraspecies groups were indicated as I-IX.

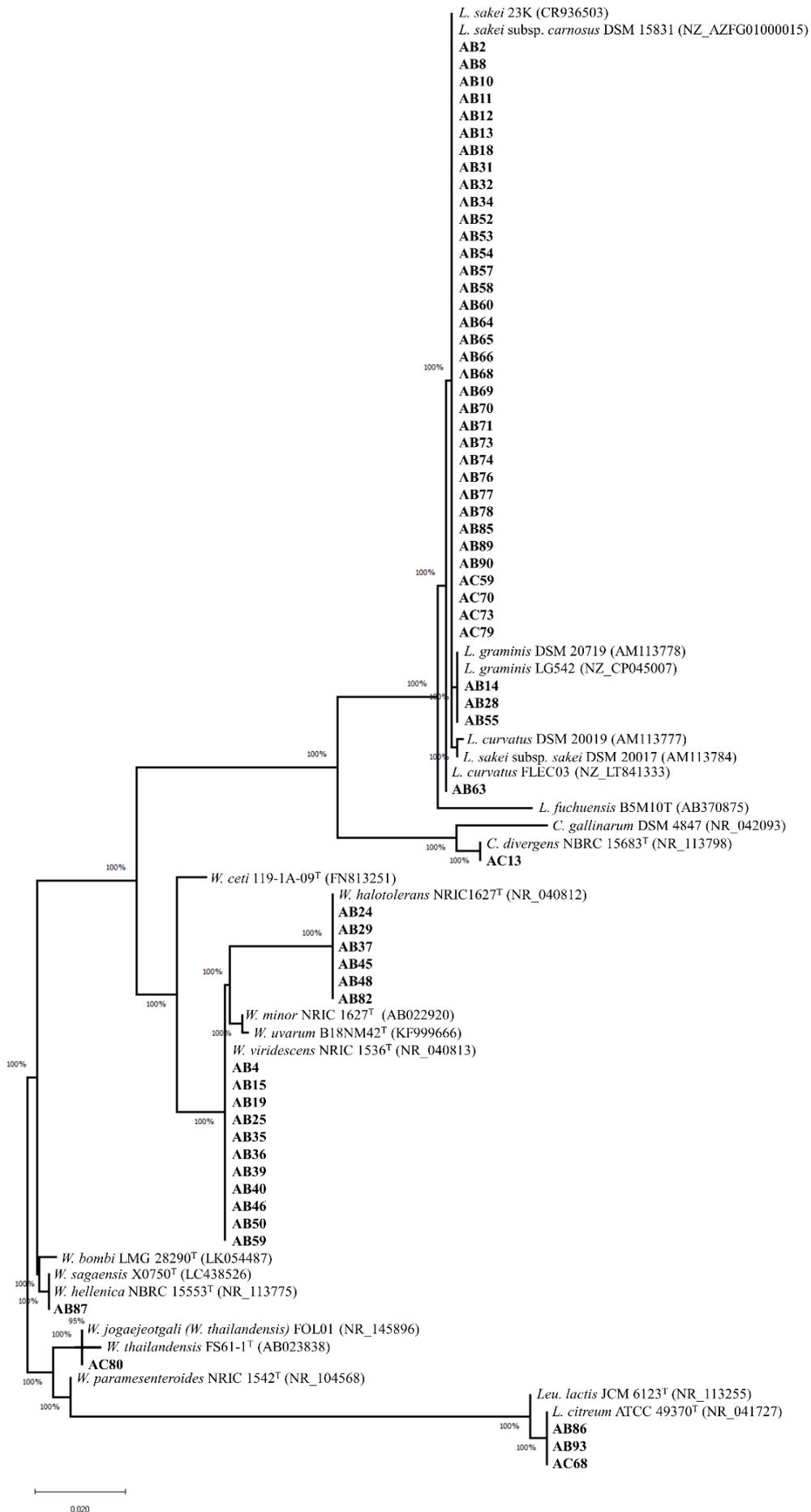


Figure 3 The phylogenetic analysis of LAB isolates and closely related type strains using 16S rRNA gene sequence. The analysis involved 830 nucleotides of 16S rRNA gene.

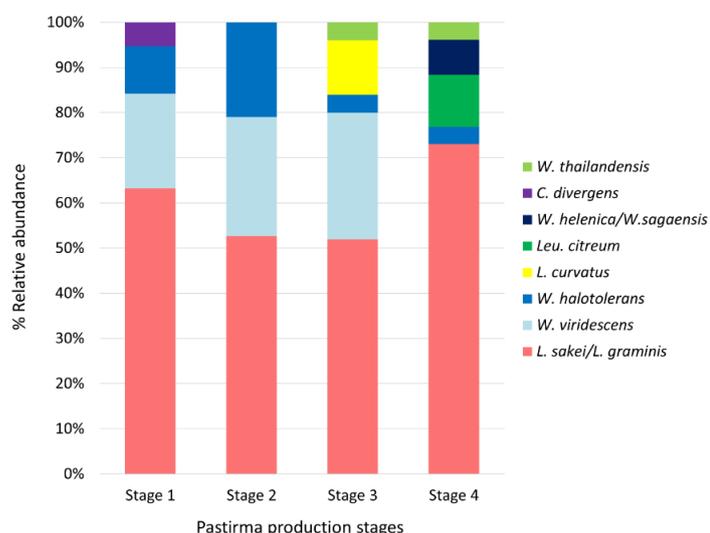


Figure 4 Relative abundance of LAB species in each production stage of pastirma

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_w decreased 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/L. graminis* group in all production stages analyzed maintaining 52–73% RA. *L. sakei* group was followed by *W. viridescens* and *W. halotolerans*. Other *Weissella* species, *W. helenica/W. sagaensis*, and a species related to *W. thailandensis* were also observed during production. *Leu. citreum* was encountered in the final stage and speculated to have originated from çemen coated on meat. Phylogenetic analysis using 16S rRNA gene demonstrated grouping of pastirma *L. sakei* isolates with *L. sakei* subsp. *carneus* rather than *L. sakei* subsp. *sakei*. On the other hand, (GTG)5 fingerprinting analysis indicated a highly differentiated *L. sakei/L. graminis* 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 analysis 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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REFERENCES

- Aksu, M. İ., & Kaya, M. (2001). Some Microbiological, Chemical and Physical Characteristics of Pastirma Marketed in Erzurum. *Turkish Journal of Veterinary and Animal Sciences*, 25(3), 319–326.
- Albano, H., van Reenen, C. A., Todorov, S. D., Cruz, D., Fraga, L., Hogg, T., Dicks, L. M. T., & Teixeira, P. (2009). Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from “Alheira”, a traditional fermented sausage produced in Portugal. *Meat Science*, 82(3), 389–398. <https://doi.org/10.1016/j.meatsci.2009.02.009>
- Amadoro, C., Rossi, F., Piccirilli, M., & Colavita, G. (2015). Features of *Lactobacillus sakei* isolated from Italian sausages: Focus on strains from Ventricina del Vastese. *Italian Journal of Food Safety*, 4(4), 5449–5449. <https://doi.org/10.4081/ijfs.2015.5449>
- Aquilanti, L., Garofalo, C., Osmani, A., & Clementi, F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: An overview. *International Food Research Journal*, 23, 429–445.
- Benlacheheb, R., Becila, S., Sentandreu, M. A., Hafid, K., Boudechicha, H.-R., & Boudjellal, A. (2019). El Gueddid, a traditional Algerian dried salted meat: Physicochemical, microbiological characteristics and proteolysis intensity during its manufacturing process and ripening. *Food Science and Technology International*, 25(4), 347–355. <https://doi.org/10.1177/1082013219825892>
- Bonomo, M. G., Ricciardi, A., Zotta, T., Parente, E., & Salzano, G. (2008). Molecular and technological characterization of lactic acid bacteria from traditional fermented sausages of Basilicata region (Southern Italy). *Meat Science*, 80(4), 1238–1248. <https://doi.org/10.1016/j.meatsci.2008.05.032>

- Chaillou, S., Champomier-Vergès, M.-C., Cornet, M., Cruz-Le Coq, A.-M., Dudez, A.-M., Martin, V., Beaufile, S., Darbon-Rongère, E., Bossy, R., Loux, V., & Zagorec, M. (2005). The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nature Biotechnology*, 23(12), 1527–1533. <https://doi.org/10.1038/nbt1160>
- Chester, B. (1979). Semiquantitative catalase test as an aid in identification of oxidative and nonsaccharolytic Gram-negative bacteria. *Journal of Clinical Microbiology*, 10(4), 525–528.
- Çinar, K., Fettahoğlu, K., & Kaban, G. (2019). Genotypic Identification of Lactic Acid Bacteria in Pastirma Produced with Different Curing Processes. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, 25(3), 299–303. <https://doi.org/10.9775/kvfd.2018.20853>
- Cocolin, L., Dolci, P., & Rantsiou, K. (2011). Biodiversity and dynamics of meat fermentations: The contribution of molecular methods for a better comprehension of a complex ecosystem. *Meat Science*, 89(3), 296–302. <https://doi.org/10.1016/j.meatsci.2011.04.011>
- Cocolin, L., Dolci, P., Rantsiou, K., Urso, R., Cantoni, C., & Comi, G. (2009). Lactic acid bacteria ecology of three traditional fermented sausages produced in the North of Italy as determined by molecular methods. *Meat Science*, 82(1), 125–132. <https://doi.org/10.1016/j.meatsci.2009.01.004>
- Cocolin, L., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C., & Cocolin, L. (2005). Characterisation of naturally fermented sausages produced in the North East of Italy. *Meat Science*, 69(3), 381–392. <https://doi.org/10.1016/j.meatsci.2004.08.007>
- Dincer, E., & Kivanc, M. (2012). Characterization of lactic acid bacteria from Turkish Pastirma. *Annals of Microbiology*, 62(3), 1155–1163. <https://doi.org/10.1007/s13213-011-0355-x>
- Doğruer, Y., Güner, A., Gürbüz, Ü., & Uçar, G. (2003). Sodyum ve potasyum nitratın üretim periyodu süresince pastirmanın kalitesine etkisi. *Turkish Journal of Veterinary and Animal Sciences*, 27(4), 805–811.
- Doğruer, Y., Gürbüz, Ü., Nizamlioğlu, M., Yalçın, S., & Atasever, M. (1997). Bromelin uygulamasının pastirmanın kimyasal, mikrobiyolojik ve duyuşal kalitesine etkisi. *Veteriner Bilimleri Dergisi . Eurasian Journal of Veterinary Sciences*, 13(2), 83–89.
- Durá, M. A., Flores, M., & Toldrá, F. (2004). Effect of *Debaryomyces* spp. On the proteolysis of dry-fermented sausages. *Meat Science*, 68(2), 319–328. <https://doi.org/10.1016/j.meatsci.2004.03.015>
- Eisenbach, L., Geissler, A. J., Ehrmann, M. A., & Vogel, R. F. (2019). Comparative genomics of *Lactobacillus sakei* supports the development of starter strain combinations. *Microbiological Research*, 221, 1–9. <https://doi.org/10.1016/j.micres.2019.01.001>
- Eisenbach, L., Janßen, D., Ehrmann, M., & Vogel, R. (2018). Comparative genomics of *Lactobacillus curvatus* enables prediction of traits relating to adaptation and strategies of assertiveness in sausage fermentation. *International Journal of Food Microbiology*, 286, 37–47. <https://doi.org/10.1016/j.ijfoodmicro.2018.06.025>
- Fessard, A., & Remize, F. (2017). Why Are *Weissella* spp. Not Used as Commercial Starter Cultures for Food Fermentation? *Fermentation*, 3(3), 38. <https://doi.org/10.3390/fermentation3030038>
- Fettahoğlu, K., Cinar Topcu, K., & Kaya, M. (2019). Biodiversity and characterization of Gram-positive, catalase-positive cocci isolated from pastirma produced under different curing processes. *Turkish Journal Of Veterinary And Animal Sciences*, 43, 68–75. <https://doi.org/10.3906/vet-1805-66>
- Fontana, C., Fadda, S., Coconcelli, P. S., & Vignolo, G. (2012). Lactic Acid Bacteria in Meat Fermentations. In *Lactic Acid Bacteria: Microbiological and Functional Aspects* (Fourth edition, pp. 247–264). CRC Press. <https://doi.org/10.1201/b11503-14>
- Franciosa, I., Ferrocino, I., Giordano, M., Mounier, J., Rantsiou, K., & Cocolin, L. (2021). Specific metagenomic asset drives the spontaneous fermentation of Italian sausages. *Food Research International*, 144, 110379. <https://doi.org/10.1016/j.foodres.2021.110379>
- Fusco, V., Quero, G. M., Cho, G.-S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W., & Franz, C. M. A. P. (2015). The genus *Weissella*: Taxonomy, ecology and biotechnological potential. *Frontiers in Microbiology*, 6, 155. <https://doi.org/10.3389/fmicb.2015.00155>
- Guimarães, L. C., Florczak-Wypianska, J., de Jesus, L. B., Viana, M. V. C., Silva, A., Ramos, R. T. J., Soares, S. de C., & Soares, S. de C. (2015). Inside the Pan-genome—Methods and Software Overview. *Current Genomics*, 16(4), 245–252. <https://doi.org/10.2174/1389202916666150423002311>
- Guner, A., Gonulalan, Z., & Dogruer, Y. (2008). Effect of tumbling and multi-needle injection of curing agents on quality characteristics of pastirma. *International Journal of Food Science & Technology*, 43(1), 123–129. <https://doi.org/10.1111/j.1365-2621.2006.01401.x>
- Gürbüz, Ü., Doğruer, Y., Yalçın, S., Nizamlioğlu, M., & Güner, A. (2003). Pastirma yapım teknolojisinin geliştirilmesinde sıcak dumanlama uygulanması ve kaliteye etkisi. *Veteriner Bilimleri Dergisi . Eurasian Journal of Veterinary Sciences*, 19(1–2), 57–66.
- Hazar, F. Y., Kaban, G., & Kaya, M. (2017). The effects of different processing conditions on biogenic amine formation and some qualitative properties in

- pastırma. *Journal of Food Science and Technology*, 54(12), 3892–3898. <https://doi.org/10.1007/s13197-017-2845-8>
- Hu, Y., Wang, H., Kong, B., Wang, Y., & Chen, Q. (2021). The succession and correlation of the bacterial community and flavour characteristics of Harbin dry sausages during fermentation. *LWT*, 138, 110689. <https://doi.org/10.1016/j.lwt.2020.110689>
- Inat, G. (2008). Determination of contamination sources and investigation of improving conditions in pastırma manufacturing. *Uludağ Üniversitesi Veteriner Fakültesi Dergisi*, 27(1–2), 53–59.
- Kaban, G. (2009). Changes in the composition of volatile compounds and in microbiological and physicochemical parameters during pastırma processing. *Meat Science*, 82(1), 17–23. <https://doi.org/10.1016/j.meatsci.2008.11.017>
- Kaban, G. (2013). Sucuk and pastırma: Microbiological changes and formation of volatile compounds. *Meat Science*, 95(4), 912–918. <https://doi.org/10.1016/j.meatsci.2013.03.021>
- Kesmen, Z., Yetiman, A. E., Gulluce, A., Kacmaz, N., Sagdic, O., Cetin, B., Adiguzel, A., Sahin, F., & Yetim, H. (2012). Combination of culture-dependent and culture-independent molecular methods for the determination of lactic microbiota in sucuk. *International Journal of Food Microbiology*, 153(3), 428–435. <https://doi.org/10.1016/j.ijfoodmicro.2011.12.008>
- Kim, E., Yang, S.-M., Kim, D., & Kim, H.-Y. (2021). Real-time PCR method for qualitative and quantitative detection of *Lactobacillus sakei* group species targeting novel markers based on bioinformatics analysis. *International Journal of Food Microbiology*, 355, 109335. <https://doi.org/10.1016/j.ijfoodmicro.2021.109335>
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16(2), 111–120. <https://doi.org/10.1007/BF01731581>
- Koort, J., Vandamme, P., Schillinger, U., & Björkroth, K. (2004). *Lactobacillus curvatus* subsp. *melibiosus* is later synonym of *Lactobacillus sakei* subsp. *carnosus*. *International Journal of Systematic and Evolutionary Microbiology*, 54, 1621–1626. <https://doi.org/10.1099/ijs.0.63164-0>
- Kroeckel, L. (2013). The role of lactic acid bacteria in safety and flavour development of meat and meat products. In J. M. Kongo (ed.), *Lactic Acid Bacteria - R & D for Food, Health and Livestock Purposes* (pp. 129–152). IntechOpen.
- Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, 35(6), 1547–1549. <https://doi.org/10.1093/molbev/msy096>
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In E. Stackebrandt & M. Goodfellow (Eds.), *Nucleic acid techniques in bacterial systematics* (pp. 115–175). John Wiley and Sons.
- Lebert, I., Leroy, S., & Talon, R. (2014). Microorganisms in Traditional Fermented Meats. In F. Toldrá, Y. H. Hui, I. Astiasarán, J. G. Sebranek, & R. Talon (Eds.), *Handbook of Fermented Meat and Poultry* (pp. 97–105). John Wiley & Sons, Ltd.
- Leistner, L. (1985). Hurdle Technology Applied to Meat Products of the Shelf Stable Product and Intermediate Moisture Food Types. In D. Simatos & J. L. Multon (Eds.), *Properties of Water in Foods: In Relation to Quality and Stability* (pp. 309–329). Springer Netherlands. https://doi.org/10.1007/978-94-009-5103-7_19
- Li, Y. Q., Tian, W. L., & Gu, C. T. (2020). *Weissella sagaensis* sp. nov., isolated from traditional Chinese yogurt. *International Journal of Systematic and Evolutionary Microbiology*, 70(4), 2485–2492. <https://doi.org/10.1099/ijsem.0.004062>
- Lorenzo, J. M. (2014). Changes on physico-chemical, textural, lipolysis and volatile compounds during the manufacture of dry-cured foal “cecina.” *Meat Science*, 96(1), 256–263. <https://doi.org/10.1016/j.meatsci.2013.06.026>
- Lorenzo, J. M., García Fontán, M. C., Cachaldora, A., Franco, I., & Carballo, J. (2010). Study of the lactic acid bacteria throughout the manufacture of dry-cured lacón (a Spanish traditional meat product). Effect of some additives. *Food Microbiology*, 27(2), 229–235. <https://doi.org/10.1016/j.fm.2009.10.003>
- Lorenzo, J. M., García Fontán, M. C., Franco, I., & Carballo, J. (2008). Biochemical characteristics of dry-cured lacón (a Spanish traditional meat product) throughout the manufacture, and sensorial properties of the final product. Effect of some additives. *Food Control*, 19(12), 1148–1158. <https://doi.org/10.1016/j.foodcont.2007.12.005>
- Martín-Platero, A. M., Valdívila, E., Maqueda, M., & Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein “salting-out” procedure. *Analytical Biochemistry*, 366(1), 102–104. <https://doi.org/10.1016/j.ab.2007.03.010>
- Montanari, C., Barbieri, F., Magnani, M., Grazia, L., Gardini, F., & Tabanelli, G. (2018). Phenotypic diversity of *Lactobacillus sakei* strains. *Frontiers in Microbiology*, 9, 2003–2003. <https://doi.org/10.3389/fmicb.2018.02003>
- Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., & Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiology*, 151(7), 2141–2150. <https://doi.org/10.1099/mic.0.27840-0>
- Öz, E., Kaban, G., Barış, Ö., & Kaya, M. (2017). Isolation and identification of lactic acid bacteria from pastırma. *Food Control*, 77, 158–162. <https://doi.org/10.1016/j.foodcont.2017.02.017>
- Seri, M., & Metin, B. (2021). Mycobiota of Konya mold-ripened (Kufllu) Tulum cheese and the diversity of *Penicillium roqueforti* isolates. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, 68(4), 349–354. <https://doi.org/10.33988/auvfd.778788>
- Özdemir, H., Şireli, U. T., Sarimehmetoğlu, B., & İnat, G. (1998). Investigation of the microbial flora of pastırma marketing in Ankara. *Turkish Journal of Veterinary and Animal Sciences*, 23, 57–62.
- Özdemir, H., & Sırken, B. (1996). Pastırmadan izole edilen laktobasillerin bazı biyokimyasal ve fizyolojik özellikleri-Some biochemical and physiological characterization of lactobacilli isolated from pastırma. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, 43(03), 307–310. https://doi.org/10.1501/Vetfak_0000000688
- Ozturk, I. (2015). Presence, changes and technological properties of yeast species during processing of pastırma, a Turkish dry-cured meat product. *Food Control*, 50, 76–84. <https://doi.org/10.1016/j.foodcont.2014.08.039>
- Pateiro, M., Franco, D., Carril, J. A., & Lorenzo, J. M. (2015). Changes on physico-chemical properties, lipid oxidation and volatile compounds during the manufacture of celta dry-cured loin. *Journal of Food Science and Technology*, 52(8), 4808–4818. <https://doi.org/10.1007/s13197-014-1561-x>
- Petrova, I., Aasen, I. M., Rustad, T., & Eikevik, T. M. (2015). Manufacture of dry-cured ham: A review. Part 1. Biochemical changes during the technological process. *European Food Research and Technology*, 241(5), 587–599. <https://doi.org/10.1007/s00217-015-2490-2>
- Powers, E. M. (1995). Efficacy of the Ryu nonstaining KOH technique for rapidly determining Gram reactions of food-borne and waterborne bacteria and yeasts. *Applied and Environmental Microbiology*, 61(10), 3756–3758. <https://doi.org/10.1128/aem.61.10.3756-3758.1995>
- Samelis, J., Maurogenakis, F., & Metaxopoulos, J. (1994). Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology*, 23(2), 179–196. [https://doi.org/10.1016/0168-1605\(94\)90051-5](https://doi.org/10.1016/0168-1605(94)90051-5)
- Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J. M., Velázquez, E., & Flores-Félix, J. D. (2020). Identification of species and subspecies of lactic acid bacteria present in spanish cheeses type “Torta” by MALDI-TOF MS and *pheS* gene analyses. *Microorganisms*, 8(2), 301. <https://doi.org/10.3390/microorganisms8020301>
- Scannell, A. G. M., Kenneally, P. M., & Arendt, E. K. (2004). Contribution of starter cultures to the proteolytic process of a fermented non-dried whole muscle ham product. *International Journal of Food Microbiology*, 93(2), 219–230. <https://doi.org/10.1016/j.ijfoodmicro.2003.11.007>
- Sha, K., Lang, Y.-M., Sun, B.-Z., Su, H.-W., Li, H.-P., Zhang, L., Lei, Y.-H., Li, H.-B., & Zhang, Y. (2017). Changes in lipid oxidation, fatty acid profile and volatile compounds of traditional Kazakh dry-cured beef during processing and storage. *Journal of Food Processing and Preservation*, 41(4), e13059. <https://doi.org/10.1111/jfpp.13059>
- Terán, L. C., Coeuret, G., Raya, R., Zagorec, M., Champomier-Vergès, M.-C., & Chaillou, S. (2018). Phylogenomic Analysis of *Lactobacillus curvatus* reveals two lineages distinguished by genes for fermenting plant-derived carbohydrates. *Genome Biology and Evolution*, 10(6), 1516–1525. <https://doi.org/10.1093/gbe/evy106>
- Tremonte, P., Sorrentino, E., Pannella, G., Tipaldi, L., Sturchio, M., Masucci, A., Maiuro, L., Coppola, R., & Succi, M. (2017). Detection of different microenvironments and *Lactobacillus sakei* biotypes in Ventricina, a traditional fermented sausage from central Italy. *International Journal of Food Microbiology*, 242, 132–140. <https://doi.org/10.1016/j.ijfoodmicro.2016.11.009>
- Urso, R., Comi, G., & Cocolin, L. (2006). Ecology of lactic acid bacteria in Italian fermented sausages: Isolation, identification and molecular characterization. *Systematic and Applied Microbiology*, 29(8), 671–680. <https://doi.org/10.1016/j.syapm.2006.01.012>
- Vargas-Ramella, M., Domínguez, R., Pateiro, M., Franco, D., Barba, F. J., & Lorenzo, J. M. (2020). Chemical and physico-chemical changes during the dry-cured processing of deer loin. *International Journal of Food Science & Technology*, 55(3), 1025–1031. <https://doi.org/10.1111/ijfs.14342>
- Versalovic, J., Schneider, M., Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, 5, 25–40.
- Vilar, I., García Fontán, M. C., Prieto, B., Tornadizo, M. E., & Carballo, J. (2000). A survey on the microbiological changes during the manufacture of dry-cured lacón, a Spanish traditional meat product. *Journal of Applied Microbiology*, 89(6), 1018–1026. <https://doi.org/10.1046/j.1365-2672.2000.01210.x>
- Virgili, R., Saccani, G., Gabba, L., Tanzi, E., & Soresi Bordini, C. (2007). Changes of free amino acids and biogenic amines during extended ageing of Italian dry-cured ham. *LWT - Food Science and Technology*, 40(5), 871–878. <https://doi.org/10.1016/j.lwt.2006.03.024>
- Zagorec, M., & Champomier-Vergès, M.-C. (2017). *Lactobacillus sakei*: A starter for sausage fermentation, a protective culture for meat products. *Microorganisms*, 5(3), 56. <https://doi.org/10.3390/microorganisms5030056>

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'Toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. In *International Journal of Systematic and Evolutionary Microbiology* (Vol. 70, Issue 4, pp. 2782–2858). Microbiology Society. <https://doi.org/10.1099/ijsem.0.004107>