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Use of *Staphylococcus xylosus* as a starter culture in dried sausages: effect on the biogenic amine content

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Abstract

The main objective of this work was to investigate if the use of *Staphylococcus xylosus* S81 as a starter culture in sausage production can influence the amount of histamine during ripening, and the concentration of the other most important biogenic amines, by influencing the activity of the microbial amino oxidases as well as the aminoacid decarboxylases. The results confirm that the biogenic amines presence in foods is the consequence of a complex equilibrium between the composition of the medium and the enzymatic activities of the microbial population. In addition, the results suggest that the presence and relative activity of amino oxidases should be considered as an important characteristic in the selection of starter cultures used in the production of fermented foods. © 2002 Published by Elsevier Science Ltd.

Keywords: Biogenic amines; Amine oxidases; *Staphylococcus xylosus*; Sausages

1. Introduction

Biogenic amines (BA) in foods are mainly formed by microorganisms able to decarboxylate aminoacids. The amount of BA can be high in spoiled food products and also in fermented products derived from raw materials with an high protein content, such as sausages and cheeses, the microbial growth often leads to high concentration of BA (Silla Santos, 1996). The production of BA is a characteristic of several groups of microorganisms such as Enterobacteriaceae, *Pseudomonas* spp., Micrococcaceae, enterococci and lactic acid bacteria (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994)

Due to its pharmacological activity, histamine is probably the most important biogenic amine, and can be involved in allergies and inflammatory processes (Anderson, Hasan, McCrodden, & Tipon, 1993). In sausages, histamine is often only detected at low level (Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 1999; Masson, Talon, & Montel, 1996), but can be occasionally produced in high amounts (Parente, Martuscelli, Gardini, Grieco, Crudele, & Suzzi, 2001). Given that histamine production increases in the poor growth conditions (i.e. the absence of fermentable substrates), it has been suggested that histidine decarboxylation can be an additional pathway for energy generation (Konings, Lolkema, Bolhuis, Van Veen, Poolman, & Driessen, 1997). The presence of this amine in foods, particularly if associated with tyramine or other BA, can be of health concern especially for consumers with reduced monoamine oxidase (MAO) or diamine oxidase (DAO) activity, due, for example, to medical treatment. In fact, BA are physiologically inactivated by amine oxidases, which are enzymes found in bacteria, fungi, plant and animal cells and are able to catalyse the oxidative deamination of amines with the production of aldehydes, hydrogen peroxide and ammonia (Cooper, 1997). In bacteria the presence of these enzymes allows them to use substituted amines as carbon and nitrogen sources. In fact, the sequential action (in the presence of an electron acceptor such as O_2) of an amine oxidase and an aldehyde dehydrogenase leads to the production of

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an acid and ammonia, which can be used to support microbial growth (Parrot, Jones, & Cooper, 1987).

MAO and DAO activity has been described in higher organisms as well as in bacteria (Ishizuka, Horinouchi, & Beppu, 1993; Murooka, Doi, & Harada, 1979; Murooka, Higaschiura, & Harada, 1976). Differences can be seen between microbial amine oxidases in terms of substrate specificity and location, as stated by Cooper (1997).

Due to the health risks associated with high concentrations of BA, studies have been carried out on the potential of microorganisms involved in food fermentations and exhibiting amine oxidase activity, to prevent or reduce the accumulation of these undesirable compounds (Leuschner & Hammes, 1998).

Leuschner, Heidel, and Hammes (1998) tested in vitro potential amine degradation by many bacteria isolated from foods and, in particular, in strains belonging to the genera *Lactobacillus, Pediococcus, Micrococcus,* as well as to the species *Staphylococcus carnosus* and *Brevibacterium linens,* and found that this enzymatic activity can be present at very different levels. The tyramine oxidase activity of the microbial strains tested was dependent on pH (with an optimum at 7.0), temperature and NaC1, as well as glucose and hydralazine concentration. Moreover, this enzyme was characterised by an higher potential activity under aerobic compared with anaerobic conditions.

In a previous study carried out by Martuscelli, Crudele, Gardini, and Suzzi (2000), many strains of *Staphylococcus xylosus,* isolated from artisanal fermented sausages in Southern Italy, had the ability to degrade BA in vitro. Among the strains tested, *S. xylosus* S81 showed the highest ability to oxidise histamine and was able to degrade, a part of tyramine.

The main objective of this work was to investigate if the use of *S. xylosus* S81 as a starter culture in sausage production can influence the amount of histamine produced during ripening as well as the concentration of the other most important BA by influencing the activity of the microbial amino oxidases and the aminoacid decarboxylases.

2. Materials and methods

2.1. Starter cultures

The strains used in this study were *Lactobacillus sakei* G20 and *S. xylosus* S81, belonging to the culture collection of Department of Biologia, Difesa e Biotecnologie Agroforestali of the University of Basilicata. The two strains were previously isolated from fermented sausages of Southern Italy. *L. sakei* G20 was grown in MRS medium (Oxoid, Basingstoke, UK) and *S. xylosus* S81 in BHI medium (Oxoid) incubated overnight at 30 and 37 °C, respectively. The starter cultures for raw sausages were harvested after centrifugation and resuspended in lactose 7.5% (w/w) solution.

2.2. Sausage preparation

Sausages were produced in a semi-industrial plant in Potenza (Italy) using the traditional technology adopted for manufacturing Soppressa Lucana, a typical sausage of the Lucania Region (Southern Italy). Nine kilograms of chilled (4 °C) pork meat, fillet and ham (95%) and fat (5%) were used for sausage manufacture. The minced meat was mixed with NaCI (2.2% w/w), L-ascorbic acid (0.06%), $NaNO_2$ (0.01%), and KNO_3 (0.01%), milk powder (0.6%), dextrose (0.6%) and black pepper (0.4%) were also added. The mixture obtained was divided into three batches: in batch A starter cultures and histamine were not added; batch B was characterised by the addition of histamine (150 mg kg^{-1}) in the absence of starter cultures; for the preparation of the batch C both histamine (150 mg kg^{-1}) and starter cultures (*L. sakei* G20 and *S. xylosus* S81) were added before casing. Natural casings were filled with the mixture to obtain sausages of 50 mm diameter and a weight of about 300–350 g. The sausages were then transferred to a controlled environment room for drying and ripening.

2.3. Sampling

Samples for chemico-physical and microbiological analyses were taken immediately after casing and during ripening after 1, 2, 5, 8, 15 and 21 days.

2.4. Microbiological analyses

For the microbiological analysis of each sample, 10 g of sausage were diluted with 90 ml of sterile peptone water and homogenised with a Stomacher Lab Blender (Seward Medicals, London, UK).

Lactic acid bacteria were enumerated by pour plating appropriate dilutions onto MRS agar (Oxoid, Basingstoke, UK) with cycloheximide (100 mg 1^{-1}) and bromochresol purple (160 mg 1^{-1}; Sigma, Bellefonte, USA) added followed by incubation at 30 °C for 48 h. Microstaphylococci were enumerated on Baird Parker Medium (Oxoid) supplemented with Egg Yolk Tellurite and incubated at 37 °C for 36 h. *Enterobacteriaceae* were enumerated by pour plating 1 ml of an appropriate dilution onto Violet Red Bile Glucose Agar (Oxoid) incubated at 37 °C for 24 h. Three replicates were carried out for each microbiological count.

2.5. pH, a_w and biogenic amines determinations

The determination of pH was performed for each sample by inserting a spear tip electrode of 3 mm

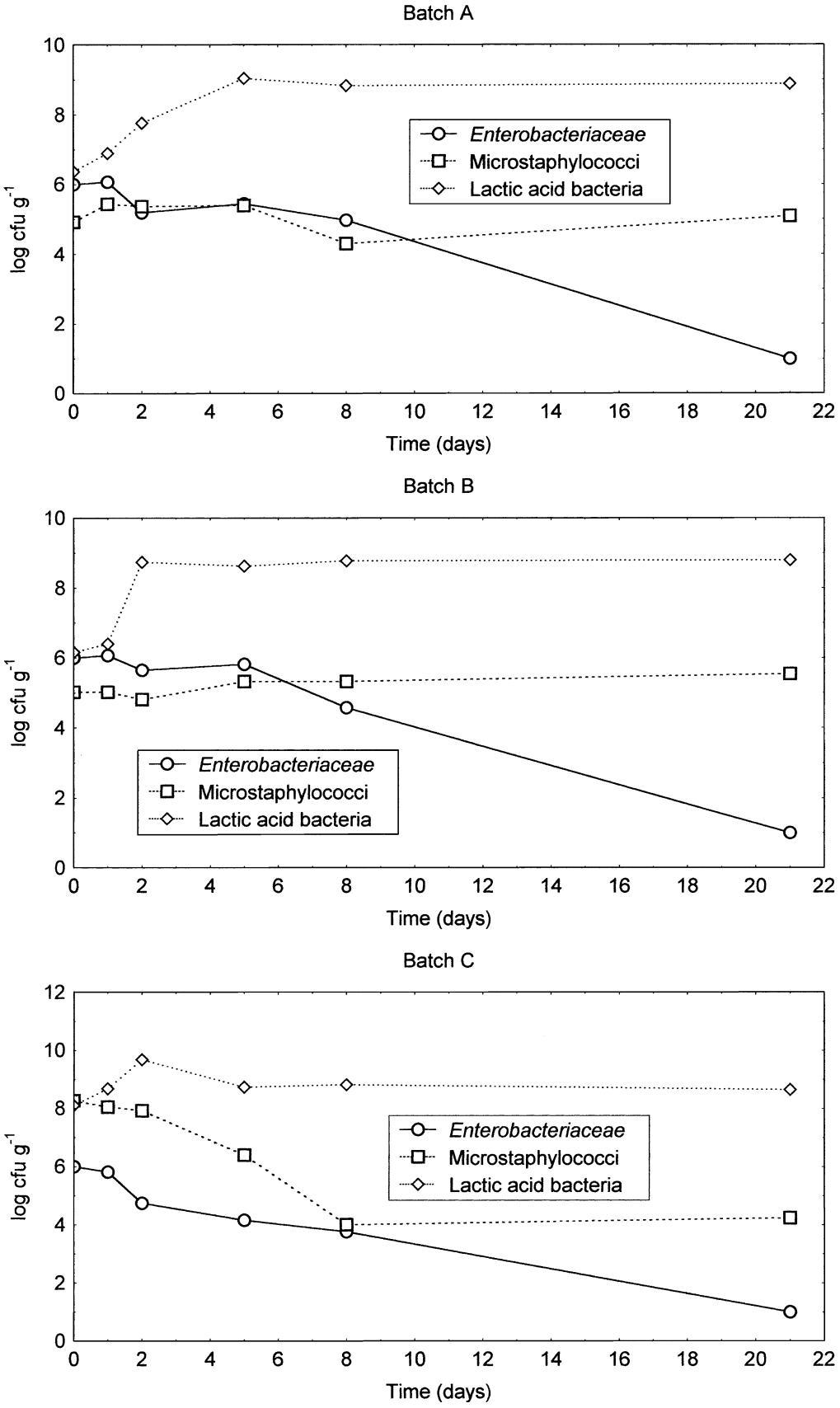

Fig. 1. Microbial counts monitored during ripening in the three batches of sausages: batch A (control), batch B (+ histamine) and batch C (+ histamine and starter cultures).

diameter (Orion Research, Beverly, USA), connected to a pH-meter model 8517 (Hanna Instruments, Padova, Italy), in three different positions of each sausage and the result expressed as the mean of the three determinations.

Water activity was measured on three replicated samples at 25 °C using a Rotronic Hygroskop BT (International PBI, Milan, Italy).

Biogenic amines were determined in the homogenised samples by HPLC, following the method proposed by Moret and Conte (1996), after amine derivatisation (Eerola, Hinnkkalnenn, Linfors, & Hirvi, 1993)

3. Results and discussion

3.1. Microbial growth and physico-chemical parameters

Using the same raw meat, three batches of sausages were prepared to evaluate if the presence of histamine can change BA production during ripening and to exploit the potential of *S. xylosus* S81 to influence the BA balance in fermented meat products. In particular, batch A was prepared without starter cultures and histamine. Batch B was manufactured with the addition of histamine (150 mg kg^{-1}) but without starter cultures. Finally, both starter cultures (*S. xylosus* S81 and *L. sakei* G20) and histamine (150 mg kg^{-1}) were added during the preparation of the sausages in the batch C. *L. sakei* G20 was added as a starter culture to ensure the traditional characteristics to the sausages. This strain is known to have no aminoacid decarboxylase activity and, consequently, is not able to form any of the BA monitored in this work.

The microbiological results are shown in Fig. 1. In all the three batches, *Enterobacteriaceae* counts were characterised by high initial concentrations which significantly decreased during ripening, independently on the presence of starter cultures. At the beginning of fermentation, the number of lactic acid bacteria was obviously higher if *L. sakei* G20 was added as a starter culture. This microbial group rapidly increased after casing and reached values of about 10^9 cfu g^{-1} in all the sausages, even in the samples to which starter cultures were not added. These high values remained relatively constant during ripening.

By contrast, in the absence of starter cultures, microstaphylococci slightly increased their number in the first days of fermentation in the batches A and B, reaching final concentrations of about 10^5 cfu g^{-1}. In the batch C, due to the high inoculum level, microstaphylococci were present at an initial concentration of about 10^8 cfu g^{-1} and rapidly decreased during ripening to reach a concentration of about 10^5 cfu g^{-1} after 8 days. A similar evolution of microstaphylococcal population in sausages was described by Leuschner and Hammes (1998).

Fig. 2 shows the changes, during ripening, of the pH values which did not vary greatly between batches, although, as expected, the drop in the first days of fermentation was more rapid and pronounced in the samples with added starter cultures. The a_w values during ripening were not significantly different in the three batches.

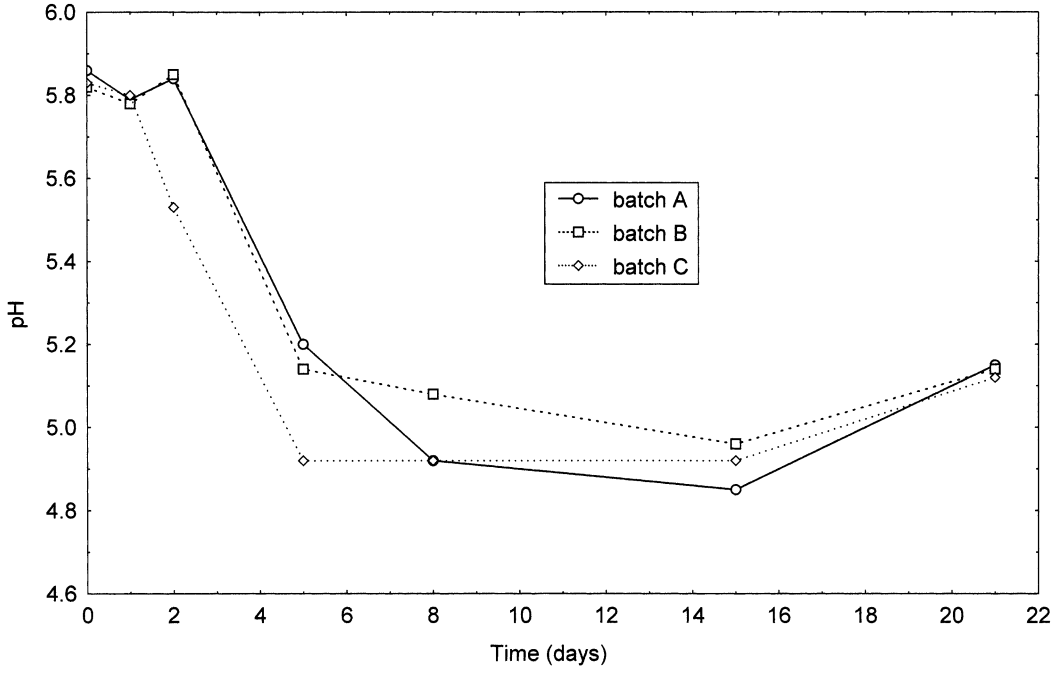

Fig. 2. pH values of sausages during ripening in the three batches: batch A (control), batch B (+ histamine) and batch C (+ histamine and starter cultures).

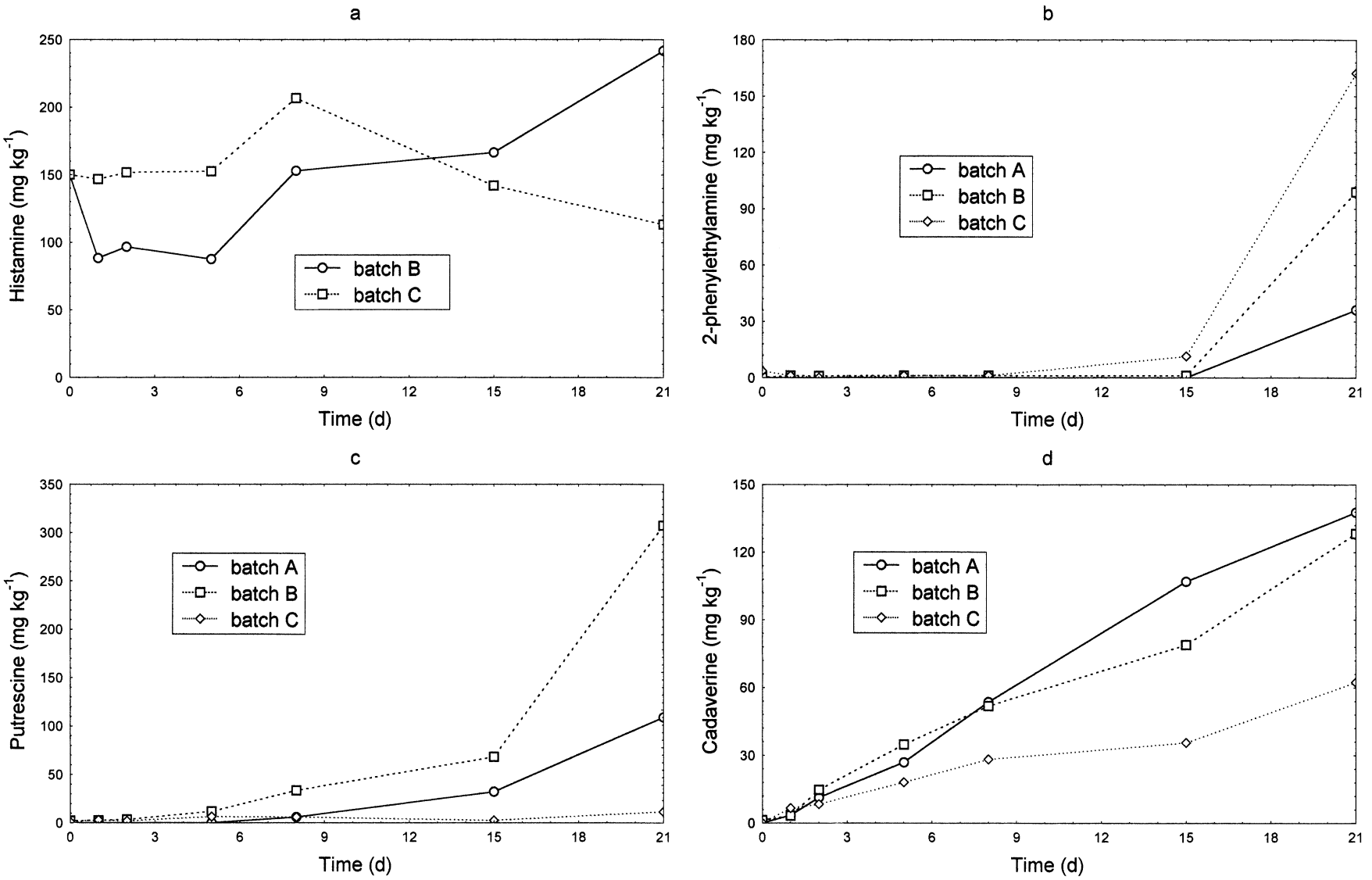

Fig. 3. The concentration of biogenic amines in sausages during ripening: (a) histamine, (b) 2-phenylethylamine, (c) putrescine and (d) cadaverine. The concentrations are relative to batch A (control), batch B (+ histamine) and batch C (+ histamine and starter cultures). No histamine was detected in batch A.

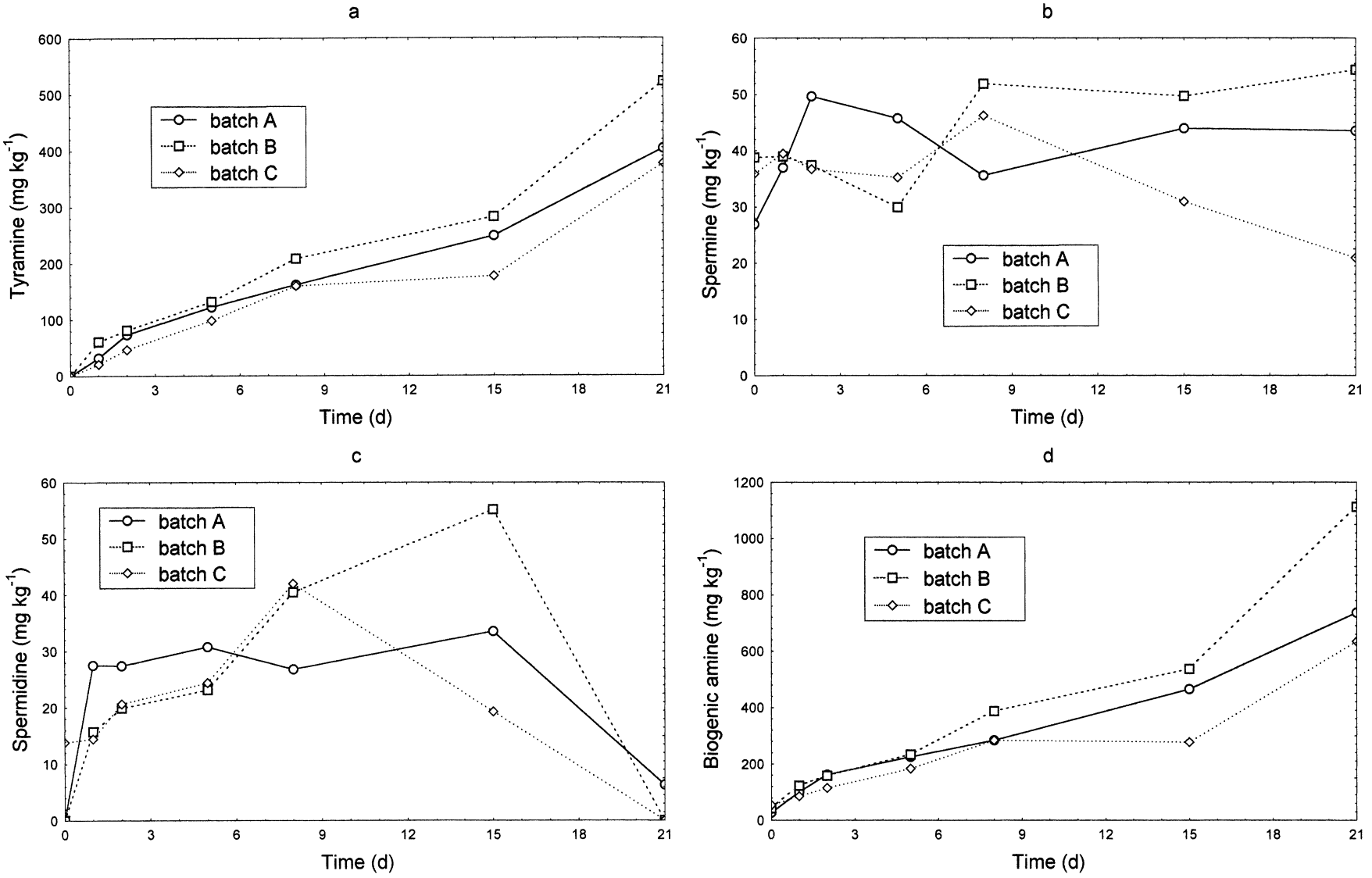

Fig. 4. The concentration of biogenic amines in sausages during ripening: (a) tyramine, (b) spermine, (c) spermidine and (d) total biogenic amines with the exclusion of histamine. The concentrations are relative to batch A (Control), batch B (+ histamine) and batch C (+ histamine and starter cultures).

3.2. Biogenic amines

The BA content of the sausages were analysed immediately after casing and during ripening. The samples were monitored for the presence of 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine. The concentrations of the different BA during ripening is shown in Figs. 3 and 4.

Histamine (Fig. 3a) was not produced in sausages during ripening by the natural microflora (batch A). Moreover, the histamine oxidising activity shown in vitro by *S. aureus* S81 (Martuscelli et al., 2000) was not marked in the sausages (batch C). In fact, only limited fluctuations in histamine contents were observed during ripening. Histamine concentrations fluctuated also in the sausages not inoculated with starters (batch B), and the amount of this amine at the end of ripening in this batch is higher than in batch C. In fact, in the absence of starter cultures (batch B), histamine, initially present at a concentration of 150 mg kg^{-1}, decreased in the first days of fermentation, and then increased to reach its maximum concentration (241 mg kg^{-1}) after 21 days. When *L. sakei* G20 and *S. xylosus* S81 were added as starter cultures, histamine levels remained constant in the first 5 days. After this period, they reached the maximum concentration (206 mg kg^{-1}), which decreased at the end of the ripening process, to a value of 113 mg kg^{-1}. The absence of histamine in the batch A and its fluctuations in the batches B and C suggests that the presence of an exogenous source of amine can influence the activities of both aminoacid decarboxylase and amine oxidase enzymes. Moreover, many authors, including Omure, Price, and Olcott (1978), Vidal and Marine, (1984) and Kurihara, Wagatuma, Jujii, and Okuzumi (1993), observed that high levels of histamine inhibited the histidine decarboxylase enzyme suggesting the need for a further study of the parameters influencing these enzymatic activities in vivo. In fact, histamine levels depend on free histidine concentrations in the medium and the balance between histamine production and histamine destruction by the microflora (Ferencik, 1970). This would suggest an important role for the final product on the enzymatic kinetics. In other words, the inhibition or acceleration of the process can depend on the microorganisms present as well as on the environmental conditions. Nevertheless, in our experimental conditions, the addition of histamine and starter cultures during sausage manufacture markedly influenced the fate of the other BA. The amount of 2-phenylethylamine is rather low during the first 2 weeks, but it markedly increased at the end of ripening if histamine was added to the sausages (Fig. 3b). In fact, the amount of this amine than batch A (36 mg kg^{-1}) after a 21 days ripening were about three times higher in batch B (99 mg kg^{-1}) and five times higher (162 mg kg^{-1}) when starter cultures were also added (batch C). Also putrescine concentrations at the end of ripening (Fig. 3c) were three times higher in batch B (307 mg kg^{-1}) compared with batch A (109 mg kg^{-1}) but the amount of this amine was dramatically reduced (11 mg kg^{-1}) in the presence of *S. xylosus* S81 (batch C). The addition of starters also reduced the content of the diamine cadaverine (Fig. 3d) and decreased the concentration of tyramine (Fig. 4a). Tyramine was quantitatively the most important Ba in all the batches and when starter cultures were added reached a concentration of about 375 mg kg^{-1} at the end of ripening, lower than the concentration detected in batch A (404 mg kg^{-1}) and B (523 mg kg^{-1}). The content of spermine (Fig. 4b) was constant during ripening in all the three batches, but decreased in concentration at the end of ripening (Fig. 4c).

Finally, Fig. 4d shows also the behaviour of the total BA contents (with the exclusion histamine) during ripening. In the presence of starter cultures and histamine (batch C), the BA contents were lowest, while the addition of histamine alone (batch B) seemed to induce a marked production of BA, which reached, at the end of ripening, considerably higher values than those produced in batches A and C.

4. Conclusion

Both aminoacid decarboxylation and BA oxidation can have important roles in nutritionally poor environments. In fact, bacterial decarboxylation systems can generate a translocation of charge across the cytoplasmatic membrane, influencing the membrane potential (Konings et al., 1997). Examples of these systems are malolactic and citrolactic fermentation, and also histidine decarboxylation can result in the net movement of a positive charge to the outside (Molenaar, Bosscher, Ten Brink, Driessen, & Konings, 1993).

The presence of starter cultures is undoubtedly one of the most important factors affecting the quantitative presence of BA in fermented foods (Chander, Batish, Babu, & Singh, 1989; Maijala, Eerola, Lievonen, Hill, & Hirvi, 1995). Some authors reported that starter cultures affect the formation of cadaverine and putrescine, because their formation depends primarily on the presence of contaminant bacteria such as enterobacteria. On the other hand, the same starter cultures have been reported not to inhibit the formation of histamine, tyramine and 2-phenylethylamine (Roig-Sagués, Hernández-Herrero, Rodriguez-Jerez, López-Sabater, & Mora-Ventura, 1997).

As a result of this study, it is possible to state that the BA content in food depends on an equilibrium between produced and oxidised amines, i. e. between decarboxylating and amine oxidasing activities, as already observed in fish products by Okuzumi, Yamanaka, Kubozuka, Ozaki, and Matsubara (1984). Ferencik

(1970) reported that the presence of histidine in tuna flesh homogenate inoculated with *Morganella morgani* stimulates the production of histamine. This, in turn, promotes amine oxidase activity which soon drastically reduces the content of the amine. In particular, the activation of microbial amine oxidases with different levels of specificity and activity can influence the fate of various BA in fermented food. Even if the activity in vitro of microorganisms having MAO and DAO enzymes is not quantitatively reproducible in vivo (probably due to the more severe conditions and, in particular, to the low O_2 tension, pH and salt concentration), slight reductions of histamine contents has been obtained in the presence of amine oxidase positive staphylococcal starter cultures. Nevertheless, the reduction of histamine concentration observed in this work is comparable with that observed for tyramine by Leuschner and Hammes (1998). Important changes have been observed in the concentration of the other BA, in particular tyramine and putrescine. Bover-Cid et al. (1999) demonstrated that the use of *Staphylococcus* strains as starter cultures yielded lower tyramine amounts compared with lactic acid bacteria. Moreover, the same authors observed that tyramine varied widely between batches fermented by different *Staphylococcus* species. Bover-Cid, Hugas, Izquierdo-Pulido, and Vidal-Carou (2001) suggested that BA production in synthetic medium might be strain dependent rather than species related. Moreover, enzymatic decarboxylase activity depends not only on the availability of substrate, but on other factors (such as pH, a_w, nutrients, food composition and technological conditions) which should be studied further to determine those manufacturing and ripening conditions that favour a good fermentation process and limit BA accumulation (Gardini et al., 2001).

In a recent paper, Bover-Cid, Izquierdo-Pulido, and Vidal-Carou (2001) observed that the concentration of the diamines putrescine and cadaverine in sausages was greatly reduced by using a mixed starter culture containing a tyrosine-producing *L. curvatus* and a proteolytic *S. xylosus*. In addition they found no relationship between the extent of proteolysis and tyramine production. The lack of such a correlation led the authors to suggest that not only the presence of free aminoacids and decarboxylase activity are important for BA production, but such factors as water activity and the interaction between different microbial groups, might modify BA production by bacteria. The results of this work confirm that the presence of BA in foods is the consequence of a complex equilibrium between the composition of the medium and the enzymatic activities of the microbial population. In addition, these results suggest that the presence and relative activity of amino oxidases should be considered as an important characteristic in the selection of starter cultures used in the production of fermented foods.

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