Influence of Curing Salts and Storage Conditions in Proteolysis and Lipid Oxidation Stability of a Low Acidity Dry Fermented Sausage Produced with DFD Meat

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Abstract

The effects of the addition of curing salts and storage periods (3, 6 and 12 months) and temperature (2-4°C vs 15-18°C) on proteolysis and oxidative stability of dry fermented sausage produced with high pH pork raw materials were evaluated. Final products with curing salts showed higher concentrations in total volatile basic nitrogen and total Free Amino Acids (FAA) (P<0.05), comparatively to counterparts free of curing salts. Despite the similarity in the FAA profile of sausages from both formulations, products having curing salts had significantly higher levels in FAA groups associated to acidic and aged tastes/flavours. At any storage period, products held at 2-4°C showed lower values for most parameters (P<0.05) associated to proteolysis and also in acidity, irrespective of the curing salt condition. Differently, hexanal and the sum of straight chain aldehydes were not affected by both factors in products stored for 3 (S4) and 12 months (S6) while in SS samples (6 months of storage), the higher the temperature the greater the oxidation occurred. Sausages having curing salts held at storage under 2-4°C presented lower total biogenic amines than counterparts stored at 12-15°C (P<0.05).

Keywords: Dry fermented sausage; Low acidity; Proteolysis; Oxidative stability; Biogenic amines

Introduction

Traditionally, home manufactured raw meat products for self-consumption used to be stored for extended periods (up to 1 year) after processing, immersed in melted pork fat or in olive oil to avoid excessive drying and to prevent rancid development. Under such storage conditions [1], the proteolytic and lipolytic phenomena would anyway go on [2,3], giving rise to distinct quality profiles and, namely, to different flavour characteristics along the time spent before consumption. Flavour attributes in dry-cured fermented sausages have been related to intrinsic enzymatic systems, which are implicated in degradation of nitrogen and lipid constituents of muscle and fat tissues, during processing and storage [3-6], generating free fatty acids [7,8], small peptides and amino acids [9-11] and several volatile compounds [12-15]. At present, since there is a consumer-driven demand for these long ripened products, the meat processing industry is interested in mimicking the production of old fashion deep ripened dry fermented sausages. However, the information concerning the formation of compounds related with the eating quality attributes after such long ripening process is still scarcely available in small diameter (2 cm) and, consequently, faster dried products, among which “chouriço de carne” is the most consumed, expectedly differing from those with larger diameter (5 cm) [2,3].

According to the rules in practice for “Chouriço de Carne de Estremoz e Borba” PGI production, the addition of nitrite and nitrate as well as ascorbic and citric salts is not allowed, despite the recognized positive effect of these additives in microbial safety control and color/ fat oxidation stability, respectively. Does this interdiction make sense in terms of final eating quality of products, if they were used in minimal concentrations? In the other hand, the maintenance of end products under refrigeration (lower microbial risk in this type of products without starters’ addition) or room (economical advantages) temperatures will influence the rate and extent of the ripening phenomena occurring during this phase. How different would that impact be?

The present study was undertaken to characterize critical physico-chemical and biochemical aspects occurred in this economically relevant meat product during processing and under vacuum storage phases, investigating their trends when added of nitrite/nitrate and ascorbic/citric acids salts and stored under vacuum packaging at 2 different temperatures (chilling 2-4°C vs room temperature 12-15°C) for 3, 6 and 12 months.

Materials and Methods

Preparation of chouriço type dry fermented sausage

Products were manufactured at a regional industrial plant from a mixture of minced lean pork (80%) and pig fat (20%) (2 cm in size) obtained from carcasses of “Alentejano” breed animals. Raw materials were mixed for about 5 minutes under vacuum and, during this operation, NaCl (2.5%), paprika paste (5%), raw garlic paste (1%), ice cooled tap water (15%) and, when under testing, industrial additive preparations [Vitacure: 0.11% (NaNO2 5%; KNO3 5 %; NaCl 90%) and L-Redominix: 0.11% (E301; E330; E331- relative composition unknown)] were added. In the meantime (last mixing instants), the mixture was cooled down through the admission of small amounts of carbon dioxide and then stuffed in natural hog casings (small intestine), having a diameter of about 2.3 cm. Raw sausages weighting around 350 g were placed in a traditional smoking and drying room (temperature and
relative humidity profiles depicted in (Figure 1) during approximately 9 days, corresponding to a weight loss of about 30-35%. To evaluate the effect associated to curing salts addition, 2 different sub-batches of about 75 kg each were prepared from the same initial 150 kg raw materials mixture. Attending to the size of the batches produced under competitive environment and the expectable variation in the composition of each “chouriço” piece, different individual products were considered replicates having been analysed three for each experimental condition.

**Sampling**

Samples were picked up at distinct processing stages, which included raw meat/fat mixtures just after mixing and seasoning operations (R0) and stuffed products after 2 (S1), 6 (S2) and 9 (S3) days of drying and smoking. In relation to the storage phase, analysis were carried out in products packaged under vacuum (BB4L, 30 cm3 m-2 24 h-1 bar-1 at 23°C and 0% relative humidity, Cryovac, Grace, SA. Spain) and held for 3 (S4), 6 (S5) and 12 (S6) months, at chilling (2-4°C) and room temperature (15-18°C). For each condition studied duplicates were analysed.

**Physico-Chemical parameters**

Water activity (a_w) of samples was determined with Rotronic Hygromer using a probe AwVC-DIO (Rotronic AG, Switzerland). Dry Matter (DM) was measured by drying the samples at 103 ± 2°C to constant weight [16]. The pH was measured with a 654 pH meter (Metrohm Herisau, Switzerland) equipped with combined pH glass electrode (Mettler Toledo, Switzerland) [17].

Determinations of Non-Protein Nitrogen (NPN), Free Amino Acids Nitrogen (FAAN) and Total Volatile Basic Nitrogen (TVBN) fractions were carried out according the procedure described by Roseiro et al. [3]. The nitrogen fraction contents were expressed as mg.100 g -1 DM of sample. In the text, the terms “sweet”, “bitter”, “acidic” and “aged” flavour amino acids correspond to the sum of glycine (Gly), alanine (Ala), serine (Ser), threonine (Thr), proline (Pro), histidine (His), arginine (Arg), methionine (Met), valine (Val), leucine (Leu), isoleucine (Ile) and phenylalanine (Phe), glutamic acid (GlU), aspartic acid (Asp) and histidine, lysine (Lys), tyrosine (Tyr) and aspartic acid, respectively [14].

Biogenic amines (BAs) were determined by HPLC according to Eerola et al. [20]. Eight grams of the samples were homogenized in 40 mL 0.4 M perchloric acid with a Polytron homogenizer and centrifuged for 10 min at 800 xg and the supernatant rinsed into a 100 mL bottle through filter paper. The extraction was repeated with 40 mL 0.4 M perchloric acid. The supernatants were combined and made up to 100 mL with 0.4 M perchloric acid. A volume of 1 mL of the sample extract was derivatized with dansyl chloride by incubation for 40 min. The samples were dissolved in acetonitrile and filtered through a Acrodisc membrane 25 mm GHP, GF 0.45 µm (Gelman Sciences, Inc.). 1, 7-diaminopentane was used as the internal standard.

Biogenic amines (tryptamine, b-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) were separated using a Waters HPLC system consisting of a quaternary pump (Waters model 510 HPLC pump), automatic sampler (Waters 717Plus autosampler), and diode array detector (Waters 2996). Separation was performed on a reverse phase Spherisorb ODS2 cartridge, 5 µm, 4.0x125 mm. A gradient elution program with a mixture of 0.1 M ammonium acetate as solvent A and acetonitrile as solvent B was used. The gradient began at 50% and finished at 90% acetonitrile in 19 min, 10 min equilibration before the next analysis. Samples were analysed in duplicate. The biogenic amines content was expressed as mg.kg -1 DM of sample.

**Figure 1:** Temperature and relative humidity profiles achieved during the smoking/drying phase of “Chouriço de Carne” processing.
Straight chain aldehydes

Volatile straight-chain aldehydes (SCA) were concentrated in a purge and trap system (HP 7695, Hewlett Packard, USA) and analyzed by gas chromatography-mass spectrometry. Portions of 1g from each finely comminuted sample (Grindomix GM 200, Retsch, Germany), were taken and stored at -18°C for further analysis. Frozen samples were directly placed in the flask from Purge and Trap system and 5 µl were taken and stored at -18°C for further analysis. Frozen samples were pre-purged for 5 min, before heating at 45°C (5 min) and the released volatile compounds were purged for 10 min, with a 43 cm 2-hexanone) were added. Samples were pre-purged for 5 min, before heating at 45°C (5 min) and the released volatile compounds were purged for 10 min, with a 43 cm 2-hexanone) were added. Samples were pre-purged for 5 min, before heating at 45°C (5 min) and the released volatile compounds were purged for 10 min, with a 43 cm

Gas chromatography was performed on a Thermo Finnigan Trace GC Ultra (Thermo Electro Corporation, Austin USA), equipped with a DB 23 column (0.32 mm x 0.25 µm x 30 m, from J&W Scientific, USA) and coupled to an ion trap mass spectrometer Polaris Q (Thermo Finnigan). The chromatographic conditions were as follows: initial oven temperature was kept at 35°C (8 min), and programmed at 10°C. min -1 from 35°C to 220°C. Injector temperature was 210°C. Helium was used as carrier gas (70 kPa). The mass spectrometer conditions were as follows: MS transfer line temperature was 250°C; start scan events at 0, 5 min; mass range from 25-350 amu; ion source temperature 200°C; electron impact at 70 eV.

Tentative identification was carried out by matching mass spectral data of sample components with those of known compounds in MAINLIB, REPLIB, WILEY 6 and NIST® demo libraries. Aldehyde standards were run in order to confirm the tentative identifications. All aldehydes were from Interchim, Paris (TK kit-151 and TK kit-155). The identification of compounds, for which no standards were available, was considered consistent only if the identification by the four libraries matched. When one of the identifications from the libraries was different, it was considered as the most probable identification.

An estimation of the concentration (mg.kg -1) was essayed, based on the ratio of individual peaks and internal standard (3-methyl-2-hexanone) areas, on the quantity of are added to the samples and on the sample weight. In the text, total SCA means the sum of pentanal, hexanal, heptanal, octanal and nonanal.

Microbiological analysis

Twenty grams of each sample were mixed with 180 mL of tryptone-salt broth (Biokar) and homogenized in a rotary homogenizer for 1.5 min. Serial ten-fold dilutions were made in the same diluent and the following analysis were carried out: total aerobic mesophylic flora on plate count agar (Biokar) incubated at 30°C for 3days [21]; Enterobacteriaceae on violet red bile glucose agar (Biokar) incubated at 37°C for 24h [22]; Enterococci on kanamycin aesculin azide agar (Liofilchem) incubated at 37°C for 2days, according to standard procedures for bacterial enumeration by colony-count method; Lactic acid bacteria (LAB) on MRS agar (Biokar) pH 6.2 and Lactobacillus on MRS agar pH 5.7 (Biokar) incubated at 30°C for 3days [23]; Total aerobic psychrotrophic flora on plate count agar (Biokar) incubated at 6.5°C for 10days [24]; Micrococcaceae on manitol salt agar (Biokar) incubated at 30°C for 3days, according to standard procedures for bacterial enumeration by colony-count method; Yeasts and molds on rose Bengal chloramphenicol agar (Biokar) incubated at 25°C for 5days [25].

Statistical analysis

The effects of curing salts(C), ripening time (t) and storage temperature (T) on the physico-chemical and biochemical characteristics of different products were evaluated by two-way analysis of variance (ANOVA). The Tukey post hoc test was used for comparison of mean values, with differences being considered significant at p<0.05. (Statistica 6.0 – StatSoft Inc., 2001).

Results and Discussion

Physico-chemical parameters

Physico-chemical characteristics of dry fermented sausage (Chouriço de Carne), influenced by the factors considered in the present study or their interactions, are shown in Tables 1 and 2, respectively. Curing additives influenced significantly the evolution of NPN and TVBN fractions (P<0.001) as well as that of ammonia (P<0.05), while the time elapsed at the drying and smoking stage affected all parameters determined (P<0.001), except acidity (Table 1). The interaction of factors also had a significant impact in pH, NPN

| R0 | S1 | S2 | S3 | SE | Time (t) | Curing salts (C) | t°C |
|----|----|----|----|----|---------|----------------|-----|
| a_n | 0.96 a | 0.92 c | 0.92 c | 0.88 b | 0.89 a | 0.89 a | 0.004 *** | ns | ns |
| pH | 6.50 a | 6.35 c | 6.39 b | 6.32 c | 6.25 a | 6.07 b | 6.11 b | 0.003 *** | ns | *** |
| NPN | 0.36 a | 0.55 b | 0.61 a | 0.56 a | 0.59 a | 0.52 c | 0.52 c | 0.006 *** | *** | *** |
| TVBN | 5.60 a | 6.40 c | 6.30 b | 4.80 a | 4.50 a | 53.00 b | 54.00 c | 0.0002 *** | *** | *** |
| Ammonia | 0.07 a | 0.33 c | 0.30 d | 0.40 b | 0.44 a | 0.43 a | 0.44 b | 0.001 *** | * | * |
| FAAN | 0.14 a | 0.17 c | 0.12 b | 0.30 a | 0.25 a | 0.28 a | 0.35 b | 0.0001 ns | ns | ns |
| Hexanal | 3.51 c | 2.91 c | 5.16 a | 1.54 b | 1.84 a | 1.25 a | 1.58 a | 0.31 *** | ns | ns |
| SCA | 8.56 c | 14.98 a | 20.56 a | 12.64 a | 12.51 a | 9.32 a | 9.44 a | 1.53 ** | ns | ns |

In same row, means with different letters are significantly different. ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001.

SE- Standard error.

SCA = 2 of pentanal, hexanal, heptanal, octanal and nonanal.

Table 1: Changes in a_n, pH, nitrogen fractions (g.100 g^-1 DM), ammonia (g.100 g^-1 DM), acidity (g.100 g^-1 fat), hexanal (mg.kg^-1) and sum of straight chain aldehydes (mg. kg^-1) during the processing of “Chouriço de Carne” manufactured with and without curing salts.
and TVBN (P<0.001). In relation to the storage period (Table 2), interaction of factors generally impacted significantly on the results, except in the case of C*T for the NPN fraction, hexanal content and the sum of straight chain aldehydes (SCA) and t*C*T for SCA.

The pH (6.50) of raw materials mixture (R0), and its evolution up to the end of processing in both sub-batches with mean values above 6.0, clearly indicates that the meat used in the formulation had a general DFD (Dark, Firm and Dry) condition and that the sausages may have undergone through a typical fermentation process. Despite the deviation from the “choriço” representative quality profile, the case may promote some kind of selection in natural proteolysis as well as lipolysis and lipid oxidation indicators, since the level determined in the final product is more than 10 fold higher that observed in an intermediate processing stage. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data. This quite unique general singularity and scarce availability of data.

The changes in pH, nitrogen fractions (g.100 g-1 DM), ammonia (g.100 g-1 DM), acidity (g.100 g-1 fat), hexanal (mg.kg-1) and sum of straight chain aldehydes (mg.kg-1) during storage of “Chouriço de Carne” manufactured with and without curing salts.

**Table 2:** Changes in pH, nitrogen fractions (g.100 g-1 DM), ammonia (g.100 g-1 DM), acidity (g.100 g-1 fat), hexanal (mg.kg-1) and sum of straight chain aldehydes (mg.kg-1) during storage of “Chouriço de Carne” manufactured with and without curing salts.

|          | S4 No curing salts | S4 With curing salts | S5 No curing salts | S5 With curing salts | S6 No curing salts | S6 With curing salts | SE | Interactions |
|----------|-------------------|---------------------|-------------------|---------------------|-------------------|---------------------|----|--------------|
| pH       | 5.77a             | 5.78a               | 5.61a             | 5.96a               | 5.89a             | 5.71a               | 5.95a | 5.55a | t*C | 9.96c  |
| NPN      | 0.74i             | 0.97i               | 0.57j             | 0.89i               | 0.80i             | 1.30j               | 0.81j | 1.23i | t*C | 0.63i  |
| TVBN10-3 | 75.00oa           | 84.00oa             | 74.00oa           | 80.00oa             | 72.00oa           | 172.00oa           | 82.00oa | 167.00oa | t*C | 79.00oa |
| FAAN     | 0.22i             | 0.39i               | 0.17j             | 0.39j               | 0.25i             | 0.65i               | 0.22i  | 0.54i  | t*C | 0.35i  |
| Ammonia  | 0.15i             | 0.19i               | 0.11i             | 0.16i               | 0.11i             | 0.21i               | 0.13i  | 0.22i  | C*T  | 0.19i  |
| Acidity  | 1.11a             | 2.83d               | 1.09a             | 2.89f               | 2.16e             | 6.29f               | 2.20g  | 6.31f  | C*T  | 4.14h  |
| Hexanal  | 0.67d             | 3.76a               | 0.81a             | 0.69d               | 2.51i             | 1.91d               | 1.42i  | 6.64j  | C*T  | 1.00d  |
| SCA      | 9.77i             | 31.36i              | 6.20f             | 5.67h               | 13.58i            | 43.81i              | 16.57i  | 44.37i  | C*T  | 7.41i  |

In same row, means with different letters are significantly different. ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001.

SE - Standard error.

SCA = Σ of pentanal, hexanal, heptanal, octanal and nonanal.
t - time; C - curing salts; T - temperature.

Nd - Not determined

The storage temperature had an utmost effect in lipolysis and in lipid oxidation stability of a Low Acidity Dry Fermented Sausage Produced with DFD Meat. J Food Process Technol 3:153. doi:10.4172/2157-7110.1000153
chain aldehydes depended on the storage time considered: S4 - higher (P<0.05) at 15-18°C for those which had no curing salts addition; S5 - higher (P<0.05) at 15-18°C for both additive conditions; S6 - no significant differences. A similar erratic behaviour was found for the hexanal evolution. Trends found in our study corroborate those referred by [36-39].

Despite differences in relation to the pH condition among products, in general, storing products at 12-18°C speeds up lipolysis and auto-oxidation processes in comparison to those kept under refrigeration, with differences being more visible when curing salts are not used and the storage period is up to 3 months. For long (6 months) and very long (12 months) ripened products, the impact of storage temperature and curing salts addition is less visible and absent at all, respectively.

Generally, curing salts did not affect significantly the acidity level in products stored at both temperatures for any of the periods assayed, except a small impact (P<0.05) in S6, specially in those stored at 12-18°C. Navarro et al. [40,41], found distinct lipolysis profiles with nitrite or nitrate additions in products submitted to different fermentation processes, with those fast fermented and added of nitrates not differing from those with nitrite. However, since those authors did not compare with products having no curing salts addition, and otherwise, differences in raw materials pH and in the concentration of curing salts also existed, the results between our studies are difficult to compare and discuss. According to our results, nitrate and nitrite concomitantly added, within the contents assayed, seem not to affect lipolysis. This is in agreement with Motilva and Toldrá [42], who stated that nitrate added, within the contents assayed, seem not to affect lipolysis. This and discuss. According to our results, nitrite and nitrate concomitantly differing from those with nitrite. However, since those authors did not

### Table 3: Changes in free amino acids content (mg.100g⁻¹ DM) at different processing stages of "Chouriço de Carne" manufactured with and without curing salts.

| Amino acids | R0 | S1 | S2 | SE | Time (t) | Curing salts (C) | t°C |
|-------------|----|----|----|----|----------|-----------------|-----|
| Lys | 8.32⁺ | 24.25⁺ | 17.66⁺ | 30.16⁺ | 29.11⁺ | 32.13⁺ | 36.56⁺ | 0.43 | *** | *** | *** |
| His | 43.98⁺ | 6.99⁺ | 5.4⁺ | 10.82⁺ | 11.04⁺ | 11.29⁺ | 13.47⁺ | 0.23 | *** | * | * |
| Arg | 5.41⁺ | 36.45⁺ | 29.14⁺ | 18.82⁺ | 32.31⁺ | 13.29⁺ | 27.35⁺ | 0.31 | *** | *** | *** |
| Asp | 5.59 | 0.02 | 0.01 | 1.20 | 1.23 | 0.65 | 0.73 | 0.06 | ns | ns | ** |
| Thr | 41.39 | 9.17 | 7.91 | 15.15 | 13.81 | 15.36 | 16.11 | 0.25 | *** | * | ns |
| Ser | ND | 49.23⁺ | 62.6⁺ | 64.01⁺ | 64.58⁺ | 62.45⁺ | 68.18⁺ | 0.74 | *** | ** | *** |
| Glu | 0.54⁺ | 25.01⁺ | 29.69⁺ | 44.43⁺ | 41.88⁺ | 43.02⁺ | 49.63⁺ | 0.63 | *** | *** | ** |
| Pro | 7.8⁺ | 22.04⁺ | 14.06⁺ | 18.04⁺ | 17.16⁺ | 17.79⁺ | 19.37⁺ | 0.15 | *** | *** | *** |
| Gly | 11.1 | 17.47 | 16.45 | 21.84 | 20.80 | 22.82 | 22.14 | 0.40 | *** | ns | ns |
| Ala | 39.77⁺ | 64.98⁺ | 64.01⁺ | 68.70⁺ | 71.98⁺ | 71.16⁺ | 77.26⁺ | 0.94 | *** | ns | * |
| Val | 4.19 | 30.35 | 25.28 | 29.15 | 27.99 | 32.13 | 34.33 | 0.69 | *** | * | ns |
| Met | ND | 8.54⁺ | 4.85⁺ | 9.19⁺ | 8.74⁺ | 10.18⁺ | 10.00⁺ | 0.20 | *** | *** | *** |
| Ile | 0.68 | 23.58 | 19.88 | 24.18 | 22.97 | 23.85 | 23.61 | 0.63 | *** | ns | ns |
| Leu | 7.64⁺ | 34.83⁺ | 22.89⁺ | 31.15⁺ | 29.82⁺ | 34.42⁺ | 36.83⁺ | 0.47 | *** | *** | *** |
| Tyr | 0.86 | 1.1 | ND | ND | ND | ND | ND | 0.09 | ns | ns | ns |
| Phe | 2.43⁺ | 6.37⁺ | 3.88⁺ | 12.13⁺ | 12.03⁺ | 13.89⁺ | 16.32⁺ | 0.27 | *** | *** | * |
| Tau | 56.32⁺ | 126.88⁺ | 130.58⁺ | 111.61⁺ | 117.41⁺ | 106.3⁺ | 114.54⁺ | 1.01 | *** | ns | ** |
| Total AA | 235.49⁺ | 487.29⁺ | 454.46⁺ | 510.58⁺ | 522.88⁺ | 510.80⁺ | 566.36⁺ | 6.45 | *** | *** | *** |

**Flavours**

| Flavours | R0 | S1 | S2 | SE | Time (t) | Curing salts (C) | t°C |
|----------|----|----|----|----|----------|-----------------|-----|
| Sweet¹ | 100.04 | 165.04 | 162.9 | 188.33 | 187.75 | 189.58 | 203.04 | 2.34 | *** | ns | ns |
| Bitter² | 14.93⁺ | 76.75⁺ | 103.67⁺ | 101.57⁺ | 105.80⁺ | 114.46⁺ | 121.07⁺ | 1.76 | *** | *** | *** |
| Acidic³ | 49.91⁺ | 35.29⁺ | 32.02⁺ | 54.15⁺ | 56.44⁺ | 54.96⁺ | 63.82⁺ | 0.89 | *** | *** | *** |
| Ageded | 14.48⁺ | 17.65⁺ | 25.37⁺ | 30.15⁺ | 31.35⁺ | 32.78⁺ | 37.28⁺ | 0.49 | *** | *** | *** |

In same row, means with different letters are significantly different. ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001. SE: Standard error.

¹ Bitter flavour = Σ of leucine, valine, isoleucine, methionine and phenylalanine. Sweet flavour = Σ of alanine, glycine, threonine, serine and proline. Acid flavour = Σ of glutamic acid, aspartic acid and histidine. Aged flavour = Σ of lysine, tyrosine and aspartic acid. ND – not detected.
detected in the products under processing had been already present in R0. The evolution observed in total FAA along the processing time confirmed that referred for the FAAN fraction, with the formation rate also being greater at the fermentation stage (2 fold higher levels in S1 than that determined in R0) and then slowing down further on (S2: + 11% the concentration found in S1; S3: + 4% the concentration found in S2). This could be related to the higher smoking and drying temperature at the beginning of processing, applied to speed up the drying out of raw products, influencing both the boom of bacteria development and the intrinsic and extrinsic enzyme systems. Among the amino acids, taurine appeared as the most concentrated for every processing time analysed, followed consistently by alanine and serine and then by glutamic acid, leucine, valine, arginine, lysine and valine, a profile somewhat different from those referred to other dry cured and fermented sausages from many countries [31,44-46]. Such differences do not surprise since processing conditions and procedures, as well as breeds and slaughter age of pigs used to get the raw materials were not surprising since processing conditions and procedures, as well as breeds and slaughter age of pigs used to get the raw materials were different and would influence the results [47,48]. Among all detected amino acids, histidine, aspartic acid, threonine and tyrosine were the only compounds with higher concentrations in R0 than those found along the processing stage. Regarding histidine and tyrosine, the decrease observed may be associated to the decarboxylation undergone by natural flora and respective biogenic amines formation (discussed later). In general, the addition of curing salts increased the amount of total free amino acids (P<0.05) accumulated in S3 samples (end products), with this trend also being confirmed for most single FAA detected. Despite the similarity of the profile represented by the 10 most concentrated compounds in both products, the amounts of the FAA groups associated to the sweet, bitter, acidic and aged flavours were all greater in end products (S3) having additives, with differences regarding the 2 last characteristics being significant (P<0.05). Nevertheless, such influence seems not to affect the sensorial judgement of products by trained panellists (results in preparation).

The time products were held under both storage temperatures, substantially increased total amino acids accumulated, which reached in S4 at 15-18°C and 2-4°C average levels 138% and 50% higher than those found in S3, respectively. The extension of the storage time up to 6 months still produced a similar evolution under both storage conditions, but the balance between the formation of free amino acids and their further intrinsic and/or extrinsic metabolism just induced a slight increase in the concentration at 15-18°C (+28%) or even a

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**Table 4:** Changes in free amino acids content (mg.100g^-1 DM) at different storage periods of “Chouriço de Carne” manufactured with and without curing salts. In same row, means with different letters are significantly different. ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001. SE - Standard error.

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small decrease in those stored at 2-4°C. However, holding the products in storage for 1 year (S6) increased again the concentration of total free amino acids in both environmental conditions (about +73.0%), confirming the physico-chemical stability of most muscle proteolytic enzymes [49,50] and the influence of mild environmental temperatures on their activity [51]. Oppositely to the trend seen in processing phase, products with curing salts had lower total FAA than free additive counterparts during storage. Except for arginine, all individual FAA also increased their concentrations under both temperatures evaluated. The case of arginine, could be due to the dynamics observed in microbial BA production. Lysine, glutamic acid, alanine, taurine and leucine, without any quantitative particular order, were the most concentrated in both manufactured product types, for any of the storage periods analyzed, with their sum in products at 2-4°C and 15-18°C averaging 53.9% and 58.5% of the total amount, respectively.

Irrespective of storage duration and curing salts “status”, products held at 2-4°C were less concentrated in all flavor FAA groups (Table 4) than counterparts stored at 15-18°C. With no curing salts addition, products held at 2-4°C for 6 months (S5) were the less concentrated in FAA groups associated to sensorial attributes described in the scope of this study, with those stored for 3 months occupying an intermediate position. In those stored at 15-18°C the aged FAA group followed the same pattern (S5<S4<S6), but regarding the other FAA groups, the concentration varied directly with storage time.

With curing salts, products held at 2-4°C presented greater concentrations with the time elapsed at storage, excepted for the group representing the aged flavor, which maintained the same trend referred in those without additives. Differently, in products stored at 15-18°C, those kept for 6 months showed the highest concentration in all FAA flavor groups, with S4 and S6 being not significantly different.

### Biogenic amines and microbiological characteristics

In fermented dry ripened sausages, biogenic amines formation inevitably occurs since the conditions for microbial decarboxylation of corresponding amino acids usually do exist [52]. Toxicological events resulting from consumption of foods having high concentrations of BA have been referred [53,54], with histamine and tyrosine repercussions denoting a particular interest due to their health impacts. Despite the lack of serious outbreaks reported to meat products consumption, the present interest for long aged products makes the evaluation of these quality/safety parameters absolutely crucial. So, under the conditions assayed, raw materials presented low total BA content (44.34 mg.kg⁻¹), with tryptamine as the most concentrated (27.65 mg.kg⁻¹) followed by tyramine and putrescine (Table 5). Endogenous polyamines (sperrmine and spermidine), as well as phenylethylamine, cadaverine and histamine, were not present, which could be related to the quality condition of meat (exhaustion of energy compounds and precursors) used in the formulation [55]. The total BA level represented by compounds other than spermine and spermidine, increased with the processing time (P<0.05) in products having or not having curing salts,

#### Table 5: Biogenic amine concentrations (mg.kg⁻¹ DM) at different processing stages of “Chouriço de Carne” manufactured with and without curing salts.

| Biogenic amine | S1 | S2 | S3 | SE | Time (f) | Curing salts (C) |
|----------------|----|----|----|----|----------|---------------|
| **Tryptamine** | 27.65 | ND | ND | 27.47 | ND | 0.10 |
| **Phenylethylamine** | ND | 22.00 | 13.27 | 17.46 | 35.48 | 2.14 |
| **Putrescine** | 7.09 | 26.29 | 36.58 | 288.33 | 59.18 | 16.53 |
| **Cadaverine** | ND | 2.70 | 19.62 | 35.39 | 25.55 | 2.59 |
| **Histamine** | ND | 0.22 | 13.68 | 21.04 | 21.38 | 1.53 |
| **Tyramine** | 9.68 | 5.59 | 19.36 | 36.93 | 77.62 | 11.98 |
| **Spermidine** | ND | ND | 0.22 | ND | 0.04 |
| **Spermine** | ND | 60.90 | 45.24 | 64.93 | 77.62 | 11.98 |
| **Total BA** | 44.34 | 117.69 | 317.79 | 596.54 | 392.25 | 40.01 |

In same row, means with different letters are significantly different. ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001. SE - Standard error. ND – not detected.

#### Table 6: Biogenic amine concentrations (mg.kg⁻¹ DM) at different storage periods of “Chouriço de Carne” manufactured with and without curing salts.

| Biogenic amine | S4 | S5 | SE | Interactions |
|----------------|----|----|----|--------------|
| **Tryptamine** | ND | 13.85 | 19.56 | 1.36 |
| **Phenylethylamine** | 31.08 | 104.48 | 17.76 | 19.56 |
| **Putrescine** | 86.65 | 1277.85 | 1951.04 | 2.03 |
| **Cadaverine** | 39.59 | 386.80 | 375.25 | 3.46 |
| **Histamine** | 31.45 | 35.16 | 34.67 | 1.35 |
| **Tyramine** | 281.05 | 946.11 | 358.90 | 16.01 |
| **Spermidine** | 2.83 | 49.46 | 32.84 | 2.40 |
| **Spermine** | 119.91 | 96.10 | 78.06 | 4.66 |
| **Total BA** | 591.65 | 2911.86 | 3092.52 | 40.25 |

In same row, means with different letters are significantly different. ns = not significant; * = P<0.05; ** = P<0.01; *** = P<0.001. SE - Standard error. ND – not detected. t - time; C - curing salts; T - temperature.
denoting a higher accumulation rate between S1 and S2, coincident with the boom in microbial development (Table 7). At this point, tyramine was the most concentrated, followed by putrescine. The fast increase in LAB counts promoted, in a first instance, the tyramine as the highest concentrated amine, but the concomitant development of Enterobacteriaceae also increased of putrescine and cadaverine levels, with the later being in most samples the third higher concentrated, in agreement with the results of Miguélez-Arrizado et al. [56].

Curing salts condition of products also influenced significantly most of the BA analysed, individually or resulting from $t^\circ C$ interaction (Table 5), probably expressing the effect operated in the Enterobacteriaceae population (Table 7), which showed to be longer viable in samples without additives. In end products, those with curing salts denoted less putrescine and cadaverine but greater amounts of phenylethylamine and histamine ($P<0.05$) than counterparts. The most visible difference was in putrescine, with values reaching a level almost 5 fold lower in the former formulation. No effect in tyramine formation was detected. In general, these results confirm those reported for Italian dry sausages by [57]. However, the addition of curing salts, in the conditions assayed at the present study, never caused a 2-3 fold increase in histamine concentration as referred by those authors, but only a slightly higher level. This confirms that BA formation is an extremely complex phenomenon, without univocal rules linking the multiple variables involved.

Certainly responding to the general reduction in microbial flora numbers, the increasing verified in total BA amounts along the storage period studied was not significant, for both temperatures and formulation types (Table 6). However, the environmental temperature under which products were stored significantly affected the total BA amounts. In general, lower levels were achieved in comparison to products held at 15-18°C, with differences being significant for phenylethylamine, putrescine, cadaverine and tyramine and for cadaverine, histamine and tyramine, when the formulation not included or had curing salts, respectively. The results obtained in S5 confirmed the above tendency, with small remarks in the individual BA significantly affected. These trends corroborate those evidenced by Bover-Cid et al. [58], who stated that at 15°C decarboxylases might remain active, even if most microorganisms during storage already reached the stationary growth or even the death phase.

Quantitatively, by decreasing order, putrescine, tyramine and cadaverine mostly prevailed at the storage periods assayed, followed by phenylethylamine, histamine and tryptamine, at much lower concentrations (Table 6). The large variability in individual BA concentrations occurred between storage temperatures and manufacture types, even when similar microbiological profiles existed (Tables 7 and 8), evidencing that their formation results from a complex interaction of factors [52], being strain rather than species dependent.

**Conclusions**

Storing low acidity dry sausages under vacuum packaging, whatever the temperature considered in the scope of the present study, determined an increasing expression of chemical parameters related with proteolysis and lipolysis, which are expected to improve the sensory attributes of processed end products. At storage under 15-18°C, the formation of compounds responsible for tasting and flavouring characteristics speed up comparatively to those held at 2-4°C, without affecting lipid oxidation considerably. However, the former condition will give rise to increased BA concentrations; if the necessary processing corrections were not implemented (e.g. starter cultures addition having not specific FAA decarboxylase ability).

The use of curing salts in the conditions assayed (small amounts and higher pH raw materials), promoted a higher total FAA and lower BA formation in final processed products. If stored for 3, 6 and 12

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**Table 7:** Variability of microbial counts (log c.f.u. g$^{-1}$) at different processing stages of “Chouriço de Carne” manufactured with and without curing salts.

|                | R0   | S1   | S2   | S3   | SE  |
|----------------|------|------|------|------|-----|
|                | No curing salts | With curing salts | With curing salts | With curing salts | SE  |
| TAMF           | 6.2 ± 0.1 | 4.57 ± 0.6 | 3.49 ± 0.4 | 7.15 ± 0.5 | 6.99 ± 0.9 | 7.73 ± 0.3 | 7.19 ± 0.5 | 0.018 ± 0.001 | *** | *** | *** |
| Enterococci    | <1.00 ± 0.2 | 1.30 ± 0.2 | 1.30 ± 0.2 | 4.69 ± 0.4 | 4.21 ± 0.4 | 4.69 ± 0.4 | 4.03 ± 0.4 | 0.056 ± 0.001 | *** | *** | * |
| LAB            | 2.56 ± 0.2 | 3.53 ± 0.2 | 3.56 ± 0.2 | 7.10 ± 0.4 | 7.15 ± 0.4 | 7.75 ± 0.4 | 7.31 ± 0.4 | 0.022 ± 0.001 | *** | * | ** |
| Micrococcaceae | 0.81 ± 0.2 | 2.64 ± 0.2 | 2.77 ± 0.2 | 6.67 ± 0.4 | 5.43 ± 0.4 | 5.44 ± 0.4 | 5.41 ± 0.4 | 0.230 ± 0.001 | *** | ns | ns |
| Enterobacteriaceae | 2.11 ± 0.2 | 1.30 ± 0.2 | 1.60 ± 0.2 | 1.78 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | ns ± 0.001 | ns | ns | ns |

In same row, means with different letters are significantly different. ns – not significant; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. SE - Standard error.

|                | S4   | S6   | Interactions |
|----------------|------|------|--------------|
|                | No curing salts | With curing salts | SE | $t^\circ C$ | $t^\circ T$ | $C^\circ T$ | $t^\circ C*T$ |
| TAMF           | 7.77 ± 0.2 | 7.89 ± 0.2 | 7.36 ± 0.2 | 7.50 ± 0.2 | 7.03 ± 0.2 | 4.37 ± 0.2 | 4.57 ± 0.2 | 4.04 ± 0.2 | 0.15 ± 0.001 | *** | *** | *** | *** |
| Enterococci    | 4.78 ± 0.2 | 4.94 ± 0.2 | 3.87 ± 0.2 | 4.20 ± 0.2 | 3.75 ± 0.2 | ND | ND | ND | 0.18 ± 0.001 | *** | *** | *** | *** |
| LAB            | 7.80 ± 0.2 | 8.05 ± 0.2 | 7.70 ± 0.2 | 7.48 ± 0.2 | 6.94 ± 0.2 | 3.89 ± 0.2 | 1.70 ± 0.2 | ND | 0.20 ± 0.001 | *** | *** | ** | ** |
| Micrococcaceae | 6.52 ± 0.2 | ND | 6.60 ± 0.2 | 6.11 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | 0.29 ± 0.001 | ns | ns | ns | ns |
| Enterobacteriaceae | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | ns ± 0.001 | ns | ns | ns | ns |

In same row, means with different letters are significantly different. ns = not significant; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. SE - Standard error. TAMF - total aerobic mesophytic flora. ND - Not detected. t - time; C - curing salts; T - temperature.

|                | S4   | S6   | Interactions |
|----------------|------|------|--------------|
|                | No curing salts | With curing salts | SE | $t^\circ C$ | $t^\circ T$ | $C^\circ T$ | $t^\circ C*T$ |
| TAMF           | 7.77 ± 0.2 | 7.89 ± 0.2 | 7.36 ± 0.2 | 7.50 ± 0.2 | 7.03 ± 0.2 | 4.37 ± 0.2 | 4.57 ± 0.2 | 4.04 ± 0.2 | 0.15 ± 0.001 | *** | *** | *** | *** |
| Enterococci    | 4.78 ± 0.2 | 4.94 ± 0.2 | 3.87 ± 0.2 | 4.20 ± 0.2 | 3.75 ± 0.2 | ND | ND | ND | 0.18 ± 0.001 | *** | *** | *** | *** |
| LAB            | 7.80 ± 0.2 | 8.05 ± 0.2 | 7.70 ± 0.2 | 7.48 ± 0.2 | 6.94 ± 0.2 | 3.89 ± 0.2 | 1.70 ± 0.2 | ND | 0.20 ± 0.001 | *** | *** | ** | ** |
| Micrococcaceae | 6.52 ± 0.2 | ND | 6.60 ± 0.2 | 6.11 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | 0.29 ± 0.001 | ns | ns | ns | ns |
| Enterobacteriaceae | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | <1.00 ± 0.2 | ns ± 0.001 | ns | ns | ns | ns |

In same row, means with different letters are significantly different. ns = not significant; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$. SE - Standard error. TAMF - total aerobic mesophytic flora. ND - Not detected. t - time; C - curing salts; T - temperature.
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