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Portuguese Traditional Dry-Fermented Sausages Processed with Liquid Smoke Flavoring: How This Alternative Technology Affects Proteolysis and Biogenic Amines Profile

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Abstract: To avoid carcinogenic polycyclic aromatic hydrocarbons, liquid smoke flavoring (LSF) is widely used by the meat industry, yet wood smoking remains a deep-rooted practice among Portuguese traditional dry-fermented producers. In this study, the use of LSF was compared with traditional smoking. In addition, two different ways of using LSF were also tested: spraying and mixing (during seasoning). The profiles of amino acids (AA) and biogenic amines (BA) were studied at pre-scheduled moments of processing and storage. When compared to smoked products, LSF did not affect total AA content; however, when mixed during seasoning, it inhibited the accumulation of arginine and lysine (precursors of BA) in final products. The conventional smoking replacement, by mixing LSF during seasoning, turned out to be critical to lessen BA of bacterial origin accumulation as well, especially for putrescine, cadaverine and tyramine. The benefits of spraying LSF over smoking were higher for final products than for storage, as differences between them tended to fade with time (except for tyramine). These results also demonstrate that a simple change in traditional dry-fermented sausage processing, such as mixing LSF during product seasoning, significantly contributes to the safety of these products through the reduction in undesirable BA.

Keywords: biogenic amines; liquid smoke flavoring; dry-fermented sausage; traditional meat products

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1. Introduction

The production of dry-fermented sausages relies on a complex conjugation of reactions triggered by the microbiota, along with the endogenous enzymes (both proteases and lipases) that naturally occur in meat. Such meat enzymes play a major role in organoleptic characteristic development. Proteolysis begins with endopeptidase (mainly cathepsin) activity on sarcoplasmic and myofibrillar proteins, where they are responsible for breaking non-terminal bonds, releasing peptides and oligopeptides. Exopeptidases, in turn, continue protein degradation by acting on terminal peptide bonds, with the release of small amino acids (AA) or peptides.

Apart from changes in texture, the release of the elementary constituents of proteins, AA, as well as small peptides, give meat products and dry-fermented sausages, in particular, unique sensory characteristics. Together with condiments, additives and smoking, AA are responsible for the development of the typical aromas and flavors found in these products.

Simultaneously, some of the released AA can be converted into biogenic amines (BA), which are nitrogenous low-molecular-weight compounds that participate in several biological functions in animals, plants and microorganisms. Among others, they may act as neurotransmitters or participate in blood pressure regulation. Despite their physiological role, when ingested in higher amounts BA may pose a potential risk to consumers' health. Due to their vasoactive and psychoactive activities, BA toxicological effects can affect the nervous and vascular systems and cause allergic reactions. Moreover, these amines are precursors of N-nitrosamines, which are known for their carcinogenic activity.

Today's consumers show a renewed interest in traditionally produced fermented meat products, especially in Europe, where this market has a great economic impact [1]. At the same time, the demand for safer, healthier products of added nutritional value is also higher and represents an important challenge for the meat industry. In view of this, a number of technologies have been introduced aiming, among other things, to reduce the salt and fat content often associated with meat products [2–4]. Another example of concern for human health regards the use of liquid smoke flavoring (LSF) as an alternative to the conventional smoking process, which is known to be the main route of chemical contamination of these products with carcinogenic polycyclic aromatic hydrocarbons. On the other hand, the processing with LSF also ensures a greater consistency from batch to batch and greater control over microbial growth as well as the fermentation that takes place [5].

Despite the advantages concerning LSF and its widespread use, studies of Portuguese meat products processed with LSF and the consequences of its application on the proteolytic activity, as well as on the formation of BA, are still lacking. In this context, the present work aimed to investigate the effect of the use of LSF on these parameters, with special focus on BA accumulation, as the latter may directly impact consumer health. The proteolytic activity was evaluated based on the quantification of the nitrogen fractions and the free AA profile.

2. Materials and Methods

2.1. Experimental Design

The experimental design set up for this investigation compared the traditional processing (designated as "Control") with two LSF processing technologies: (1) spraying the product surface and (2) mixing with raw meat and fat (similar to other ingredients). Meat products were analyzed (1) after product formulation ("RM"), at the end of processing (final product, "FP") and after 1 and 3 months of storage ("1M" and "3M", respectively). Attending to the smoking chamber dimensions, two independent batches were processed. Figure 1 shows the experimental design followed for the production of dry-fermented sausages.

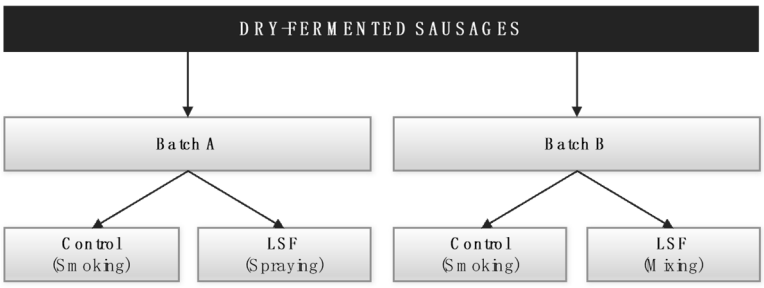

Figure 1. Experimental design for dry-fermented sausage processing.

2.2. Sausage Technology and Sampling Procedures

A blend of pork meat (60%) containing different pork parts, including belly, shoulder, and leg, was mixed with fat (40%), minced and seasoned with red pepper (*Capsicum annuum* L.) paste (3.5%), garlic (*Allium sativum* L.) paste (3%), salt (1%), water (2.5%), Palatinata Cure curing salts [0.25%, with $NaNO_3$ (4.9%) and KNO_3 (5%)] and Jabarot antioxidant mix (0.15%). Based on the instructions provided by the supplier (Formulab, Leça do Balio, Portugal), an amount of 0.5% (w/w) of LSF was also added for "LSF" products from Batch B. This mixture was then stuffed in natural sheep casings. Dry-fermented sausages were mainly dried in a controlled environment chamber set to a 6 °C temperature and 80% relative humidity for 6 days. After this period, the temperature was set to 10 °C and relative humidity to 75% until the end of the drying stage. In turn, "LSF" products from Batch A were sprayed with a freshly prepared diluted solution [10% (v/v)] of LSF on the 2nd, 3rd and 7th days after processing. Whenever products were sprayed with LSF solution, they were left to air for approximately two hours, before being reintroduced into the chamber, in order to eliminate the excess of moisture. Since these samples denoted a lower color development when compared to smoked counterparts, the amount of LSF applied was reinforced: first with a second spray (on the 13th, 14th and 15th days) and then with a 20% (v/v) solution of LSF on the last day of processing (17th day). Dry-fermented sausages, designated as "Control", were smoked using *Quercus ilex* L. wood for periods of 4 h/day. When both LSF and smoked products reached a 35% weight loss, they were vacuum-packaged. Products were stored at 4 °C for predetermined times (1 and 3 months), after which they were deep-frozen at −80 °C until analysis.

2.3. Physicochemical and Proteolysis Characterization

The samples' pH, non-protein nitrogen (NPN) and total volatile basic nitrogen (TVBN) were assessed as described by Roseiro et al. [6].

2.4. Amino Acid Profile

The AA extracts were obtained using the analytic protocol described by Aristoy and Toldrá [7], derivatized with ortho-phtalaldehyde [8] and injected into the HPLC system.

Chromatographic analysis was performed using an HPLC Alliance Separation Module 2695 (Waters, Milford, MA, USA), coupled to a Multi-λ Fluorescence Detector 2475 (Waters, Milford, MA, USA) set to 338/425 nm (Ex/Em wavelengths). Free AA separation was performed on a reversed-phase Spherisorb column 5 µm ODS2, 4.6 × 250 mm (Waters, Milford, MA, USA). The elution program combined different proportions of solvent A, containing phosphate buffer (50 mM, pH 5.5), methanol and tetrahydrofuran (80:19:1, ($v/v/v$)) and solvent B, with methanol and phosphate buffer (80:20, (v/v)). The elution started with a flow rate of 0.5 mL/min in solvent A at 100% that increased in the first minute to 1.0 mL/min. At 10 min, the solvent ratio was ramped up to 75% of solvent A and 25% of solvent B and maintained for 2 more minutes. From 13 to 20 min, the flow increased once more to 1.5 mL/min. During the next 30 min, the gradient changed linearly to 0% of solvent A and 100% of solvent B.

2.5. Biogenic Amines Profile

The BA quantification was carried out by HPLC as described by Roseiro et al. [9].

2.6. Statistical Analysis

The data were tested for normality and the homogeneity of variance using Kolmogorov–Smirnov's test and Levene's F-test, respectively. Once these assumptions were confirmed, results were then tested using a full factorial ANOVA, with three independent variables:

processing technology, time, and batch. Differences between means were determined by the Honest Significant Differences test (HSD Tukey) for a significance level of 0.05. Statistical analysis was computed in Statistica 10 (StatSoft Inc., 1984–2011, Hamburg, Germany).

3. Results

3.1. Physicochemical and Proteolysis Characterization

The results concerning the physicochemical and proteolysis parameters, shown in Table 1, highlight differences ($p < 0.05$) depending on the considered batch. In fact, samples from Batch B showed lower pH levels, as well as higher amounts of the studied nitrogen fractions (NPN and TVBN). Differences due to the manufacturing procedures and processing stage were also observed (Table 1).

The pH levels followed the typical evolution pattern for traditional dry-fermented sausage manufacturing. The initial pH (6.05 determined for Batch A; 5.90 and 5.98 measured in "Control" and "LSF" products from Batch B) consistently decreased during the processing and storage stages. As expected, the greatest reduction occurred during dry-fermented sausage processing where the pH global decrease ranged from 0.40 up to 0.45 units. Those samples from Batch B that were sprayed with LSF were the exception. For these, the decrease in pH was less pronounced, nearly half (0.22 pH units), from 5.98 to 5.76. Regardless of the considered batch, "Control" samples showed lower pH levels when compared to those observed in "LSF" samples. After 3 months of storage, the pH levels measured in "Control" products were 5.56 and 5.51 (Batch A and B, respectively), while in "LSF" the pH levels were 5.64 and 5.70 (Batch A and B, respectively).

3.2. Amino Acid Profile

The results regarding mean free AA content (Table 2) show a consistent increase during the whole trial, but more markedly in the processing stage. Indeed, among the three investigating factors, the effect of time was the most relevant in terms of AA content. In Batch A samples, total AA amounts almost doubled, from 550.26 up to 1058.83 and 985.20 mg/100 g on a dry matter basis (DM) ("Control" and "LSF", respectively). An increase of the same order of magnitude was also verified in Batch B products whose initial content rose from 591.03 to 1183.98 mg/100 g DM.

A closer look at Table 2 highlights alanine as the most abundant AA in the raw material, corresponding to nearly 40% of total AA (mean values for both batches), with mean levels ranging from 210.93 up to 318.66 mg/100 g DM (in Batch A and "LSF" products from Batch B, respectively). Alanine content remained globally unchanged through processing and storage stages.

In turn, arginine, a precursor of putrescine, was the second AA found in greater amounts. Its initial content of about 100 mg/100 g DM promptly increased during processing and later on during product storage. In fact, the highest amounts of arginine occurred after 3 months of storage in smoked products from Batch B (311.54 mg/100 g DM). Within the products stored for a similar period, those from Batch A processed with "LSF" resulted in the lowest arginine amounts (225.68 mg/100 g DM).

A similar evolution pattern was observed for other AA, including the BA precursors. For example, the levels of lysine, a precursor of cadaverine, increased during the processing by two to five times. Once more, the smoked dry-fermented sausages stood out with the greatest lysine amounts, at 129.26 and 149.75 mg/100 g DM (Batches A and B, respectively), while those processed with LSF, in turn, showed 99.36 and 113.28 mg/100 g DM (Batches B and A, respectively).

Table 1. Mean pH values and content of studied nitrogen fractions in dry-fermented sausages (expressed in DM).

	Batch A								**Batch B**							
	Control				**LSF (Spraying)**				**Control**				**LSF (Mixing)**			
	RM	**FP**	**1M**	**3M**	**RM**	**FP**	**1M**	**3M**	**RM**	**FP**	**1M**	**3M**	**RM**	**FP**	**1M**	**3M**
pH	$6.05^{j} \pm 0.01$	$5.60^{b,c,d} \pm 0.00$	$5.57^{b,c} \pm 0.02$	$5.56^{b} \pm 0.01$	$6.05^{j} \pm 0,01$	$5.61^{c,d} \pm 0.00$	$5.63^{d} \pm 0.01$	$5.64^{d,e} \pm 0.02$	$5.90^{h} \pm 0.00$	$5.49^{a} \pm 0.01$	$5.49^{a} \pm 0.01$	$5.51^{b} \pm 0.02$	$5.98^{i} \pm 0.01$	$5.76^{g} \pm 0.01$	$5.68^{e,f} \pm 0.02$	$5.70^{f} \pm 0.00$
NPN (mg/g)	$6.24^{a,b} \pm 0.11$	$6.01^{a} \pm 0.02$	$6.57^{c,d} \pm 0.08$	$7.21^{h,i} \pm 0.12$	$6.24^{a,b} \pm 0.11$	$6.11^{a} \pm 0.07$	$6.53^{c,d} \pm 0.03$	$6.78^{d,e,f} \pm 0.04$	$6.68^{d,e} \pm 0.07$	$7.00^{f,g,h} \pm 0.04$	$6.86^{e,f,g} \pm 0.03$	$7.85^{j} \pm 0.05$	$6.40^{b,c} \pm 0.04$	$6.93^{e,f,g} \pm 0.01$	$7.10^{g,h} \pm 0.07$	$7.47^{i} \pm 0.05$
TVBN (mg/g)	$0.25^{a} \pm 0.00$	$0.47^{c,d} \pm 0.02$	$0.51^{d,e,f} \pm 0.02$	$0.54^{f,g} \pm 0.00$	$0.25^{a} \pm 0.00$	$0.43^{c} \pm 0.02$	$0.51^{d,e,f} \pm 0.00$	$0.62^{h,i} \pm 0.01$	$0.48^{c,d,e} \pm 0.00$	$0.57^{g,h} \pm 0.02$	$0.75^{j} \pm 0.02$	$0.78^{j} \pm 0.02$	$0.37^{b} \pm 0.03$	$0.51^{d,e,f} \pm 0.00$	$0.53^{e,f,g} \pm 0.02$	$0.64^{i} \pm 0.02$

Results are presented as mean ± SD. In the same line, mean values followed by different letters are significantly different ($p < 0.05$). NPN: non-protein nitrogen, TVBN: total volatile basic nitrogen; RM: seasoned raw material; FP: final product; 1M and 3M: dry-fermented sausages after 1 and 3 months of storage, respectively.

Table 2. Mean content of free amino acids in dry-fermented sausages (expressed in DM).

	Batch A								Batch B							
	Control				LSF (Spraying)				Control				LSF (Mixing)			
	RM	FP	1M	3M	RM	FP	1M	3M	RM	FP	1M	3M	RM	FP	1M	3M
Aspasrtic acid	ND	ND	$0.33^{a,b} \pm 0.10$	$2.38^{c} \pm 0.52$	ND	$0.18^{a,b} \pm 0.07$	$0.35^{a,b} \pm 0.12$	$1.02^{b} \pm 0.60$	ND	ND	$0.01^{a} \pm 0.00$	$0.82^{a,b} \pm 0.10$	ND	$0.09^{a} \pm 0.01$	$0.16^{a} \pm 0.05$	$0.15^{a} \pm 0.07$
Glutamic acid	$7.94^{a} \pm 0.27$	$28.44^{c,d} \pm 2.61$	$35.70^{e} \pm 1.50$	$51.75^{h} \pm 3.48$	$7.94^{a} \pm 0.27$	$26.98^{c} \pm 2.94$	$34.58^{d,e} \pm 2.00$	$45.76^{g,h} \pm 3.15$	$14.83^{a,b} \pm 1.51$	$35.05^{d,e} \pm 2.90$	$36.69^{e,f} \pm 2.36$	$43.58^{f,g} \pm 2.13$	$17.56^{b} \pm 1.74$	$33.07^{c,d,e} \pm 2.35$	$43.62^{f,g} \pm 3.32$	$51.55^{h} \pm 1.87$
Serine	$10.55^{a} \pm 0.90$	$33.41^{d} \pm 1.77$	$46.73^{e,f} \pm 1.85$	$67.39^{g} \pm 4.41$	$10.55^{a} \pm 0.90$	$38.33^{d,e} \pm 4.19$	$48.00^{f} \pm 4.47$	$61.57^{g} \pm 7.25$	$15.41^{a,b,c} \pm 2.04$	$16.39^{a,b,c} \pm 0.99$	$21.12^{b,c} \pm 1.95$	$24.09^{c} \pm 0.52$	$17.74^{a,b,c} \pm 1.78$	$17.81^{a,b,c} \pm 1.74$	$12.99^{a,b} \pm 0.79$	$18.34^{a,b,c} \pm 0.84$
Glutamine	$112.10^{g} \pm 6.49$	$90.98^{f} \pm 8.15$	$78.68^{e,f} \pm 4.26$	$67.31^{d,e} \pm 5.05$	$112.10^{g} \pm 6.49$	$91.13^{f} \pm 7.43$	$81.47^{e,f} \pm 1.44$	$57.74^{c,d} \pm 11.51$	$73.66^{d,e,f} \pm 3.70$	$38.43^{b} \pm 0.48$	$25.19^{a,b} \pm 2.47$	$12.71^{a} \pm 0.43$	$74.70^{d,e,f} \pm 6.59$	$39.53^{b,c} \pm 7.79$	$29.30^{a,b} \pm 8.50$	$15.70^{a} \pm 0.55$
Histidine	$6.68^{a} \pm 0.35$	$28.13^{b,c,d} \pm 1.76$	$34.40^{c,d,e} \pm 1.37$	$51.64^{h} \pm 3.71$	$6.68^{a} \pm 0.35$	$26.32^{b,c} \pm 2.71$	$34.21^{c,d,e} \pm 3.44$	$44.67^{g,h} \pm 6.56$	$12.29^{a} \pm 1.73$	$35.69^{d,e,f} \pm 2.82$	$41.75^{e,f,g} \pm 3.15$	$49.70^{g,h} \pm 1.40$	$13.13^{a} \pm 1.31$	$23.28^{b} \pm 2.16$	$29.93^{b,c,d} \pm 2.92$	$42.84^{f,g} \pm 1.66$
Threonine	$9.98^{a} \pm 0.57$	$30.11^{d,e} \pm 1.83$	$35.50^{e} \pm 1.31$	$50.15^{f} \pm 3.40$	$9.98^{a} \pm 0.57$	$28.50^{c,d,e} \pm 2.87$	$35.95^{e} \pm 3.17$	$47.43^{f} \pm 9.50$	$15.75^{a,b} \pm 2.80$	$17.58^{a,b} \pm 0.65$	$19.61^{a,b,c} \pm 3.27$	$22.89^{b,c,d} \pm 4.02$	$18.50^{a,b,c} \pm 2.67$	$24.08^{b,c,d} \pm 3.49$	$24.43^{b,c,d} \pm 2.23$	$31.66^{d,e} \pm 2.37$
Glycine	$23.17^{a} \pm 1.59$	$44.10^{c,d,e,f} \pm 2.90$	$47.48^{d,e,f,g} \pm 2.37$	$61.82^{h} \pm 4.49$	$23.17^{a} \pm 1.59$	$37.65^{b,c,d} \pm 4.28$	$47.66^{d,e,f,g} \pm 5.43$	$55.61^{f,g,h} \pm 8.45$	$26.12^{a,b} \pm 5.67$	$48.36^{d,e,f,g} \pm 3.33$	$53.11^{e,f,g,h} \pm 3.73$	$59.77^{g,h} \pm 1.44$	$31.73^{a,b,c} \pm 2.96$	$37.67^{b,c,d} \pm 5.20$	$42.05^{c,d,e} \pm 3.03$	$52.48^{e,f,g,h} \pm 2.34$
Taurine	$10.47^{e} \pm 0.58$	$2.90^{a,b,c} \pm 0.19$	$2.34^{a,b} \pm 0.08$	$2.37^{a,b} \pm 0.15$	$10.47^{e} \pm 0.58$	$1.78^{a} \pm 0.09$	$2.04^{a} \pm 0.13$	$2.19^{a} \pm 0.27$	$10.49^{e} \pm 0.15$	$3.55^{b,c,d} \pm 0.24$	$3.81^{c,d} \pm 0.18$	$3.82^{c,d} \pm 0.07$	$13.22^{f} \pm 1.34$	$3.65^{b,c,d} \pm 0.51$	$4.57^{d} \pm 0.29$	$4.75^{d} \pm 0.26$
Alanine	$210.93^{a,b} \pm 13.57$	$211.54^{a,b} \pm 12.67$	$203.33^{a} \pm 9.02$	$198.90^{a} \pm 12.21$	$210.93^{a,b} \pm 13.57$	$201.16^{a} \pm 20.76$	$221.23^{a,b,c,d} \pm 19.89$	$210.04^{a} \pm 34.09$	$213.99^{a,b,c} \pm 34.17$	$232.65^{a,b,c,d} \pm 14.89$	$235.72^{a,b,c,d} \pm 17.57$	$236.73^{a,b,c,d} \pm 3.65$	$318.66^{e} \pm 33.50$	$278.59^{b,c,d,e} \pm 40.49$	$279.87^{c,d,e} \pm 23.35$	$282.26^{d,e} \pm 15.13$
Arginine	$92.46^{a} \pm 6.12$	$176.70^{c,d,e} \pm 12.24$	$189.67^{d,e,f} \pm 7.68$	$234.02^{f,g} \pm 15.14$	$92.46^{a} \pm 6.12$	$152.71^{b,c,d} \pm 15.64$	$195.52^{d,e,f} \pm 20.70$	$225.68^{e,f,g} \pm 33.19$	$106.19^{a,b} \pm 19.55$	$263.11^{g,h,i} \pm 16.72$	$287.74^{h,i} \pm 22.20$	$311.54^{i} \pm 6.45$	$131.11^{a,b,c} \pm 13.07$	$177.96^{c,d,e} \pm 20.91$	$206.76^{e,f} \pm 12.65$	$258.51^{g,h} \pm 12.88$
Tyrosine	$3.12^{b,c} \pm 0.31$	$17.33^{e} \pm 1.25$	$14.99^{e} \pm 0.57$	$16.38^{e} \pm 0.41$	$3.12^{b,c} \pm 0.31$	$15.72^{e} \pm 1.73$	$15.34^{e} \pm 1.93$	$11.81^{d} \pm 1.32$	$0.99^{a,b} \pm 0.87$	$0.01^{a} \pm 0.01$	$0.06^{a} \pm 0.08$	$0.06^{a} \pm 0.10$	$3.77^{c} \pm 0.69$	$2.04^{a,b,c} \pm 0.18$	$0.89^{a,b} \pm 0.77$	$0.49^{a,b} \pm 0.80$
Methionine	$3.31^{a} \pm 0.15$	$20.82^{b,c} \pm 1.10$	$26.17^{d,e,f} \pm 0.73$	$36.01^{g,h} \pm 2.43$	$3.31^{a} \pm 0.15$	$18.39^{b,c} \pm 1.47$	$22.79^{c,d,e} \pm 2.10$	$27.85^{e,f} \pm 4.16$	$6.18^{a} \pm 1.02$	$29.28^{f} \pm 1.16$	$34.90^{g} \pm 3.33$	$40.71^{h} \pm 0.95$	$6.87^{a} \pm 0.59$	$16.82^{b} \pm 0.78$	$22.22^{c,d} \pm 1.24$	$30.81^{f,g} \pm 0.97$
Valine	$8.46^{a} \pm 0.47$	$41.38^{b,c,d} \pm 2.35$	$51.77^{d,e,f} \pm 2.16$	$75.04^{h,i} \pm 5.16$	$8.46^{a} \pm 0.47$	$37.65^{b,c} \pm 3.66$	$47.34^{c,d,e} \pm 4.95$	$61.73^{f,g} \pm 8.72$	$13.76^{a} \pm 1.81$	$58.07^{e,f,g} \pm 2.94$	$68.02^{g,h} \pm 6.01$	$80.93^{i} \pm 2.03$	$15.34^{a} \pm 1.42$	$34.19^{b} \pm 2.00$	$44.91^{b,c,d} \pm 3.74$	$63.57^{g} \pm 2.30$
Thryptophan	$1.49^{a} \pm 0.14$	$9.61^{c,d} \pm 0.83$	$11.87^{c,d,e} \pm 0.43$	$16.84^{f} \pm 1.49$	$1.49^{a} \pm 0.14$	$8.77^{b,c,d} \pm 0.79$	$11.35^{c,d,e} \pm 1.33$	$14.83^{e,f} \pm 2.89$	$3.21^{a} \pm 1.48$	$13.00^{d,e,f} \pm 0.32$	$16.67^{f} \pm 2.75$	$16.71^{f} \pm 0.33$	$4.14^{a,b} \pm 0.68$	$8.14^{b,c} \pm 1.31$	$10.17^{c,d,e} \pm 0.52$	$17.37^{f} \pm 3.72$
Phenylalanine	$5.81^{a} \pm 0.28$	$36.43^{c} \pm 2.16$	$46.34^{d,e} \pm 2.04$	$64.76^{g} \pm 4.25$	$5.81^{a} \pm 0.28$	$30.96^{b,c} \pm 2.98$	$39.08^{c,d} \pm 4.23$	$48.58^{d,e} \pm 6.35$	$9.70^{a} \pm 1.41$	$50.65^{e,f} \pm 2.84$	$60.06^{f,g} \pm 6.01$	$68.94^{g} \pm 1.49$	$10.91^{a} \pm 0.88$	$25.92^{b} \pm 1.81$	$35.07^{b,c} \pm 2.99$	$50.76^{e,f} \pm 1.56$
Isoleucine	$3.99^{a} \pm 0.32$	$32.66^{c} \pm 1.92$	$42.02^{d,e} \pm 1.76$	$59.91^{g,h} \pm 4.30$	$3.99^{a} \pm 0.32$	$28.98^{b,c} \pm 2.99$	$36.71^{c,d} \pm 3.79$	$46.74^{e,f} \pm 6.82$	$7.11^{a} \pm 1.04$	$44.21^{d,e} \pm 2.36$	$53.23^{f,g} \pm 4.70$	$63.87^{h} \pm 1.42$	$8.11^{a} \pm 0.87$	$23.56^{b} \pm 1.82$	$32.53^{b,c} \pm 2.89$	$47.96^{e,f} \pm 1.71$
Leucine	$9.33^{a} \pm 0.53$	$59.96^{b,c,d} \pm 3.29$	$75.67^{d,e,f} \pm 3.14$	$106.81^{h,i} \pm 7.52$	$9.33^{a} \pm 0.53$	$54.01^{b,c} \pm 5.20$	$68.10^{c,d,e} \pm 7.05$	$84.95^{f,g} \pm 10.95$	$15.68^{a} \pm 2.38$	$81.16^{e,f,g} \pm 4.17$	$96.67^{g,h} \pm 9.00$	$113.30^{i} \pm 2.42$	$17.53^{a} \pm 1.47$	$45.91^{b} \pm 3.32$	$63.08^{c,d} \pm 5.37$	$91.73^{g,h} \pm 2.90$
Ornithine	$4.94^{a} \pm 0.10$	$65.08^{d,e,f} \pm 4.45$	$76.54^{f,g} \pm 3.71$	$102.44^{h} \pm 7.41$	$4.94^{a} \pm 0.10$	$72.72^{e,f} \pm 7.01$	$89.28^{g,h} \pm 10.07$	$97.01^{h} \pm 10.60$	$7.55^{a} \pm 4.40$	$67.05^{d,e,f} \pm 2.59$	$60.13^{c,d,e} \pm 7.04$	$48.30^{b,c} \pm 1.54$	$7.69^{a} \pm 0.59$	$53.92^{b,c,d} \pm 4.46$	$44.77^{b,c} \pm 2.58$	$40.90^{b} \pm 1.41$
Lysine	$25.54^{a} \pm 1.39$	$129.26^{b,c,d} \pm 7.54$	$165.67^{e,f,g} \pm 9.78$	$247.65^{j} \pm 15.52$	$25.54^{a} \pm 1.39$	$113.28^{b,c} \pm 10.95$	$155.91^{d,e,f} \pm 19.42$	$202.99^{h,i} \pm 22.37$	$38.12^{a} \pm 10.54$	$149.75^{d,e,f} \pm 5.64$	$176.05^{f,g,h} \pm 20.83$	$226.17^{i,j} \pm 5.72$	$41.44^{a} \pm 3.56$	$99.36^{b} \pm 1.17$	$135.51^{c,d,e} \pm 7.43$	$197.45^{g,h,i} \pm 8.16$
Total AA	$550.26^{a} \pm 33.88$	$1058.83^{c,d} \pm 57.54$	$1185.20^{c,d,e} \pm 45.04$	$1513.56^{f} \pm 100.31$	$550.26^{a} \pm 33.88$	$985.20^{b,c} \pm 94.46$	$1186.90^{c,d,e} \pm 115.17$	$1348.21^{e,f} \pm 184.44$	$591.03^{a} \pm 89.93$	$1183.98^{c,d,e} \pm 59.62$	$1290.53^{d,e,f} \pm 101.02$	$1424.64^{e,f} \pm 33.04$	$752.14^{a,b} \pm 75.14$	$945.57^{b,c} \pm 85.53$	$1062.84^{c,d} \pm 61.28$	$1299.27^{d,e,f} \pm 51.00$

Results are expressed in mg/100 g, as mean ± SD. In the same line, mean values followed by different letters are significantly different ($p < 0.05$). ND—not detected; RM: seasoned raw material; FP: final product; 1M and 3M: dry-fermented sausages after 1 and 3 months of storage, respectively.

Despite this, results in Table 2 also demonstrate the reduction in some AA over time. This is the case of glutamine. Such results are of great interest, since these AA are involved in putrescine production. In Batch A, initial glutamine content was reduced from 112.10 mg/100 g DM by nearly half. In turn, in Batch B they decreased to nearly one fifth of their initial levels (from approximately 74 mg/100 g DM to 12.71 and 15.70 mg/100 g DM). Even though a decreasing trend in ornithine content was also observed in stored Batch B samples, an opposite trend was seen in Batch A counterparts.

The release of valine, phenylalanine, isoleucine, methionine and leucine followed a similar pattern, accumulating during dry-fermented sausage processing, irrespective of the considered batch (Table 2). Still, it should be pointed out that the highest accumulation of these AA was observed in smoked products. Regarding tryptophan, despite its increment during processing and storage, the final levels in "Control" and "LSF" products after 3 months of storage were similar, ranging between 14.83 and 17.37 mg/100 g DM. During the processing stage, histidine, glycine and glutamic acid almost doubled. For example, the content of histidine in the "FP" ranged from 23.28 to 35.69 mg/100 g DM (in "LSF" and "Control" from Batch B, respectively). Later on, during storage, this content increased to 42.84 and 49.70 mg/100 g DM (found in "LSF_3M" and "Control_3M" from Batch B, respectively).

Regarding threonine, serine and tyrosine, the main changes were mainly related to the use of different batches of raw material and not to processing. In products from Batch B, the found levels of these AA remained unchanged over time, while those from Batch A significantly increased immediately after processing. This effect was particularly marked for tyrosine, which ranged between 0.01 and 3.77 mg/100 g DM in products from Batch B, while counterparts from Batch A exhibited between 3.12 and 17.33 mg/100 g DM. In any case, for both batches, tyrosine content remained globally unchanged during storage.

3.3. Biogenic Amines Profile

The BA content determined for the studied products, as presented in Table 3, show that it was drastically ($p < 0.001$) influenced by the raw material batch, processing technology, and storage time. Higher total BA content was determined in products from Batch B. Moreover, while BA levels increased with processing and storage, the processing with LSF helped to counteract their accumulation. Although total BA levels determined in "RM" samples were comparable between batches (ranging between 78.68 and 93.96 mg/kg DM), differences arose during processing and subsequent storage. In "FP" of Batch B, total BA reached 279.32 and 621.70 mg/kg DM (samples "LSF" and "Control", in the same order), while those from Batch A accounted for 113.82 and 127.74 mg/kg DM ("LSF" and "Control" samples, respectively). After a 3-month storage, total BA in Batch B products were three to six times higher than those initially found in "RM" samples, reaching up to 982.30 and 691.84 mg/kg DM (in the same order samples "Control_3M" and "LSF_3M", respectively). On the other hand, in products from Batch A, BA levels corresponded to 329.62 and 281.53 mg/kg DM in "Control_3M" and "LSF_3M", respectively.

As expected, endogenous amines, spermine and spermidine, clearly prevailed in "RM" samples (Table 3). Their preponderance, however, progressively decreased with the accumulation of bacterial-origin amines (including tyramine and putrescine), which became the most abundant. In global terms, the found levels of spermine in "RM" samples from both batches decreased during processing and storage to final levels ranging between 51.34 and 67.02 mg/kg DM (in Batch A "LSF_3M" and "Control_3M", respectively).

On the contrary, spermidine content initially determined in "RM" samples (0.26 and 1.23 mg/kg DM ("LSF" from Batch B and Batch A)) increased with processing and storage. Even though similar content was found in dry-fermented sausages ("FP") in both

batches (4.32–7.47 mg/kg DM in "LSF" and "Control" samples from Batch A), a significant increase took place during the storage of products from Batch B, reaching up to 22.44 and 20.32 mg/kg DM in "Control_3M" and "LSF_3M", respectively.

For putrescine, cadaverine and tyramine, the results show that their evolution was also dependent on the studied factors. In Batch B "FP" products, tyramine content corresponded to 285.86 and 124.82 mg/kg DM ("Control" and "LSF", in the same order). For "Control" counterparts, tyramine levels remained stable until the end of storage. Nevertheless, a different trend was registered for "LSF" products, where this amine continuously increases during the entire storage (up to 246.28 mg/kg DM). Also, for "Control" products, tyramine levels increased from 32.74 mg/kg DM in "FP" up to 180.70 mg/kg DM determined in "3M", whilst in "LSF" samples these levels varied from 29.77 mg/kg DM in "PF" products to 138.70 mg/kg DM found in dry-fermented sausages stored for 3 months.

Regarding putrescine and cadaverine, these amines were absent or present at low levels in seasoned raw material ("RM" samples). However, the accumulation of cadaverine rose in "FP" from Batch B (126.72 mg/kg DM, which is 7 to 20 times higher than in other "FP" products). In any case, the highest cadaverine levels were always registered in stored products: after the first month for smoked dry-fermented sausages (23.40 and 167.75 mg/kg DM, Batches A and B, respectively), or after 3 months of storage for those produced with "LSF" (33.53 and 56.52 mg/kg DM, Batches A and B, respectively). Such discrepancies related to the batch were also perceived for putrescine. Once more, the "FP" from Batch B showed the highest levels (131.30 mg/kg DM in "Control" samples and 48.17 mg/kg DM in "LSF" samples). On the other hand, putrescine content in "FP" from Batch A was always lower than 10 mg/kg DM. After a 3-month storage period, levels of putrescine ranged from 50.46 and 52.39 mg/kg DM ("Control" and "LSF", respectively) found in Batch A, while in Batch B values as high as 418.84 and 305.51 mg/kg DM ("Control" and "LSF", respectively) were observed.

In order to give a better perspective of the risk associated with the consumption of these products, vasoactive amine content estimated on a wet weight basis (WW) was also included in Table 4. With regard to β-phenylethylamine, it was detected only in a few samples and always in small amounts, never exceeding 10 mg/kg on a wet weight basis (WW). Considering the sum of vasoactive amines, it was almost exclusively due to tyramine. In global terms, the highest amounts of these amines were determined in products from Batch B, especially in smoked products, ranging between 190.38 and 204.65 mg/kg WW, found in "FP" and "3M", respectively. In LSF products from the same batch, the vasoactive amines ranged from 82.57 to 164.43 mg/kg WW ("FP" and "3M", respectively). Lower contamination levels were registered in products from Batch A, where vasoactive content was always below 130 mg/kg WW.

Table 3. Mean content of biogenic amines in dry-fermented sausages (expressed in DM).

	Batch A								Batch B							
	Control				LSF (Spraying)				Control				LSF (Mixing)			
	RM	FP	1M	3M	RM	FP	1M	3M	RM	FP	1M	3M	RM	FP	1M	3M
Triptamine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-Phenylethylamine	1.61 ^{a} ± 2.79	ND	ND	0.88 ^{a} ± 0.95	1.61 ^{a} ± 2.79	ND	ND	ND	ND	ND	ND	ND	8.30 ^{b} ± 6.20	ND	ND	ND
Putrescine	ND	9.65 ^{a,b} ± 1.29	53.11 ^{c} ± 5.98	50.46 ^{c} ± 0.79	ND	8.66 ^{a,b} ± 0.33	21.86 ^{b} ± 7.39	52.39 ^{c} ± 3.03	ND	131.30 ^{d} ± 3.99	262.61 ^{f} ± 1.90	418.84 ^{h} ± 17.00	ND	48.17 ^{c} ± 5.31	198.58 ^{e} ± 8.26	305.51 ^{g} ± 9.86
Cadaverine	ND	6.28 ^{a,b} ± 1.17	23.40 ^{c,d} ± 3.21	20.91 ^{c} ± 1.47	ND	15.99 ^{b,c} ± 0.01	23.17 ^{c,d} ± 1.86	33.53 ^{d} ± 0.34	ND	126.72 ^{f} ± 3.94	167.75 ^{g} ± 10.66	165.49 ^{g} ± 6.18	1.47 ^{a} ± 2.34	21.83 ^{c} ± 1.45	47.06 ^{e} ± 4.85	56.52 ^{e} ± 1.84
Histamine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tyramine	ND	32.74 ^{b} ± 1.97	113.07 ^{c} ± 11.81	180.70 ^{d} ± 1.94	ND	29.77 ^{a,b} ± 0.04	58.19 ^{b} ± 2.39	138.70 ^{c} ± 4.64	ND	285.86 ^{f} ± 4.14	284.06 ^{f} ± 16.01	309.15 ^{f} ± 35.41	ND	124.82 ^{c} ± 1.80	191.51 ^{d} ± 0.73	246.28 ^{e} ± 3.32
Spermidine	1.23 ^{a,b} ± 0.88	7.47 ^{c} ± 0.98	6.93 ^{c} ± 1.74	9.66 ^{c} ± 0.80	1.23 ^{a,b} ± 0.88	4.32 ^{a,b,c} ± 0.19	8.12 ^{c} ± 1.52	5.57 ^{b,c} ± 0.31	ND	5.10 ^{a,b,c} ± 0.38	20.85 ^{d} ± 3.10	22.44 ^{d} ± 3.67	0.26 ^{a,b} ± 0.45	6.71 ^{c} ± 2.06	18.13 ^{d} ± 3.89	20.32 ^{d} ± 0.66
Spermine	86.56 ^{f} ± 8.54	71.60 ^{c,d,e} ± 2.74	68.05 ^{b,c,d} ± 6.33	67.02 ^{b,c,d} ± 2.04	86.56 ^{f} ± 8.54	55.09 ^{a,b} ± 2.25	55.77 ^{a,b} ± 2.15	51.34 ^{a} ± 1.43	78.68 ^{d,e,f} ±3.43	72.73 ^{c,d,e,f} ± 2.46	63.76 ^{a,b,c} ± 4.49	66.38 ^{b,c,d} ± 3.00	83.93 ^{e,f} ± 9.87	77.79 ^{c,d,e,f} ± 1.85	66.42 ^{b,c,d} ± 3.00	63.21 ^{a,b,c} ± 2.36
Total BA	89.40 ^{a} ± 11.19	127.74 ^{a,b} ± 6.64	264.56 ^{c} ± 28.94	329.62 ^{d} ± 7.19	89.40 ^{a} ± 11.19	113.82 ^{a} ± 2.08	167.11 ^{b} ± 5.49	281.53 ^{c,d} ± 9.02	78.68 ^{a} ± 3.43	621.70 ^{f} ± 10.20	799.04 ^{h} ± 30.35	982.30 ^{i} ±38.25	93.96 ^{a} ± 14.94	279.32 ^{c,d} ± 0.41	521.70 ^{e} ± 13.96	691.84 ^{g} ± 17.74

Results are expressed in mg/kg, as mean ± SD. In the same line, mean values followed by different letters are significantly different ($p < 0.05$). ND—not detected; RM: seasoned raw material; FP: final product; 1M and 3M: dry-fermented sausages after 1 and 3 months of storage, respectively.

Table 4. Mean content of vasoactive amines in dry-fermented sausages (expressed in WW).

	Batch A						Batch B					
	Control			LSF (Spraying)			Control			LSF (Mixing)		
	FP	1M	3M	FP	1M	3M	FP	1M	3M	FP	1M	3M
Triptamine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-Phenylethylamine	ND	ND	0.62 ± 0.55	ND	ND	ND	ND	ND	ND	ND	ND	ND
Histamine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tyramine	22.44 ± 1.11	77.35 ± 6.59	128.56 ± 1.13	20.20 ± 0.05	40.91 ± 1.15	99.85 ± 3.45	190.38 ± 2.2	185.11 ± 8.47	204.65 ± 18.76	82.57 ± 1.31	124.50 ± 0.15	164.43 ± 1.81
Vasoactive amines	22.44 ± 1.11	77.35 ± 6.59	129.18 ± 1.44	20.20 ± 0.05	40.91 ± 1.15	99.85 ± 3.45	190.38 ± 2.2	185.11 ± 8.47	204.65 ± 18.76	82.57 ± 1.31	124.50 ± 0.15	164.43 ± 1.81

Results are expressed in mg/kg, as mean ± SD. ND—not detected; FP: final product; 1M and 3M: dry-fermented sausages after 1 and 3 months of storage, respectively.

4. Discussion

With regard to pH, the main differences coincided with processing stage, resulting from the proliferation of lactic acid bacteria (LAB) that normally takes place during product processing [3,6,9,10], and the concomitant release of organic acids like lactic acid. Lactic acid is typically one of the compounds responsible for the pH decrease in dry-fermented meat products. When compared with "Control" products, those processed with LSF showed a less pronounced pH reduction, with this effect being particularly evident in products from Batch B. Since there were no differences in TVBN levels indicating the release of important amounts of nitrogen compounds, such as ammonia, that could explain the higher pH values measured in "LSF" products, these results suggest that the smoke condensate had a similar effect to that attributed by Laranjo et al. [10] to salt addition. The use of this ingredient, particularly when it was directly added during seasoning, may have favored a greater extraction of the proteins that are responsible for a buffer effect and the lower pH decrease.

In the light of current knowledge, the temperature, fermentation period, drying conditions, smoking and processing time are decisive factors that affect proteolysis dynamics. In this regard, nitrogen fractions, like NPN and TVBN, and the AA profile may provide important clues regarding proteolytic activity. The NPN is a good example, since it progressively increases during the dry-fermented sausage maturation as a result of this proteolytic activity. The NPN fraction includes the polypeptide and peptide nitrogen content, as well as AA and TVBN.

As a result of the proteolytic activity that takes place during meat product fermentation, total AA increased with the processing time. Yet AA accumulated at a lower rate during product storage, which may be attributed to two different factors: (1) the AA formation has a feedback effect, negatively affecting aminopeptidase activity; (2) AA are further converted into other compounds [11]. Indeed, once in their free form, AA can follow several transformation mechanisms, where deamination and decarboxylation are included.

Among the studied AA, alanine was the most abundant, despite its content remaining globally unchanged throughout processing and storage. In turn, arginine levels doubled when compared to their initial levels. The a_W reduction is known to contribute to the inhibition of the enzymes involved in these AA releases, the alanylaminopeptidases and arginylaminopeptidases [12]. However, arginylaminopeptidase activity seems to be less impacted by lower a_W [12], which might explain the higher arginine accumulation observed throughout processing and product storage. However, various trends have been reported in the literature regarding arginine content during meat product processing [6,13–15], suggesting that multiple factors may be simultaneously affecting the release/metabolism of this AA.

In fact, arginine is closely related to ornithine formation through the arginine deiminase pathway (ADI) [1,16], which can further be converted to putrescine. Likewise, arginine can also be converted to agmatine and later on to putrescine. As such, the amounts of arginine will depend on the balance between these pathways. For example, Virgili et al. [13] observed a negative correlation between arginine and ornithine content. Our results, however, show a positive correlation between these two AA ($r = 0.58$, $p < 0.05$). In Batch A, the ornithine content increased proportionally to that of arginine, with the ratio arginine/ornithine being quite regular, between 2.49 and 2.21 (in the "Control" and "LSF" samples, respectively). A different scenario was found in stored samples from Batch B due to the ornithine decrease during storage, coinciding with a significant increase in putrescine amounts in later storage stages. Leucine and lysine are among those AA that increased the most during processing and product storage, in line with what was observed by Bermúdez et al. [17] and Toldrá et al. [11]. In their work, Toldrá et al. [11] refer to the

fact that muscle aminopeptidases do not have the same degree of preference, depending on which AA is found in the *aminus* terminal. While alanylaminopeptidases show a broad range of specificity, acting over phenylalanine, lysine, methionine, alanine and leucine, arginylaminopeptidase activity is more restricted to AA with a basic nature, like arginine and lysine [11]. The significant leucine and lysine accumulations suggest that arginylaminopeptidase seems to be the most active. Regarding ornithine, its low levels in "RM" samples and higher amounts at the storage end are consistent with the fact that this AA is not natural in meat proteins, its formation being attributed to microbial activity [18].

The BA formation and accumulation depend on a complex interaction of factors like the processing conditions, raw material microbiological quality and handling procedures [19,20]. In this regard, results concerning BA are significantly impacted by the production batch. Amongst "RM" samples, three of them exhibited amines of bacterial origin: β-Phenylethylamine and cadaverine. Even in low amounts, the presence of these amines in dry-fermented sausages is indicative of the presence of bacteria with a decarboxylative capacity, which may trigger the accumulation of significant amounts of exogenous amines in later stages, as was confirmed (especially in Batch B products). As TVBN is often related to microbial development, higher levels observed also contributed to consolidate this conclusion.

Despite the generalized increase in exogenous amines seen during processing, tyramine was the one that stood out in samples from Batch A. In Batch B products, tyramine levels ended up being equaled, or even surpassed, by putrescine levels. Actually, Batch B stood out with the highest total levels of BA. Such a fact is indicative of differences in terms of the existing microbiota present in each batch, particularly for those microorganisms capable of producing the enzymes involved in the production of putrescine (and cadaverine). This is the case of enterobacteria that can be found in raw material and during initial processing stages [21], whose enzymes contribute to the accumulation of putrescine and cadaverine and remain active even in the absence of viable cells during storage.

The presence of tyramine in fermented products is chiefly related to LAB activity. As they are the most active bacteria participating in the fermentation process, it can be expected that this is generally the predominant amine. Similarly to other studies [22–25], tyramine was the prevalent amine in samples from Batch A, associated with lower concentrations of putrescine and cadaverine. In both batches, the initial content of tyramine precursor AA was below 4 mg/100 g DM. However, while in Batch A this value increased during processing, the same was not the case in Batch B, as the tyrosine released would be immediately decarboxylated and converted to tyramine (tyramine increased more rapidly in Batch B than in Batch A). When the two processing technologies are compared, in Batch B the higher temperatures experienced by "Control" dry-fermented sausages seem to have favored tyrosine decarboxylase enzyme activity. In contrast, in Batch A, no differences were found due to processing technology. This discrepancy can be attributed to the strains present in the raw materials, insofar as the production of BA is dependent on the strain and not on the genus of the microorganism, as mentioned by Fernandez et al. [26]. Finally, it is important to highlight that the ability to decarboxylate tyrosine is not exclusive to LAB. In fact, this activity has also been identified in microorganisms from the genera *Enterococcus* and *Staphylococcus* [27]. In this sense, the accumulation of such different amounts of tyramine is admissible, even in the presence of identical LAB counts.

Once BA are produced, they are very difficult to destroy by subsequent processing (pasteurization, cooking, etc.). As a consequence, the most efficient strategies must rely on the prevention side. Apart from the raw material quality and rigorous control of processing/storage conditions, the addition of starter culture or technological additives (like sugars, essential oils and spices) may counteract BA accumulation in fermented foods [28].

In the context of BA formation, the comparison of the two processing methods followed in this study must, necessarily, consider two fundamental assumptions: (1) an antimicrobial effect of LSF and (2) the lower temperatures to which these products are exposed. It is known that wood smoke contributes to the microbiological stability of dry-fermented sausages. Even though Brustolin et al. [29] identified a beneficial effect as an antimicrobial agent in LSF, namely in the control of foodborne pathogens like *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*, the microbial species affected may diverge, and thus different levels of BA may arise. On the other hand, differentiated drying conditions will inevitably affect the dehydration rate, the reduction in water activity and, concomitantly, the microbiological profile and related aminogenic activity. The detection of higher levels of BA in smoked products was demonstrated for both batches. The higher temperatures to which these products are exposed may have contributed to this, as they potentiate the proteolysis (and AA release) as well as the decarboxylase enzyme activity. The enzymes responsible for ornithine, lysine and tyrosine decarboxylation have optimal activities at a temperature of 37 °C [30]. For this reason, even in the presence of greater microbial contamination, the decarboxylative capacity is reduced at lower temperatures, which translates into a lower release of putrescine, cadaverine and tyramine, respectively. In Batch A, the differences between the "Control" and "LSF" samples appeared during storage, while in Batch B they were readily observed in "FP". Thus, when the two technologies are compared, it can be concluded that the exposure of "LSF" dry-fermented sausages to lower temperatures was a decisive factor in minimizing the decarboxylase activity. Yet, the impact of drying technology was strongly conditioned by raw material quality.

The way the BA profile evolved over time is also explained by the observed proteolytic process, as demonstrated by several statistical correlations that have been identified. Concerning putrescine, its formation can occur through several metabolic pathways that take place simultaneously, having glutamine, arginine and agmatine as its AA precursors. Based on Stadnik and Dolatowski [31], glutamine, by conversion into ornithine, represents one of the indirect precursors of this amine. The same was seen in our investigation, where glutamine exhibited a strong negative correlation with putrescine ($r = -0.85$, $p < 0.05$). Concerning the arginine conversion into putrescine, it may occur through two possible metabolic pathways: (1) the ADI pathway, and (2) via arginine decarboxylation to agmatine followed by deamination to putrescine [32]. In fact, the main arginine catabolic pathway performed by LAB is the ADI, which has already been described for *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc* [33], which are frequently found in meat products. The results obtained in this investigation did not allow a direct correlation to be established between ornithine and putrescine; however, a significant correlation between this AA and arginine was observed ($r = 0.58$, $p < 0.05$). It should also be noted that in "FP" samples from Batch A, until the end of storage, there was an almost constant ratio of approximately 2.35 between arginine and ornithine. In Batch B, due to the sharper increase in arginine, this ratio increased progressively, both for "Control" and "LSF" dry-fermented sausages, to 3.61, 4.70 and 6.39 ("FP", "1M" and "3M" samples, respectively). A significant correlation between arginine and putrescine ($r = 0.80$, $p < 0.05$) was also noticed, which may also indicate the relevance of this AA. As agmatine was not one of the studied BA, it is not possible to draw conclusions about the relevance of this pathway in putrescine formation. In addition, spermine and spermidine are interconvertible with each other and the latter into putrescine. Thus, the presence of these two endogenous amines may also contribute for the formation of putrescine in dry-fermented sausages. In his work, Landete et al. [34] found that the production of putrescine in response to spermine and spermidine addition was dependent on the involved microorganism. Our results showed a progressive spermine decrease over time, as opposed to an increase in spermidine, accompanied by an increase

in putrescine concentration. This observation is statistically supported by the correlation found between spermine and spermidine ($r = -0.40$, $p < 0.05$) and the latter with putrescine ($r = 0.90$, $p < 0.05$).

In global terms, the levels of amines from bacterial origin found in our study are comparable to the results that have been reported by several authors, in the most varied traditional meat products, whether produced in Portugal or other countries [3,15,35–39]. From the point of view of consumer safety, there is currently no legislation that determines maximum levels of BA applicable to dry-fermented sausages. Therefore, the levels at which toxicological effects are observed are often considered as a reference, i.e., 100 mg/kg WW for histamine as well as for tyramine, 30 mg/kg WW for β-phenylethylamine and 200 mg/kg WW for vasoactive amines [40]. With the exception of "Control_3M" dry-fermented sausages from Batch A, the presence of vasoactive amines was exclusively due to tyramine and never surpassed the content of 100 mg/kg WW recommended for this BA. A different scenario was observed for Batch B products, where this level was attained already in "FP" or after "1M" of storage ("Control" and "LSF", respectively). Concerning the sum of vasoactive amines, the content of 200 mg/kg was only exceeded in "Control_3M" samples from Batch B.

As endogenous polyamines, spermine and spermidine, are involved in several physiological processes, they occur naturally in meat. Hence, these are usually the most predominant in early processing stages, similarly to what was found in this research. Spermine is more abundant in foods of animal origin, a fact that justifies the observed prevalence over spermidine. Overall, the found levels for spermine and spermidine were slightly higher than those reported by Laranjo et al. [3], Kalač [41], Genccelep et al. [42] and Papavergou et al. [43] but still slightly lower than those found by Roseiro et al. [39]. When compared to their initial levels found in "RM" samples, during the processing stage, spermine levels decreased, while spermidine levels increased. In the opinion of several authors, the levels of these polyamines are not usually affected by processing [41,44,45]. In turn, Durak-Dados et al. [46] also state that spermine content may slightly decrease during processing. However, the levels of these polyamines depend on several factors that are not yet fully explained, namely their use as a source of nitrogen by some microorganisms, but also the conversion of spermine into spermidine [9,47].

5. Conclusions

The use of LSF in dry-fermented sausages was characterized by a tendency towards overall higher pH levels, impacting the extent of the proteolytic process, which turned out to be lower in these products. The higher proteolysis that took place in smoked products proved to be more relevant for arginine and lysine. It was also the smoked samples that showed higher levels of BA contamination, which can be attributed to a convergence of several factors, including greater availability of precursor AA, as well as higher drying temperatures. On the contrary, the reduced temperatures in LSF processing were beneficial in preventing the accumulation of higher amounts of BA in traditional dry-fermented sausages. As it was not possible to confirm the microbiological quality of the raw material, this effect constituted an additional safeguard in preventing the formation of these chemical contaminants.

The processing modification proposed here, such as mixing LSF during seasoning, does not require any further investments from traditional producers, making it an extremely advantageous technology, especially with the benefits already mentioned in terms of reducing polycyclic aromatic hydrocarbons, as well as BA.

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