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EVOLUTION OF SAFETY AND QUALITY ASPECTS OF DRY-CURED HAM DURING AGING UNDER NATURAL CLIMATIC CONDITIONS

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Abstract

An in-depth study of the "aging duration" is a challenge in order to adapt traditional air-dried meat products to modern food safety and quality optimization requirements. The aim of this research was to determine the effect of aging time in natural climatic rooms on the safety and quality complex of traditional dry-cured ham, manufactured without nitrite/nitrate.

Three groups of hams as a result of different aging period (12, 24 and 36 months) were evaluated for various physicochemical and biochemical parameters in concern of their proteolytic changes and safety issues, including: carbonyls, biogenic amines and amino acids content, as well as microbial scene. Identification and quantification of amino acids and biogenic amines were carried out by HPLC techniques. Standard or validated methods were used to determine the microbiological characteristics.

Results for carbonyls showed that not significant oxidized processes were observed in the fraction of proteins during extended ageing time. The amino acids - histidine, alanine, lysine, isoleucine, phenylalanine, asparagine and serine were dominant in all three types of aged hams. In the samples with the highest ripening time (36 months), the amino acids - asparagine, isoleucine, alanine and proline were determined in greater quantities. Despite the increased quantitative accumulation of their precursor amino acids during aging, the amount of biogenic amines - putrescine, cadaverine, histamine and tyramine, was reduced, and their amounts in test samples except cadaverine, were below the minimum required level to ensure product safety. Microbiologically, no pathogenic bacteria were detected in all three samples. In the most aged samples, despite the unfavorable conditions for bacterial growth, few staphylococci and micrococci and residual enterococci and coliforms were detected.

Obtained results allow completing the knowledge about chemical processes and microbiological hazards in the air-died ham that occur at the absence of nitrite and nitrate. More researches are needed to evaluate the pattern, regarding biogenic amine formation, which is important in food safety perspective.

***Key words**: Traditional Bulgarian meat product, Natural climatic aging, Oxidized protein carbonyls, Biogenic amines, Aminoacids.*

1. Introduction

Traditional dry-cured meat products are well-known, much sought after and widely distributed types of food. Their production technology is closely related both to the environmental conditions and resources in the region [1] and to the food practices of the different civilizations which settled in the area [2]. The variations in the climatic conditions are undoubtedly among the main factors that have contributed to the creation of the large variety of meat products. For instance, the dry curing of meat is more common in countries with a warm climate such as Italy, Spain, and Portugal, whereas the meat products manufactured in Northern Europe (Germany and the Scandinavian countries) are mainly subjected to wet curing and smoking

[3, 4]. There is no single technology in Europe for the manufacture of dry-cured pork ham; on the contrary, each country and even area has reached its traditional know-how on the basis of experience and regional customs and by use of different raw materials and technological combinations of: shaping, curing, aging temperatures and times, with or without smoking. The temperature and duration of the production stages is an important determinant not only for the development of the quality characteristics of dry-cured hams [5 - 8] but also for ensuring their food safety. Although traditional dry-cured meat products have good microbial safety background, the preliminary risk assessment shows that their production is only possible with more flexible interpretation of the hygiene principles stipulated in the effective legislation and reliable control of hazards [3]. On the one hand, some authors [9], believe that more severe hygiene requirements in the production of dry cured meat products and the direct addition of nitrites have led to loss of the traditional taste and aroma of these products since they reduce the quantity and growth of the desired authentic microflora. Factors like the temperature at which the aging of dry cured ham takes place (usually between 7 - 28 ^{0}C), the microorganisms present, and the presence of additives, accompanied by proteolysis, influence the formation of biogenic amines (BA) [10, 11] are affecting product safety. Moreover, more BA are produced at higher pH, which is likely for these kinds of products in view of the higher microbial cell counts [10].

On the other hand, the formation of protein carbonyls is one of the most notable modifications resulting from protein oxidation. The latter can lead to quality defects such as color deterioration and texture hardness, as well as reduced nutritional value and protein digestibility [12]. Sodium nitrite has a significant negative correlation with carbonyl levels and due to the facilitated proliferation of proteolytic bacteria results in a positive correlation with free amine levels [13].

There are numerous studies on the physicochemical and quality properties of a number of South European hams and the variations of these properties in relation to the traditional technology used [14 - 18]. Nevertheless, no attempts have been made in the available sources at a combined study of the effect of aging time on the quality and safety aspects of traditional dry cured ham. The formation of biogenic amines in these traditional meat products, which is a challenge in view of their adaptation to modern food safety requirements, has been insufficiently studied. Therefore, the aim of the this study was to determine the effect of the aging duration of a traditional meat product produced without the addition of nitrites and nitrates in a natural drying chamber upon its proteolytic and microbiological changes with regard to the safety and quality issues.

2. Materials and Methods

2.1 Materials

The studies were made with Elena ham, a traditional meat product for the Elena region of the Balkan mountain produced from the hind leg of pigs according to a technology similar to that of other European dry-cured hams. Only hind pork legs from swine raised on the Terahib AD pig farm in the village of Nikola Kozlevo (Shumen region) and slaughtered at 12 months of age were used for the purpose. The hams were separated through a cut between the last lumbar vertebra and the first sacral vertebra and a cut through the tarsometatarsal joint. The subsequent technological operations connected with the shaping, salting and aging of the Elena ham were performed at the Biltrans EOOD Company, town of Elena. Cooled hams were additionally shaped by carefully releasing the hip bone, leaving only a small part of the ilium (2 - 3 cm). Then the soft and pelvic fat on the inside was removed, and on the outside the skin was preserved. The ham obtained had an average weight of 11.567 ± 0.404 kg and pH of 5.78 ± 0.05 was measured in *musculus semimembranosus*. Raw ham was salted with large amounts of ordinary cooking salt at t = 0 - 3 ^{0}C and relative air humidity of 80 - 90% for a period of 45 days. After salting, the legs were mechanically cleaned of the salt mixture adhering to their surface, washed with clean drinking water, drained and hung for aging and curing in the natural drying chambers under the specific mountainous climatic conditions in the town of Elena (Figure 1). Two

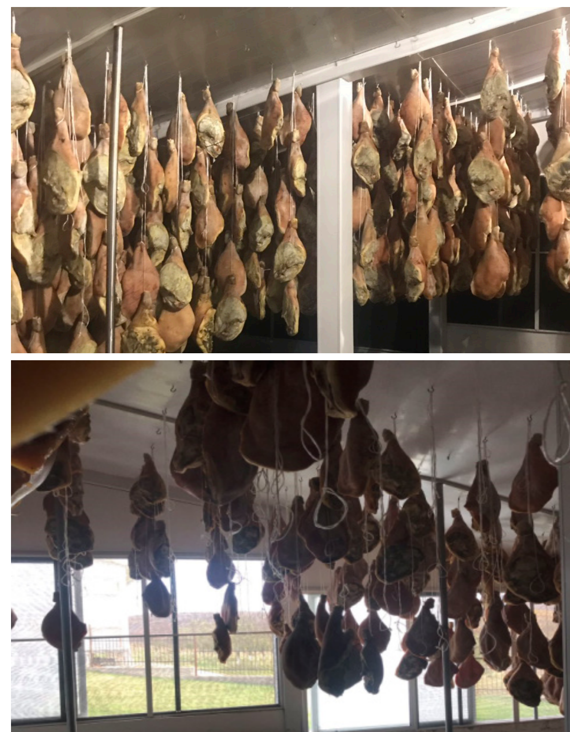

Figure 1. Natural drying chambers for Elena ham at the Biltrans EOOD Company

dry-cured legs cured and aged for 8, 18 and 36 months under the above conditions were used for the studies, and the samples for the analysis included *m. semimembranosus, m. semitendinosus* and *m. biceps femoris*.

2.2 Methods

2.2.1 Proteolytic index and amino acids content

The proteolysis index (PI) was obtained as the ratio between nonprotein nitrogen (NPN) and total nitrogen (TN) determined by the Kjeldahl method and multiplied by 100 [19]. The amino acid content of muscle tissue of the samples was evaluated according to the procedure described by Sarbon *et al.*, [20]. For the purpose, a 0.01 g meat sample pre-dried to constant weight was hydrolyzed with 5 mL 6N HCl at 105 ^{0}C for 24 h. Then the liquid was evaporated under vacuum at t = 40 - 50 ^{0}C. The sediment remaining after triple rinsing with distilled water and evaporation was diluted with 10 mL 20 mM HCl. The solution obtained was filtered through filter paper (Watman 1). The sample derivatization was performed by adding 70 μL of borate buffer to 10 μL of the filtrate. The test tubes were vortexed for 1 - 2 s. 20 μL of the IccQ Fluor Reagent solution prepared in advance were added to each test tube. The AccQ-Fluor amino acid derivatives were analysed on a liquid chromatography ELITE LaChrome (Hitachi) system fitted with a diode-array detector (DAD) and ELITE LaCHrome software and a C 18 AccQ-Tag (3.9 mm x 150 mm) reversed-phase column, Waters A-buffer (WATO52890) and mobile phase B: 60% acetonitrile. The amino acids were detected at 254 nm. The results were presented as g amino acid in 100 g of protein.

2.2.2 Evaluation of the biogenic amine content through HPLC

Ten grams of a ground average sample were weighed and transferred into a 50 mL conical flask with 10 mL 0.4M of perchloric acid, then homogenized for 30 min. The homogenized sample was centrifuged for 10 min at 3,000 rpm; then the separated supernatant was filtered through filter paper (Watman 1). 50 μL solution of the internal standard (1,000 ppm solution of 1,7-diaminoheptane), 200 μL 2N of sodium hydroxide, 300 μL saturated solution of sodium bicarbonate and 2 mL solution of dansyl chloride (10 mg/mL) were added to 1 mL of the extract obtained. The sample was subjected to dark incubation at room temperature for 15 min, then 100 μL NH_4OH were added to it to remove the residual dansyl chloride. After 5 min at room temperature, the volume of the reaction mixture was brought to 5 mL using acetonitrile and the mixture was centrifuged for 5 min at 2,500 g. It was filtered through a syringe filter with a PVDF membrane (pore size 0.45 μL). The filtered sample (20μL) was injected into the injector of the ELITE LaChrome (Hitachi) HPLC system fitted with a diode-array detector (DAD), ELITE LaCHrome software and a Purospher STAR(C18) column (4.6 x 150 mm, 5 μm particle size). A mixture of acetonitrile and water in a 70 : 30 volume ratio was used as a mobile phase. The mobile phase was pre-filtered through a membrane filter with pore size of 0.45 μm and subjected to degassing in an ultrasonic bath. Flow rate was 0.8 mL/min. A 10 min waiting period was needed before the next analysis in order to restore the balance in the column. The absorption of the dansyl derivatives of the biogenic amines was reported at 254 nm by the UV detector of the HPLC system, and the final amounts were calculated using standard curves for five biogenic amines: putrescine, cadaverine, tyramine, histamine and 1,7-diaminoheptane. The results obtained were presented as mg per kg of dry matter.

The protein carbonyl content was determined using the 2,4-dinitrophenylhydrazine (DNPH) derivatization method, as described by Oliver *et al.*, [21]. An absorption coefficient of 22,000 $M^{-1}cm^{-1}$ was used at 370 nm [13]. The results were expressed as nanomoles of carbonyls fixing DNPH in 1 milligram of protein (nmol carbonyls/mg protein).

2.2.3 Microbiological analysis

The microbiological characteristics were determined according to the following methods: 1. Mesophilic aerobic and facultative anaerobic microorganisms by ISO 4833; 2. Coliforms through inoculations in a 1 mL quantity of ten-fold dilutions on HiChrome Coliform Agar w/SLS (HiMedia) incubated at 37 and 44 ^{0}C for 48 hours, validated under ISO 5541; 3. Lactic acid bacteria according to ISO 13 721 upon MRS agar; 4. Enterococci through inoculations in a 1 mL quantity of pre-made ten-fold dilutions prepared in advance on bile esculn azide agar (HiMedia) after 48 hours at 30 ^{0}C; 5. Quantitative determination of staphylococci (non-pathogenic and pathogenic) and micrococci by inoculations in a 1 mL quantity of pre-made ten-fold dilutions upon Manitol salt agar (HiMedia) at 30 ^{0}C for 48 h; 6. Mold and yeast through surface inoculations on a selective medium for yeast and mold cultivated at 20 ^{0}C for 3/7 days; 7. Presence of *Salmonella* spp. in 25 g according to ISO 6579; and 8. Presence of *Listeria monocytogenes* in 25 g according to ISO 11290-1. The samples were prepared for the microbiological examinations according to ISO 6887.

The data obtained were statistically processed using the STATGRAFICS XVI software. One-way ANOVA analysis was applied for evaluation of the effect of the aging duration (factor I) on the parameters studied. The calculations were made at confidence level α = 0.05. The mean values of the samples were compared using Duncan's multiple range test. The experiments were made with three-fold repetitions of three samples for

each ham, and the data in the tables and graphs are arithmetic means ± the standard deviation (SD). Statistically significant differences between the mean values were found at probability lower than 0.05.

3. Results and Discussion

3.1 Proteolytic index and protein carbonyls

Constant increase in the proteolytic index of Elena ham was a direct indication to the further development of the proteolytic changes in the fraction of microfibrillar proteins [22], in the course of aging (Table 1). Its highest value in the ham aged for 36 months under natural climatic conditions was expected. These results are in good correlation with the results reported by other authors who studied different types of dry-cured hams [23].

Comparison of the data on the oxidative changes in proteins showed that the protein carbonyls obtained were statistically indistinguishable from each other (Table 1). The stabilization of their quantity may have been due to the fact that the protein oxidation did not occur predominantly in the process of aging and drying of dry-cured hams. Thus, for instance, Koutina *et al.*, [24], also reported a tendency for stabilization of the formation of protein radicals and protein carbonyls toward the final stages of aging of Parma dry-cured ham. Contrariwise, Ventanas *et al.*, [25, 26] established higher protein carbonylation as a result of protein and lipid oxidation, as the ripening and drying conditions of hams became longer and more severe. An explanation of the contradictory results reported by the different authors may be sought not only in the specific character of the technology used for the production of the hams studied but also in the nitrites, if any, used for their curing [13].

3.2 Biogenic amines

Quantities of biogenic amines found in the samples studied (Table 1) were significantly lower than the minimum defined with a view to ensuring the food safety (except for cadaverine in the 8-month sample). The maximum mean values reported were as follows: for histamine: 1.31 mg x kg^{-1} with reference value of 50 mg x kg^{-1}, for tyramine: 2.07 mg x kg^{-1} with reference value of 600 mg x kg^{-1} for healthy individuals and 50 mg x kg^{-1} or 6 mg x kg^{-1} for people undergoing treatment with monoamine oxidase (MAO) inhibitors depending on their type; for putrescine: 1.29 mg x kg^{-1} with safety limit under 300 mg x kg^{-1}; and for cadaverine: values ranging between 0.83 and 203 mg x kg^{-1} with admissible safety level below 100 mg x kg^{-1} according to EU Regulation 2470/2017 [27]. Normally, putrescine and tyramine are the biogenic amines present in the largest amounts in dry-cured meat products, and their formation is usually attributed to the microbial metabolism of lactic acid bacteria. In Elena ham, these amounts are significantly lower than those reported for a number of meat products obtained as a result of a lactic acid fermentation process [10]. Perhaps, this is directly related to the weaker growth of this microbial population in the traditional product studied and to its different technology. The higher levels of cadaverine and, to some extent, of histamine, are mainly related to the proliferation of bacteria of the *Enterobacteriaceae* and *Enterococcus* family above 3 logarithmic units (Table 3), [10]. Apart from the microbiological profile, all factors affecting the proteolytic changes in dry-cured meat products (pH, aw, salt, temperature, etc.) are of great importance to the type and quantity of biogenic amines in these products [28, 29].

This study established that the prolonged aging of the meat product was accompanied by lower amounts of the biogenic amines putrescine, cadaverine, histamine and tyramine. That was in opposition to the higher values registered for the proteolytic index (Table 1), which indicated increased proteolytic decomposition that led to the generation of free amino acids preceding the biogenic amines. These lower biogenic amine amounts in the samples that aged for a longer time could be regarded as a consequence of the Strecker degradation of the precursor amino acids in the complex meat matrix. Suzzi and Gardini, [10], established a relation between the increasing salt concentration

Table 1. Changes in the protein fraction of the Elena ham samples studied

SAMPLE	PARAMETER					
	Proteolytic Index	***nmol carbonyls/ mg protein***	***Biogenic amines, mg x kg^{-1} dry matter***			
			putrescine	cadaverine	histamine	tyramine
8 months	23.42 ± 4.88^{a}	53.97 ± 8.52^{a}	1.29 ± 0.01^{c}	203 ± 8.28^{c}	1.11 ± 0.03^{b}	2.07 ± 0.93^{a}
18 months	30.94 ± 3.32^{a,b}	70.64 ± 15.36^{a}	1.09 ± 0.08^{b}	1.11 ± 0.03^{b}	1.31 ± 0.26^{b}	ND
36 months	48.39 ± 4.11^{b}	63.80 ± 17.22^{a}	0.79 ± 0.01^{a}	0.83 ± 0.01^{a}	0.66 ± 0.00^{a}	ND

Legend: $^{a-c}$ - the values within the same column bearing a common letter designation did not differ statistically ($p > 0.05$); ND - not determined.

Table 2. Amino acid composition of the Elena Ham samples studied

AMINO ACID, g/100 g protein		SAMPLE		
		8 months	*18 months*	*36 months*
Asp	Asparagine	8.93 ± 1.87[b]	9.97 ± 1.41[b]	6.34 ± 1.44[a]
Ser	Serine	6.65 ± 0.58[b]	6.06 ± 1.07[b]	5.49 ± 0.75[a]
Gly	Glycine	3.64 ± 2.48[a]	3.83 ± 1.17[a]	8.03 ± 1.72[b]
Glu	Glutamic acid	0.65 ± 1.15[a]	0.73 ± 1.35[a]	2.68 ± 1.51[b]
His	Histidine	10.95 ± 0.80[a]	11.27 ± 0.56[a]	12.47 ± 1.60[b]
Arg	Arginine	6.55 ± 0.01[a]	6.53 ± 0.54[a]	6.53 ± 0.68[a]
Thr	Threonine	4.47 ± 0.15[a]	4.55 ± 0.59[a]	4.76 ± 0.90[a]
Ala	Alanine	7.23 ± 0.94[a]	9.08 ± 1.24[a]	8.42 ± 0.96[a]
Pro	Proline	3.26 ± 0.37[a]	3.96 ± 0.67[a]	3.80 ± 0.55[a]
Cys	Cystine	2.50 ± 0.85[a]	2.68 ± 1.08[a]	4.05 ± 0.55[b]
Tyr	Tyrosine	5.57 ± 0.79[a]	4.20 ± 1.11[a]	5.57 ± 0.49[a]
Val	Valine	3.23 ± 0.55[a]	4.14 ± 0.87[a]	4.23 ± 0.43[a]
Met	Methionine	2.76 ± 0.32[a]	2.61 ± 0.86[a]	3.22 ± 0.83[b]
Lys	Lysine	10.97 ± 0.81[a]	9.59 ± 1.03[a]	9.53 ± 1.62[a]
Ile	Isoleucine	9.53 ± 2.72[b]	9.66 ± 2.02[b]	4.88 ± 2.18[a]
Leu	Leucine	0.74 ± 0.14[a]	0.51 ± 0.58[a]	0.49 ± 0.11[a]
Phe	Phenylalanine	12.34 ± 1.44[b]	10,59 ± 0.81[b]	9.49 ± 0.27[a]

Note: The triptophane amino acid has not been included since the amino acid profile was obtained through acid hydrolysis of proteins and triptophane is obtained through alkaline hydrolysis.

and the reduced accumulation of biogenic amines in spite of the proteolytic changes. In our studies, the salt concentration in the dry matter increased with the dehydration in the natural chambers (data not shown), which could also be explained by the lower biogenic amine values with the longer aging time. Simultaneously, the microbial potential for their production was reduced with the longer aging and drying time [30], which was also evident from the microbiological results obtained (Table 3).

3.3 Amino acid profile

The amino acids shown in Table 2 were identified in all dry-cured hams studied, with different changes in their amounts in the different samples. The amino acids: histidine, alanine, lysine, isoleucine, phenylalanine, asparagine and serine were predominant in the three sample types. Amino acid composition of the samples that had aged for 18 months was more similar to the 8-month samples in terms of quantity, whereas the 36-month samples differed significantly. In the latter, the levels of the amino acids: asparagine, serine, isoleucine, and phenyl alanine were lower compared to the other samples, while the levels of glycine, glutamic acid and sulfur-containing amino acids histidine, serine and methionine were higher. At this stage, we cannot offer an explanation of this phenomenon. It could be attributed to the effect of the production time, manner and degree of salting, subsequent temperatures and drying and aging duration under natural climatic conditions, as well as to the type of raw materials used [31] and the muscle groups [32] taken for amino acid analysis.

As regards the tyrosine amino acid whose presence in dry-cured hams is relatively stable [4, 31], it was deposited in the form of visible crystals on the surface of the muscle tissue (Figure 2). This is related to the poor solubility of tyrosine in meat products which have undergone a fuller process of proteolysis and dehydration whereby tyrosine precipitates, forming tyrosine crystals [33]. These crystals are not regarded as a visual defect but rather as an indicator of a high-quality dry-cured meat product having an authentically long maturation period [33].

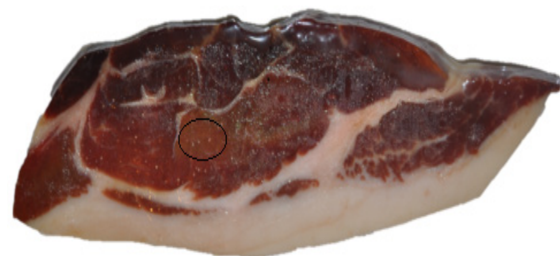

Figure 2. Picture of the sample which aged for 36 months in natural drying chambers wherein the deposit of tyrosine crystals was observed (as white spots)

Table 3. Microbiological characteristics of Elena ham samples in relation to the duration of aging

Parameter	Sample		
	8 months	18 months	36 months
Total Plate Count, log cfu/g	5.66±0.15[c]	4.79±0.36[b]	2.64±0.59[a]
Lactic acid bacteria, log cfu/g	4.59±0.14[c]	3.15±0.19[b]	0±0[a]
Staphylococci and micrococci, log cfu/g	5.63±0.17[c]	4.86±0.15[b]	4.25±0.25[a]
Enterococci, log cfu/g	3.89±0.11[c]	3.23±0.03[b]	2.00±0.01[a]
Coliforms at 37 °C, log cfu/g	4.29±0.26[c]	3.29±0.26[b]	1.9±0.05[a]
Coliforms at 44 °C, log cfu/g	1.78±0.02[b]	0±0[a]	0±0[a]
***E. coli*, 44 °C, log cfu/g**	1.78±0.02[b]	0±0[a]	0±0[a]
Mold and yeast, log cfu/g	1.78±0.3[a]	1.85±0[b]	0±0[b]
***Salmonella* spp.**	not established in 25 g of the product		
Listeria monocytogenes	not established in 25 g of the product		

Legend: [a-c] - the values within the same column bearing a common letter designation did not differ statistically ($p > 0.05$).

3.4 Microbiological characteristics

With the longer drying and aging period and the ensuing changes in water activity (data not shown), a significant effect on the microbiological characteristics of the Elena ham samples were observed (Table 3). The total number of bacteria gradually decreased, until no lactic acid bacteria, fecal coliforms, mold or yeast were found in the 36th month. Lücke and Vogeley, [3], stated that it is the longer aging of traditional air-dried sausages at lower temperatures (<15 °C) that is the main factor compensating the lack of antimicrobial potential of the lower values of pH and nitrites which are not typical of traditional dry-cured meat products. The same authors report that nitrite concentration significantly affected the Gram-positive catalase-positive cocci, which is in conformity with the results obtained by us. Only small amounts of enterococci and coliforms, and mainly staphylococci and micrococci, were established as residual microflora in the samples. During the aging period studied, the staphylococci and micrococci exceeded the other microflora, including the amount of lactic acid bacteria. These results corresponded to the dominant role of bacteria of the *Micrococcaceae* family in the production of dry-cured pork ham [34, 35] characterized by a higher sodium chloride content and higher pH. With regard to pathogenic bacteria that serve as a criterion for the safety of a product [36], *Salmonella* bacteria and *L. monocytogenes* in 25 g were not found in any of the Elena ham samples.

Thermotolerant fecal coliforms, including *E. coli* which grew at 44 °C, were no longer found between the 8th and the 18th month of the dry-cured ham maturation, probably as a result of the bacteriostatic effect of the low aw value and the increased salt concentration in the samples examined. The higher levels of this microbial group in the 8-month sample compared to the quantitative changes in the microbiological profile of hams reported by Blesa *et al.*, [37], could be attributed to the more significant growth of *Enterobacteriaceae* during the first production stages when nitrate/nitrite were not added [38].

4. Conclusions

- Under the conditions of longer aging in natural drying chambers, the microbial scene in Elena ham, though it demonstrated a downward trend, preserved the dominating staphylococci and micrococci share and lower amounts of enterococci and coliforms were identified.

- Although no pathogens such as *Salmonella* sp. and *L. monocytogenes* were found in the 8th month, as regards fecal coliforms the conditions that could guarantee the product safety were only established after 8 months of aging in natural drying chambers.

- The effect of the absence of nitrite/nitrate in the context of a risk of protein oxidation and microbiological hazards was limited due to the extended aging, which was accompanied by a prolonged bacteriostatic effect of water activity and proteolytic changes. Dry-cured ham aging affects biogenic amine accumulation, as evidenced by the quantitative differences in the detected contents at different ripening times. Regardless of the higher degree of protein hydrolysis with the longer aging and drying time of the dry-cured Elena ham, lower levels of the biogenic amines tyramine, histamine, cadaverine and putrescine were observed.

- Future research based on biogenic amine generation in dry-cured hams could be used to localize differences between technologies used in association with the raw materials, salting pattern, use of nitrites, muscle types, applied temperatures, and post-aging treatments.

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