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Mixed Starter Cultures To Control Biogenic Amine Production in Dry Fermented Sausages

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ABSTRACT

Several combinations of an amine-negative *Lactobacillus sakei* strain, along with proteolytic *Staphylococcus carnosus* or *Staphylococcus xylosus* strains, were used to study the influence of mixed starter cultures on biogenic amine production during the manufacture of dry fermented sausages. Changes in pH, water content, proteolysis, microbial counts, and biogenic amine contents were simultaneously examined in a spontaneously fermented batch and in three mixed starter-mediated batches. A double-controlled microbial charge initially inoculated as mixed starter culture of *L. sakei* and *Staphylococcus* spp. (all amine-negative strains) drastically reduced tyramine, cadaverine, and putrescine accumulation. No production of other aromatic amines such as histamine, phenylethylamine, or tryptamine was observed in any batch. The polyamines, spermine and spermidine, were found in raw materials and their levels decreased slightly in the spontaneously fermented batch. No correlation between proteolysis and biogenic amine production was observed. The use of proper technological conditions favoring starter development and the use of the raw materials with good hygienic quality make it possible to produce fermented sausages nearly free of biogenic amines.

The study of biogenic amines in food is relevant due to their potential toxicological effects to the consumers. The intake of food containing quite large amounts of biogenic amines has been related to several toxic reactions such as histaminic intoxication, food-induced migraines, and hypertensive crisis due to interaction with monoamine-oxidase inhibitor drugs *(10).* The toxic threshold for biogenic amines is difficult to establish because there are several variables. Alcohol and other biogenic amines (such as the diamines, putrescine and cadaverine) have been described as potentiating factors. Furthermore, individual sensitivity, the inactivation of detoxification mechanisms due to genetic deficiencies, gastrointestinal diseases, and monoamine-oxidase inhibitor treatments all influence the expression of toxicological reactions of biogenic amines *(20).* In meat and meat products that contain nitrites and nitrates as curing agents, the formation of carcinogenic nitrosamines could be an additional toxicological risk of biogenic amines *(24).*

Large amounts of biogenic amines are found in fermented foods, such as dry fermented sausages, and also in aged or spoiled food products *(10, 14, 23).* The main cause of biogenic amines in food is the microbial metabolism through the decarboxylation of their precursor amino acids *(10).* The active growth of several microbial populations, acidification and the proteolysis during dry sausage fermentation, make the environment particularly favorable to biogenic amine formation. Pseudomonads, enterobacteria, enterococci, and lactobacilli have been related to biogenic amine production in meat and meat products *(6, 14).* The hygienic quality of raw materials constitutes one of the most important critical control points to reduce biogenic amine production *(8, 19, 21).* The addition of selected starter cultures is recommended to prevent excessive amine accumulation. However, several studies have failed to demonstrate that some starter cultures reduce amine production during sausage fermentation *(3, 11, 21).* A wide variation in the biogenic amine profile and in their concentrations is observed in fermented sausages from the retail market *(9, 10, 16).* Such variation indicates the existence of numerous factors affecting biogenic amine production before, during, and after processing of such food products. Some samples, though only a few, from previous studies of retail fermented sausages showed extremely low amounts of biogenic amines *(9, 16).* Therefore, once the main factors that affect amine production during meat fermentation are established, it will be possible to produce, on an industrial scale, dry fermented sausages with low levels of biogenic amines.

The main bacterial populations responsible for meat fermentation are the lactic acid bacteria (LAB) and the group of gram-positive catalase-positive cocci, including micrococci and staphylococci *(18).* Metabolic activity of these microbial groups is essential for the desirable changes determining the typical attributes of dry fermented sausages. LAB, especially those originating from meat (e.g., *Lactobacillus sakei* and *Lactobacillus curvatus*), are well adapted to meat fermentation environments and are involved in all the changes occurring during ripening. Lactic acid production by LAB lowers the pH and has a preservative effect. Acidification also facilitates the drying process, the development of the typical curing color, and the cohesion of the sausages. Micrococci and staphylococci also contribute to sausage ripening by enhancing the development of

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TABLE 1. *Microbial composition of mixed starter cultures used for fermented sausage manufacture (inoculation level of 10^6 CFU/g for each strain)*

Batch	Lactobacilli strain[a]	Staphylococci strain[b]
NS	No starter	No starter
SC	*L. sakei* CTC494	*S. carnosus* LTH2102
SX-1	*L. sakei* CTC494	*S. xylosus* CTC3037
SX-2	*L. sakei* CTC494	*S. xylosus* CTC3050

[a] *L. sakei* strain was previously found as an amine-negative producer and used as a single starter culture in Bover-Cid et al. *(5).*

[b] Strains of proteolytic staphylococci are amine-negative producing bacteria and were used as single starter cultures in Bover-Cid et al. *(7).*

the characteristic flavor and color through their proteolytic and lipolytic activities. Catalase production by micrococci and staphylococci protects against color changes and rancidity, while nitrate and nitrite reductase aid reddening and reduce residual nitrite content *(13).* Starter cultures are applied to improve and stabilize the quality of the final product and to shorten the ripening period. As starter organisms should not negatively affect the product, their capacity for producing biogenic amines also needs to be assessed *(4).* The usual bacterial composition of starters consists of one (single starters) or several (mixed starters) strains of LAB (mainly lactobacilli) combined with micrococci or staphylococci strains *(18).* The technological conditions of sausage manufacture and interactions between starters and other microorganisms present in the meat mixture affect their development. The capacity of bacterial populations to produce biogenic amines and the amounts of precursor amino acids resulting from proteolysis may also be affected by such processing conditions *(4).*

In the present study, the influence of mixed starter cultures of lactobacilli and staphylococci (all amine-negative strains) on biogenic amine production during the fermentation of dry sausages was studied. These sausages were compared with sausages obtained from the same raw materials, under the same processing conditions, but spontaneously fermented without inoculation of starter.

MATERIALS AND METHODS

Fermented sausages. The meat product used was fuet, a typical small-diameter dry sausage from Catalonia (Spain), made of pork meat and fat, curing salts, pepper, sugar, and other additives. Fuet production consists of mincing the meat raw materials, mixing with other ingredients and additives, and stuffing the mixture into artificial or natural casings. A short fermentation and ripening process (from 15 to 25 days) at relatively low temperatures (between 14 and 20°C), with mold development on the surface, results in a slightly acidified fermented sausage with a characteristic flavor. Dry fermented sausages were manufactured in the pilot-plant of the Meat Technology Center (CTC-IRTA, Monells, Girona, Spain) and the Food Microbiology and Biotechnology Unit of the same center provided the starter cultures. Microbial composition of starter cultures used in the manufacture of fermented sausages is summarized in Table 1. Shoulder pork and backfat (80:20) initial mixture was ground to 6-mm particle size and divided in four batches that were mixed with the following ingredients: 2.2% sodium chloride, 2.0% lactose, 0.3% black pepper, 0.1% dextrose, 0.05% sodium ascorbate, 0.03% potassium nitrate, and 0.01% sodium nitrite (Sanofi Bio-Industries, Barcelona, Spain). Starter cultures, dissolved in 500 ml of cool water, were added to achieve a final concentration of 10^6 CFU/g sausage for each bacterial strain. Each mixture was homogenized and stuffed into natural pork casings (~250 to 300 g/sausage and diameter <4 cm). Sausages were embedded in a mold spore suspension of *Penicillium candidum* (Rhône-Poulenc Texel SA, Dangé Saint Roman, France). Ripening and drying was conducted at 14 to 16°C and 70 to 80% relative humidity for 24 days.

Sampling. Duplicate samples of raw meat mixture just before stuffing (time zero) and three sausages from each batch were sampled at different times throughout the ripening process (days 3, 9, 17, and 24). Values of pH, water content, nonprotein nitrogen (NPN), free amino nitrogen, and biogenic amine contents were measured in triplicate at each sampling point. Bacteriological assessment (counts of LAB, coagulase-negative staphylococci, enterococci, and *Enterobacteriaceae*) was performed at time zero and in the finished products (after 24 days of ripening).

Analyses. pH was measured by insertion of the electrode of a microcomputerized pH meter (Crison 507, Spain) into the sausage.

Water content was determined by drying the sample at 100 to 105°C until constant weight was reached.

NPN was determined by the Kjeldahl method. The NPN fraction was previously extracted from 5 to 10 g of sausage with 0.6 N perchloric acid *(12).*

Total free amino acids were assessed as α-amino nitrogen (AAN) according to the Sörensen method by titration with formaldehyde *(2).*

Biogenic amines were extracted from 5 to 10 g of sample with 0.6 N perchloric acid and determined spectrofluorometrically as *ortho*-phthalaldehyde derivatives by ion-pair high-performance liquid chromatography *(17).*

Microbial analysis was conducted by cutting 10 to 15 g of sausage without casing into small pieces and placing this into a sterile Stomacher bag. Sterile diluent, 0.1% of bactopeptone (Difco, Detroit, Mich.) and 0.85% of NaCl (Merck, Darmstadt, Germany) in deionized water, was added in a proportion of 1:9, and the mixture was homogenized for 2 min using a Stomacher (Lab Blander, Seward, London, UK). Serials of decimal dilutions were prepared with the same diluent. LAB were enumerated on de Man, Rogosa, and Sharpe (MRS) medium (Merck) incubated at 30°C for 48 h anaerobically; coagulase-negative staphylococci on mannitol salt agar (Difco) at 30°C for 48 h; enterococci on kanamicin aesculin azide agar (Oxoid-Unipath, Madrid, Spain) at 37°C for 48 h; and *Enterobacteriaceae* on violet red bile dextrose agar (Merck) incubated with a double layer at 30°C for 24 h.

Statistics. Statistical analyses were performed using the SPSS 9.0 for Windows software (SPSS Inc., Chicago, Ill.). Because values were not normally nor symmetrically distributed, the nonparametric Mann-Whitney *U* test was used to examine the statistical significance of the changes throughout the fermentation and of the differences between batches.

RESULTS AND DISCUSSION

Changes in water content, pH, and proteolysis parameters. Moisture and pH evolution during ripening of fermented sausages are summarized in Table 2. A similar loss of water occurred in all four batches as a result of the

TABLE 2. *Changes in mean values (and standard deviations) of water content and pH during ripening of the four batches of dry fermented sausages with different starter cultures*[a]

	Water content (%)				pH			
Day	NS	SC	SX-1	SX-2	NS	SC	SX-1	SX-2
0	65.57 (0.25)	65.38 (0.00)	65.86 (0.00)	65.91 (0.00)	5.99 (0.00)	5.96 (0.00)	5.97 (0.01)	5.69 (0.01)
3	62.75 (0.50)	62.95 (0.44)	62.56 (0.78)	63.71 (0.52)	5.99 (0.02)	5.71 (0.03)	6.30 (0.58)	5.65 (0.01)
9	50.47 (0.48)	52.27 (0.97)	53.58 (1.28)	52.11 (1.22)	6.01 (0.02)	5.35 (0.02)	5.44 (0.01)	5.33 (0.03)
17	38.30 (2.01)	39.36 (1.32)	37.94 (2.48)	37.77 (2.20)	6.10 (0.10)	5.51 (0.04)	5.57 (0.12)	5.49 (0.07)
24	32.36 A (2.72)	33.04 A (1.53)	32.27 A (1.57)	32.73 A (0.65)	6.30 A (0.02)	5.62 B (0.02)	5.69 C (0.03)	5.63 B (0.11)

[a] NS, spontaneously fermented without starter culture; SC, starter-mediated fermentation with *L. sakei* CTC494 and *S. carnosus* LTH2102; SX-1, starter-mediated fermentation with *L. sakei* CTC494 and *S. xylosus* CTC3037; SX-2, starter-mediated fermentation with *L. sakei* CTC494 and *S. xylosus* CTC3050. Different letters in the same row indicate statistically significant differences ($P < 0.05$) between batches.

drying process. A drop in pH occurred in batches manufactured with starters due to lactic acid production by the *L. sakei* strain, while a slight increase in pH was observed during ripening of spontaneously fermented sausages. Values of pH in sausages ripened at low temperature (<20°C) may eventually rise and reach similar values to those of the raw meat mixture. This may be caused by proteolytic processes and mold growth on the sausage surface *(5, 7)*. However, the low water activity (<0.80) of such products makes them safe for consumption.

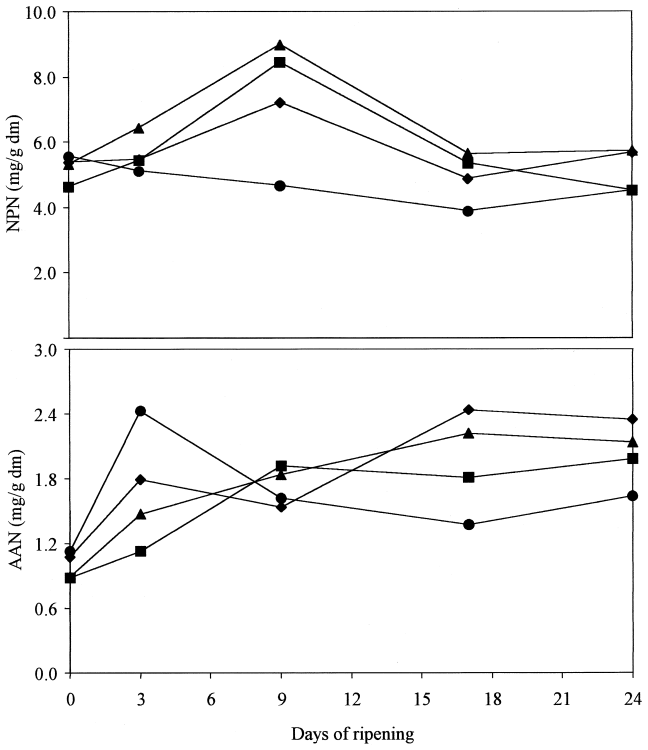

FIGURE 1. *Changes in proteolytic-related parameters NPN and AAN during ripening of spontaneously fermented sausages (●, NS) and sausages fermented through a mixed starter culture of* L. sakei *plus* S. carnosus *(■, SC) or* S. xylosus *(▲, SX-1 and ♦, SX-2).*

Changes in proteolysis-related parameters are outlined in Figure 1. Proteolytic processes during ripening of fermented sausages were influenced by the inoculation of starter cultures. Throughout the process, batches fermented with starters showed higher ($P < 0.05$) values of NPN than the spontaneously fermented batch, whose NPN content did not significantly vary during ripening. Total free AAN also followed different profiles depending on the use of the starter ($P < 0.05$). Spontaneous fermentation resulted in a rise of AAN during the first days followed by a decrease, leading to similar AAN contents at the end of the ripening as at the beginning. In contrast, starter-mediated fermentation resulted in a gradual increase of AAN throughout the whole ripening. *Staphylococcus xylosus* strains yielded higher total free amino acids than *Staphylococcus carnosus*, though the differences were not statistically significant. Larger differences in proteolysis-related parameters were detected among batches in a previous study *(7)* in which all three staphylococci (*S. carnosus* and both *S. xylosus* strains) were applied as single starter cultures. Interactions with the *L. sakei* strain as well as differences in the sausage formulation and ripening program may explain the different results obtained.

Bacteriological results. Microbial counts (Table 3) of samples from the sausage mixture (time zero) reflected the inoculation of the mixed starter culture. Both LAB and staphylococci numbers in batches with starter were approximately two logarithmic units higher than in the control batch (NS). Relatively low levels of enterococci and *Enterobacteriaceae* were detected in samples of all four batches at the beginning of the fermentation, which indicate the good hygienic quality of the raw materials. After 24 days of ripening, spontaneously fermented sausages (NS) showed high counts of coagulase-negative staphylococci, higher than counts of LAB. In the starter-mediated batches,

TABLE 3. *Microbial counts, mean of log CFU/g (and standard deviation) in meat mixture (time zero) and final fermented sausages (day 24) of the four batches manufactured with different starter cultures*[a]

Day	LAB				Coagulase-negative staphylococci				Enterococci				Enterobacteria			
	NS	SC	SX-1	SX-2	NS	SC	SX-1	SX-2	NS	SC	SX-1	SX-2	NS	SC	SX-1	SX-2
0	3.99 (0.05)	6.03 (0.01)	6.09 (0.10)	6.05 (0.06)	3.93 (0.07)	5.45 (0.13)	5.96 (0.26)	5.82 (0.05)	0.85 (0.21)	1.90 (0.08)	2.37 (0.04)	2.18 (0.10)	2.54 (0.23)	2.62 (0.06)	2.92 (0.06)	2.71 (0.03)
24	7.06 A (0.29)	9.19 B (0.21)	8.93 B (0.09)	8.98 BC (0.07)	8.32 A (0.33)	7.75 A (0.26)	8.03 B (0.21)	8.00 B (0.37)	4.65 A (0.10)	2.66 B (0.10)	2.66 B (0.22)	2.56 B (0.31)	3.30 (2.86)	<1.00 —	<1.00 —	<1.00 —

[a] Different letters in the same row indicate statistically significant differences ($P < 0.05$) between batches.

LAB achieved higher numbers than coagulase-negative staphylococci. Several authors reported the prevalence of LAB in dry fermented sausage ripening in spite of the inoculation of starter containing staphylococci or micrococci *(7, 15, 19).* Other authors, however, observed higher levels of coagulase-negative staphylococci than those of LAB at the end of the ripening process *(1).* The large development of coagulase-negative staphylococci and their proteolytic activity might have contributed to the unusually high pH found in spontaneously fermented sausages. Enterococci and *Enterobacteriaceae* counts increased considerably in the NS fermented sausages. A smaller numbers of enterococci were found in the starter-mediated batches, and their final counts were lower than in the NS batch. *Enterobacteriaceae* numbers dropped below 1 logarithmic unit in sausages of all three starter-mediated batches.

Changes in biogenic amine contents during ripening of spontaneously fermented sausages (batch NS). A sharp rise in biogenic amine production occurred between days 3 and 9 of the ripening process (Fig. 2). In noninoculated sausages (batch NS), tyramine was the main amine formed followed by cadaverine and, to a much lesser extent, putrescine. These are the three main biogenic amines commonly found in fermented sausages, tyramine usually being the major amine in amounts, followed by the diamines (either putrescine or cadaverine). In this batch, tyramine could be related to tyrosine-decarboxylase activity of wild lactic fermenting flora (lactobacilli and enterococci). Cadaverine is more often associated with *Enterobacteriaceae,* while putrescine seems to be the result of both microbial groups *(4, 6, 14).*

No histamine was produced during the ripening. The initial low levels of 0.5 mg/kg dry matter even decreased during ripening and end products showed 0.3 mg/kg dry matter. Although concentrations of up to 150 mg/kg of histamine can be found in certain retail fermented sausages and histamine formation during ripening was reported by some authors *(21),* no significant accumulation of histamine during the ripening process was observed in the present study or in other previous studies *(5, 7, 9, 15).* Phenylethylamine and tryptamine are other biogenic amines that may occasionally appear, usually at low levels, during the second part of the fermentation process. However, in the present study these minor amines remained nondetectable throughout ripening. The good hygienic quality of raw materials and proper and controlled hygienic conditions throughout sausage manufacture seem to have been critical for the lack of formation of histamine, phenylethylamine, and tryptamine during the ripening of sausages. On the other hand, the polyamines spermine and spermidine were found in the raw meat mixture at fairly high levels (Table 4), because they are of natural origin. Polyamine levels fluctuated during ripening and ended up slightly lower than in the raw meat mixture.

Biogenic amine contents in dry sausages generally vary greatly among different types of fermented meat products, manufacturers, batches, and even between different parts of a sausage due to the numerous factors that affect

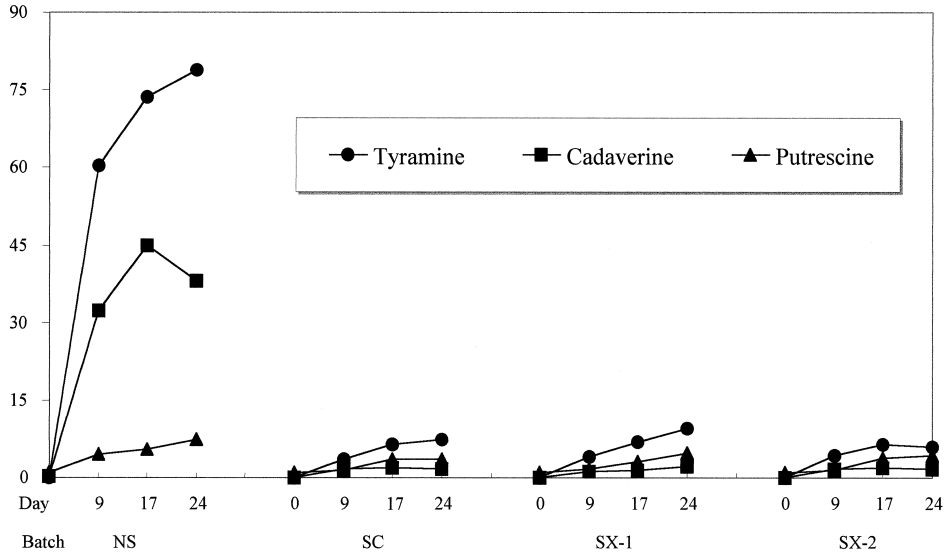

FIGURE 2. *Changes in biogenic amine contents (mg/kg dry matter) during ripening of spontaneously fermented sausages (NS) and sausages fermented through a mixed starter culture of* L. sakei *plus* S. carnosus *(SC), or* S. xylosus *(SX-1 and SX-2).*

amine production *(9, 23).* Indeed, considerable variation has been detected depending on the raw material batch used *(8, 19, 21).* However, both the profile and the concentrations of biogenic amines found in this trial were comparable with amines produced in other trials of sausages manufactured in the same pilot plant *(5, 7).* These results strongly suggest that adventitious microbial flora from the processing plant and machines significantly contributes to biogenic amine production during dry sausage fermentation, especially when raw materials used show good hygienic quality with a low microbial charge.

Influence of amine-negative mixed starter cultures on biogenic amine accumulation during sausage ripening. The combinations of two amine-negative bacteria, *L. sakei* plus *S. carnosus* (SC) or *S. xylosus* (SX-1 and SX-2), resulted in a drastic reduction of biogenic amine production in all three batches (Fig. 2). Tyramine and cadaverine accumulation was significantly ($P < 0.05$) inhibited by 90 to 95% in starter-mediated sausages compared to the spontaneously fermented sausages (NS). The low amount of putrescine in the starter-mediated batches was half that in the spontaneously fermented sausages. Therefore, the starter bacteria inoculated did not change their amine-negative character during sausage fermentation. Neither the proteolytic activity of staphylococci strains induced to higher biogenic amine production during the ripening process. Moreover, starters were able to outgrow the usually amine-positive wild microbial flora (e.g., adventitious LAB and *Enterobacteriaceae*) reducing the production of biogenic amines during ripening. Histamine contents slightly diminished to levels below 0.2 mg/kg, whereas phenylethylamine and tryptamine remained nondetectable, as in the spontaneously fermented batch (NS). The physiological polyamines, spermine and spermidine, fluctuated during the ripening, but final contents did not differ from the initial ones. Similar behavior was observed for the three starter-mediated batches (Table 4). Spermine increased slightly during the first 9 days followed by a decrease. The final levels of

TABLE 4. *Changes in mean values (and standard deviations) of polyamines spermine and spermidine contents (mg/kg dry matter) during ripening of the four batches of dry fermented sausages with different starter cultures*[a]

	Spermine				Spermidine			
Day	NS	SC	SX-1	SX-2	NS	SC	SX-1	SX-2
0	64.08 (0.61)	59.60 (2.84)	62.06 (0.23)	64.11 (3.13)	7.41 (0.27)	6.63 (0.16)	6.69 (0.10)	6.98 (0.51)
3	77.92 (7.25)	85.90 (2.87)	84.04 (5.40)	79.97 (10.37)	7.74 (0.41)	7.17 (0.15)	6.87 (0.03)	6.47 (0.81)
9	75.36 (6.25)	72.20 (6.83)	72.03 (2.43)	72.64 (1.60)	8.05 (0.83)	6.64 (0.11)	6.39 (0.18)	6.48 (0.50)
17	45.37 (3.44)	61.85 (6.61)	67.13 (4.11)	66.71 (4.40)	6.82 (0.32)	7.25 (0.24)	6.57 (1.10)	6.80 (0.26)
24	48.45 A (9.35)	61.17 AB (8.77)	67.43 B (5.33)	68.49 B (1.37)	6.89 A (0.93)	7.13 A (0.49)	7.51 A (0.40)	7.27 A (0.45)

[a] Different letters in the same row indicate statistically significant differences ($P < 0.05$) between batches.

spermine were higher in the starter-mediated batches than in the NS batch. The changes in spermidine content showed differences between the NS batch and the starter-mediated sausages, though final levels were not significantly different.

Different results have been reported about the influence of various microorganisms as single or mixed starter cultures on biogenic amine production during the manufacture of dry fermented sausages. Rice and Koehler *(22)* showed that the use of a single starter of a *Pediococcus acidilactici* strain did not cause lower tyramine levels than spontaneous fermentation. *Lactobacillus plantarum* was also tested and did not reduce biogenic amines during sausage manufacture *(11, 22).* Neither Bauer et al. *(3)* nor Paulsen and Bauer *(21)* found any influence on amine production during sausage fermentation inoculated with several combinations of lactobacilli and staphylococci/micrococci strains. However, Hernández-Jover et al. *(15)* reported a slight reduction of tyramine and cadaverine but not of putrescine production during ripening of sausages with mixed starter consisting of *Micrococcus carnosus* plus *L. plantarum* and *M. carnosus* plus *Pediococcus pentosaceus.* Likewise, Maijala et al. *(19)* found a significant decrease in tyramine, cadaverine, and histamine levels during ripening of sausages with an amine-negative mixed starter culture (staphylococci plus lactobacilli). Each of the starter microorganisms used in the present work had already been tested as single starter cultures in previous studies. All three staphylococci strains (*S. carnosus* and both *S. xylosus*) applied as single starter cultures could significantly reduce tyramine production, but the accumulation of the diamines, cadaverine and putrescine, was not affected by the addition of starter cultures *(7).* In contrast, the single addition of the *L. sakei* strain as a second-generation starter *(18)* resulted in a very competitive starter culture being able to reduce by approximately 80 to 90% the production of all biogenic amines *(5).* In the present study, the use of a double controlled and amine-negative microbial charge through a mixed starter culture resulted in an even more effective reduction of amine accumulation.

Mixed starter cultures provided the advantages of both bacterial groups, ensuring not only preservation but also the development of the typical flavor, texture, and color of the dry fermented sausages, with no significant production of biogenic amines. Nevertheless, the importance of using raw material of good hygienic quality and proper technological conditions that favor starter development needs to be highlighted, if sausages nearly free of biogenic amines are to be produced.

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