BIOGENIC AMINES IN FERMENTED FOODS

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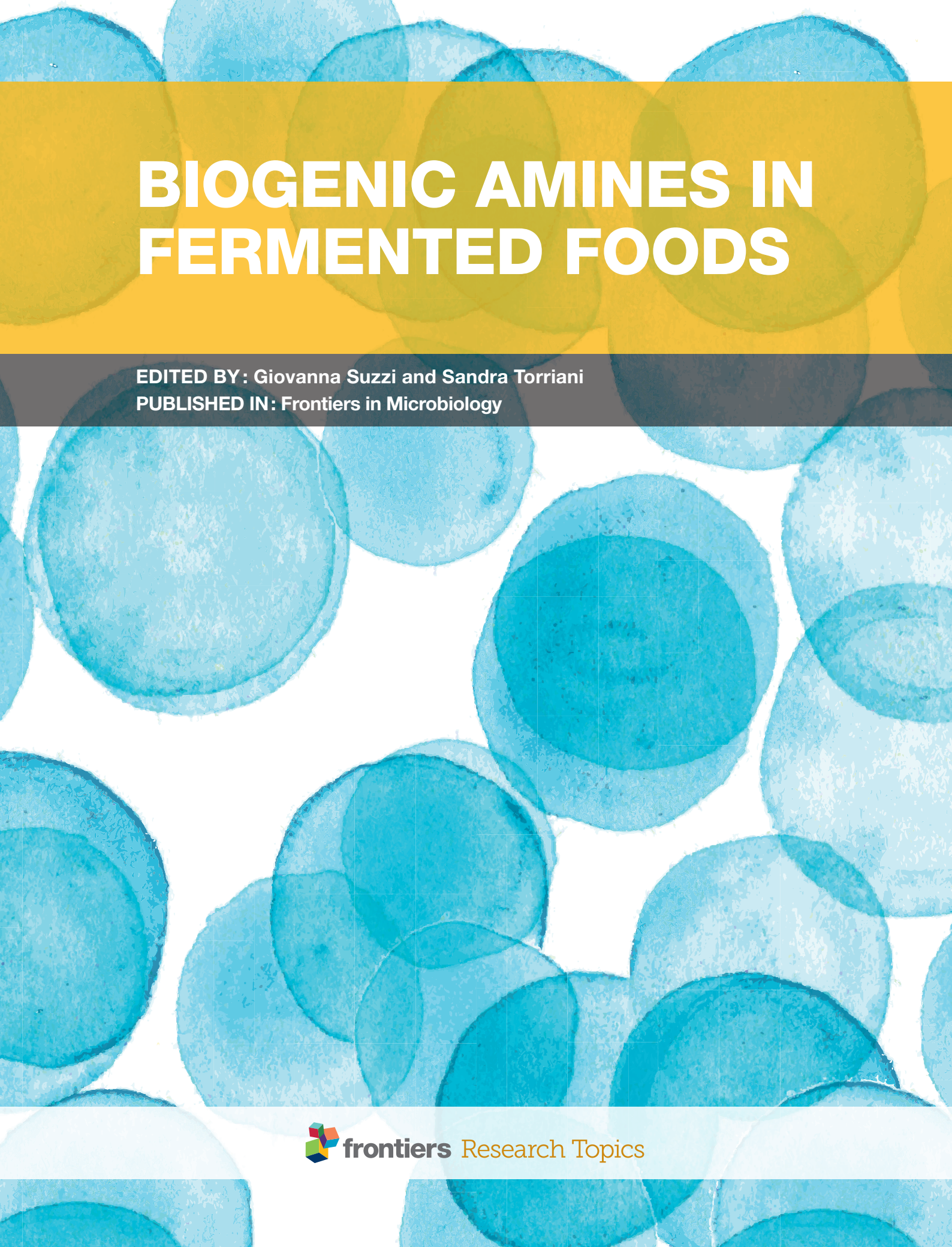

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BIOGENIC AMINES IN FERMENTED FOODS

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Biogenic amines (BA) are sources of nitrogen and precursors for synthesis of hormones, alkaloids, nucleic acids and proteins, occurring in all organisms. Under normal condition in humans the consumption of food or beverages containing these compounds have not toxic effects because they are rapidly detoxified by the activity of the amine oxidizing enzymes, monoamine (MAO) and diamine oxidases (DAO). However in presence of high BA content, in allergic individuals or if MAO inhibitors are applied the detoxification system is not capable of metabolizing dietary intake of BA. This fact can induce toxicological risks and health troubles, but the European Union established regulation for just only histamine levels in fish and fishery products.

The presence of BA in foods is due to the enzymatic decarboxylation of free amino acids by microorganisms that possess this activity. Many foods such as meat products, cheeses, fishes, fermented products and beverages could contain high levels of these compounds. Determination of BA rates in food are important as indicators of the degree of freshness or spoilage other then from the point of view of their toxicology. The content of the EBook deals the presence of BA in some fermented and non fermented foods and the measures to control their content.

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EDITORIAL
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Editorial: Biogenic amines in foods

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Keywords: biogenic amines, histamine, tyramine, sausages, fish, cheeses, wine

Biogenic amines (BA) are nitrogenous compounds of low molecular weight and are essential at low concentrations for natural metabolic and physiological functions in animals, plants, and microorganisms. Histamine, putrescine, cadaverine, tyramine, tryptamine, 2-phenylethylamine, spermine and spermidine are the most important BA in foods in which they are mainly produced by microbial decarboxylation of amino acids. Many factors influence BA production in foods, including food physico-chemical parameters (NaCl, pH and ripening temperature), storage, and distribution conditions, manufacturing processes and practices, presence of decarboxylase-positive microorganisms, raw material quality, and availability of free amino acids (Linares et al., 2012). Nonetheless, consumption of food or beverages containing high amounts of these compounds can have toxic effects such as hypertension, cardiac palpitations, headache, nausea, diarrhea, flushing, and localized inflammation; in extreme cases the intoxication may have fatal outcome. The degree of BA intoxication depends on the amount and type of BA ingested and the correct functioning of the detoxification system. In fact, after food consumption, small quantities of BA are commonly metabolized in the human gut to physiologically less active forms through the activity of the amine oxidizing enzymes, monoamine and diamine oxidases. So the toxic level of BA ingested is difficult to establish, as this depends on the individual sensitivity and health status of consumers. Moreover the malfunction or reduced activity of amine oxidase can result in high BA blood levels, whereas people taking drugs with amino oxidase inhibitor and/or alcohol show interaction with the detoxification system.

Among intoxications related with BA there is the "Scombroid poisoning" caused by histamine which is the only BA with regulatory limits, set by European Commission, up to a maximum of 200 mg/kg in fresh fish and 400 mg/kg in fishery products treated by enzyme maturation in brine (Visciano et al., 2012, 2014). After fish, cheese is the next most commonly implicated food item associated with tyramine poisoning, so called "Cheese reaction," related with its high content in aged cheeses (Schirone et al., 2012). Other potentially BA, specially histamine and putrescine are also present in milk-based fermented foods (Linares et al., 2012).

Moreover in fermented beverages, such as wine, it is very difficult to minimize content of BA, that are produced mainly through the decarboxylation of amino acids by yeasts during fermentation and/or lactic acid bacteria during malolactic fermentation. In particular vintage, grape variety, geographical region, and vinification methods such as grape skin maceration are some of the variables that can lead to an increase of precursor amino acids and subsequently the BA content in wine (Smit et al., 2012). Recently, some *Lactobacillus plantarum* strains isolated from wine and other oenological source were tested for their ability to degrade BA. Two strains were selected for their potential ability to reduce BA in wine (putrescine and tyramine) and to design malolactic starter cultures (Capozzi et al., 2012).

Among the approaches useful to control the formation of BA, such as the reduction of microbial growth through chilling and freezing or hydrostatic pressures, irradiation, controlled atmosphere packaging, or the use of food additives, etc., the use of selected starter cultures free of the potential to form BA, has been proposed as one of the best technological measures to control aminogenesis during traditional sausages production (Latorre-Moratalla et al., 2012). In fact in traditional dry sausages high content of BA can be produced by different microbial groups such as lactic acid

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bacteria and enterococci, but also by staphylococci and bacilli (Bermúdez et al., 2012).

Among the food BA, polyamines are ubiquitous substances considered to be bioregulators of numerous cells functions and are involved in tissue repair and in intracellular signaling. Although many biological functions have been attributed to polyamines, high levels of these compounds in foodstuffs can have toxicological effects; however, no safe level for the intake of polyamines in a diet as yet been established. The polyamine agmatine, derived from arginine, is present at high levels in alcoholic beverages, such as wine, beer, sake (Galgano et al., 2012).

The articles within this eBook address various issues related to the qualitative and quantitative presence of BA in cheese, dry sausages, wine, and fish. The possible inactivation and scavenging of these compounds by technological processes and amine oxidase activity of some microorganisms is also reported.

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Managing your wine fermentation to reduce the risk of biogenic amine formation

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Biogenic amines are nitrogenous organic compounds produced in wine from amino acid precursors mainly by microbial decarboxylation. The concentration of biogenic amines that can potentially be produced is dependent on the amount of amino acid precursors in the medium, the presence of decarboxylase positive microorganisms and conditions that enable microbial or biochemical activity such as the addition of nutrients to support the inoculated starter cultures for alcoholic and malolactic fermentation (MLF). MLF can be conducted using co-inoculation or an inoculation after the completion of alcoholic fermentation that may also affect the level of biogenic amines in wine. This study focused on the impact of the addition of complex commercial yeast and bacterial nutrients and the use of different MLF inoculation scenarios on the production of biogenic amines in wine. Results showed that the addition of complex nutrients to real grape must could potentially increase histamine concentrations in wine. The same experiment in synthetic grape must showed a similar trend for putrescine and cadaverine. The effect of different MLF inoculation scenarios was examined in two cultivars, Pinotage and Shiraz. Conflicting results was obtained. In the Shiraz, co-inoculation resulted in lower biogenic amine concentrations after MLF compared to before MLF, while the concentration was higher in the Pinotage. However, the production of biogenic amines was affected more by the presence of decarboxylase positive lactic acid bacteria than by the addition of complex nutrients or the inoculation scenario.

Keywords: biogenic amines, nutrients, co-inoculation, malolactic fermentation, lactic acid bacteria, wine

INTRODUCTION

Biogenic amines are basic nitrogenous compounds produced in wine mainly through the decarboxylation of amino acids by yeasts or lactic acid bacteria (LAB). The concentration of biogenic amines that can potentially be produced in wine largely depends on the abundance of amino acid precursors in the medium, the presence of decarboxylase positive microorganisms and wine parameters such as pH, alcohol, and sulfur dioxide that will impact the growth of microbes (Smit et al., 2008; Moreno-Arribas et al., 2010).

Vintage, grape variety, geographical region, and vinification methods such as grape skin maceration are some of the variables that can lead to an increase of precursor amino acids and subsequently the biogenic amine content in wine. Aging of wine on yeast lees involves autolyzing yeast cells that release vitamins and nitrogenous compounds into the wine. The latter may include amino acids that are the precursors of biogenic amines (Smit and Du Toit, 2011).

The yeasts and LAB responsible for wine fermentations have certain basic nitrogen nutrient requirements. *Saccharomyces* species can utilize the ammonium ion (NH_4^+) and free alpha amino acids as nitrogen sources. Moreover, yeasts can synthesize all required nitrogen compounds, including amino acids, from ammonium. However, if amino acids are present yeasts will use it very efficiently after ammonium has been depleted. Yeast strains display different preferences for the uptake of different amino acids, and can also secrete certain amino acids into the wine (Bely et al., 1990). In general, yeasts require at least a minimum of 140–150 mg/N/L to prevent stuck fermentations, but 200 mg/L is recommended to avoid the formation of off-flavors (Ribéreau-Gayon et al., 2006). Because ammonium alone does not meet all the nutritional requirements of yeast, many wine yeast manufacturers recommend the use of complex yeast nutrients that include a nitrogen supplement (González-Marco et al., 2006; Hernández-Orte et al., 2006). Bach et al. (2011) have shown that the addition of yeast nitrogen compounds leads to an increase in the total level of biogenic amines in wine.

Lactic acid bacteria require and are able to use only complex organic nitrogen sources, such as amino acids. They can also utilize peptides or proteins as nitrogen sources by the breakdown to amino acids by proteolytic enzyme activity (Leitão et al., 2000). It has been shown that the highest risks for the production of histamine and tyramine is during malolactic fermentation (MLF; Vidal-Carou et al., 1990a,b; Soufleros et al., 1998; van der Merwe, 2007). Generally complex malolactic nutrients include inactivated yeast cells rich in alpha amino acids as well as casein, vitamins, minerals, polysaccharides, and cellulose. As with commercial yeast preparations, commercial malolactic nutrients are recommended for use with fermentation starter cultures (selected *Oenococcus oeni* strains) unable to produce biogenic amines according to the manufacturers (Lerm et al., 2010). However, in practice winemakers

might add nutrients to sluggish spontaneous MLF or allow spontaneous MLF to proceed after complex yeast nutrients had been added during alcoholic fermentation (AF). The question arises whether residual precursor amino acids from complex nutrients could be present in the wine for the natural LAB flora, which could include decarboxylase positive strains, to use.

In this study, the influence of complex commercial yeast and bacterial nutrients on biogenic amine production by yeast and natural LAB (partially supplemented by decarboxylase positive *Lactobacillus* species) in the wine or added to a synthetic medium are evaluated. The second aim was to assess the impact of different MLF inoculation scenarios on the production of biogenic amines.

MATERIALS AND METHODS

GRAPE MUST FERMENTATIONS WITH COMPLEX NUTRIENTS

Cabernet Sauvignon grapes from the Paarl region and Shiraz grapes from the Stellenbosch region, South Africa, were used in this study. After grapes were destemmed and crushed, the skins and free-run juice were separated and homogenized. Equal amounts of free-run juice (per volume) and grape skins (per weight) were allocated to each treatment in 10 L plastic buckets. Sulfur dioxide (SO_2) was added to the must of all treatments at 20 mg/L. AF was performed by *Saccharomyces cerevisiae* strain NT202 (Anchor Yeast, South Africa) in all treatments; rehydrated and inoculated at 30 g/hL according to the instructions of the manufacturer. Sugar density was measured daily in all treatments with a Brix hydrometer to monitor the progression of AF, which was conducted at room temperature. The temperature in the cellar was not controlled, but grape must temperature was recorded daily and fluctuated between 19 and 22°C. Grape skins were punched down daily throughout AF and pressed with a hydraulic basket press to 1 bar at the completion of AF. Wines were transferred to 4.5 L glass bottles to complete MLF at 20°C. MLF was performed spontaneously by strains native to the grapes used in this study, supplemented with a culture containing confirmed decarboxylase positive strains isolated from spontaneous MLF in South African wine (Downing, 2003; Smit et al., submitted). This decarboxylase positive culture, comprised of equal cell concentrations of *Lactobacillus hilgardii* B74, *L. hilgardii* M59, and *Lactobacillus brevis* M58 (Smit et al., submitted), was inoculated into all treatments at approximately 10^6 CFU/mL after the completion of AF and prior to the start of spontaneous MLF.

Representative samples for the analysis of biogenic amines and microbial enumeration were drawn in sterile sample vials before AF (grape must), after AF, and after MLF. The presence and growth of LAB in the wine was monitored by plate counts on selective agar media (Smit and Du Toit, 2011). MLF was monitored using Fourier transform mid infrared spectroscopy (FT-MIR; WineScan FT120, FOSS Analytical, Denmark) to determine malic acid and lactic acid concentrations in the wine. All treatments were repeated in duplicate for each of the two cultivars. Treatments consisted of the addition of commercial preparations of complex yeast or bacterial nutrients to the fermenting must or wine. Yeast nutrients were added after the exponential growth phase of yeast; 1–3 days after inoculation with yeast for AF, as recommended by the manufacturers. Bacterial (malolactic) nutrients were added after AF, before the start of MLF. All complex nutrients were added to the fermenting must or wine at the maximum dosage recommended by the manufacturers. Treatment 1 contained no added nutrients, treatments 2, 3, and 4 were supplemented with complex yeast nutrients (nutrients A, B, and C), and treatments 5 and 6 were supplemented with complex bacterial nutrients (nutrients D and E).

The following descriptions of the compositions of the nutrients used in this study were provided by the manufacturers. Nutrient A contains inactivated yeast, diammonium phosphate (DAP), and ammonium sulfate. Nutrient B contains inactivated yeast, DAP, and one specific vitamin. Nutrient C contains inactivated yeast, DAP, vitamins, minerals, unsaturated fatty acids, and sterols. Nutrient D contains inactivated yeast and cellulose. Nutrient E contains inactivated yeast, cellulose, and casein. It is not clear in all cases from the manufacturers' descriptions whether vitamins, minerals (trace elements), polysaccharides, sterols, and fatty acids were added or derived from the inactivated yeast. Some manufacturers claim to use specially selected inactivated yeast.

SYNTHETIC MEDIUM FERMENTATIONS WITH COMPLEX NUTRIENTS

Synthetic grape must (MS300) described by Bely et al. (1990) was used to perform a similar experiment to confirm results under controlled conditions. The following modifications were made to the synthetic grape must: 120 mg/L ammonium chloride was included as the only nitrogen source (no amino acids were added as per the original protocol), 0.005% of pyridoxal 5′-phosphate (Sigma-Aldrich, Germany) was added to induce decarboxylase activity. All chemicals used for the preparation of the synthetic grape must were purchased from Saarchem (Merck, South Africa) except for malic acid, glucose, and ammonium chloride (Sigma-Aldrich, Germany).

The treatments in the synthetic grape must consisted of four control treatments without the addition of any complex nutrients, (1) in the absence of yeast and LAB, (2) in the presence of yeast and LAB, (3) in the absence of yeast but presence of LAB, and (4) in the absence of LAB but presence of yeast. Treatments 5–10 consisted of different combinations of three complex yeast and two bacterial nutrients; (5) nutrients A and D, (6) nutrients A and E, (7) nutrients B and D, (8) nutrients B and E, (9) nutrients C and D, and (10) nutrients C and E. Treatments 11 (nutrient D) and 12 (nutrient E) contained only bacterial nutrients.

Saccharomyces cerevisiae strain NT202 was rehydrated and inoculated at 30 g/hL for AF. Complex yeast nutrients were added to the fermenting synthetic must 48 h after the start of AF. Fermentations were performed under static conditions in 100 mL Erlenmeyer flasks fitted with airlocks, at 30°C. After 14 days AF was considered complete when CO_2 gas formation seized, and yeast cells were removed from the treatments by centrifugation at 8000 rpm for 5 min to prevent further fermentation or autolysis and release of nutrients from the yeast cells. Complex bacterial nutrients were added to the synthetic wine and 1% of precultured decarboxylase positive LAB culture was inoculated into the medium. LAB growth (with limited MLF) proceeded for a period of 14 days at 30°C.

Prior to the start of this experiment, the five nutrients tested, dissolved in synthetic must, were analyzed for biogenic amines. Samples were drawn from the fermentation medium after AF and at the end of growth of LAB for biogenic amine analysis.

CO-INOCULATION VERSUS SEQUENTIAL MALOLACTIC FERMENTATION

Malolactic fermentation was carried out in the cultivars Pinotage and Shiraz to examine the influence of different MLF inoculation times on the biogenic amine content of wine. The co-inoculation treatments were inoculated for MLF 24 h after the addition of the yeast for AF, whereas the sequential treatments were inoculated for MLF by the conventional practice, after the completion of AF. All treatments were performed in triplicate, using 9.5 kg of grapes for the co-inoculation fermentations and 4.5 L of wine for the sequential fermentations. After destemming and crushing of the grapes, sulfur dioxide was added at a concentration of 20 and 30 mg/L to the co-inoculation and sequential treatments, respectively. The *S. cerevisiae* yeast strain WE372 (30 g/hL; Anchor Yeast, South Africa) was used for AF. The juice was supplemented with diammonium phosphate (250 mg/L) on day one of AF. No additional nutrients were added to any treatment. AF was performed at 25˚C. As soon as AF was completed (residual sugar <5 g/L) the wines were moved to a 20˚C incubation room to complete MLF. After the completion of MLF (which ranged between 18 and 34 days) sulfur dioxide was added to a final concentration of between 60 and 80 mg/L, after which the wines were bottled. The MLF treatments for the Pinotage consisted of a spontaneous control, which was not inoculated for MLF, two treatments inoculated with *L. hilgardii* strains (*L. hilgardii* 1 and *L. hilgardii* 2) and one treatment inoculated with a *Lactobacillus plantarum* strain. These *Lactobacillus* cultures were isolated from South African wines and were investigated for their ability to perform MLF (unpublished data). For the Shiraz, a treatment inoculated with a commercial *O. oeni* starter culture was included for comparison to the *Lactobacillus* cultures. Samples were taken from all fermentations before inoculation for MLF and again at the completion of MLF for analyses of the biogenic amines histamine, tyramine, putrescine, and cadaverine.

Statistically significant differences between the amine concentration before and after MLF for a specific inoculation time were evaluated by one-way analysis of variance with Fisher's LSD test at a 95% significance level. The analysis was performed using Statistica (Stat Soft, Inc., USA, version 10).

BIOGENIC AMINE ANALYSES

The biogenic amines histamine, tyramine, putrescine, and cadaverine were quantified by two different methods in this study. For the small scale wine fermentations with complex nutrients, biogenic amines were analyzed by high performance liquid chromatography (HPLC) using the method described by Alberto et al. (2002) with modifications (Smit and Du Toit, 2011). Amines in the synthetic media fermentations with complex nutrients, and in co-inoculation versus sequential MLFs were determined using liquid chromatography mass spectrometry (LC-MS/MS) as described by Smit et al. (submitted).

RESULTS

GRAPE MUST FERMENTATIONS WITH COMPLEX NUTRIENTS

On average, AF was complete in 9 days in Cabernet Sauvignon and 8 days in Shiraz. **Figures 1** and **2** show the results of malic acid degradation and growth of LAB during the course of MLF in the two cultivars. In Cabernet Sauvignon (**Figure 1**), MLF initially proceeded faster in some treatments that received added complex nutrients. When comparing the three treatments that received additional complex yeast nutrients (treatments 2, 4, and 4); not only was the rate of AF enhanced (data not shown), but treatments 2 and 3 completed spontaneous MLF at a faster rate (44–51 days) than the control, treatment 1 (66 days). MLF in treatments 5 and 6 also proceeded faster than in the control treatment (59 days), suggesting that malolactic nutrients can act to increase the initial rate of spontaneous MLF. Treatment 4 (yeast nutrient C) did not enhance MLF rate in Cabernet Sauvignon. For Shiraz treatments the rate of MLF was similar in all treatments (**Figure 2**), and took approximately 40 days to be completed. There were no significant differences between LAB numbers measured in relation to the nutrient treatments.

Four of the biogenic amines commonly found in wine were analyzed (histamine, tyramine, putrescine, and cadaverine). No tyramine was detected in any of the treatments for both cultivars. Although biogenic amines were detected in the grape must (putrescine), and wines after AF and MLF (putrescine and cadaverine), levels were similar for all treatments and no effect of nutrient addition could be observed (results not shown). The only biogenic amine that showed a difference between treatments was histamine. **Figure 3** shows the concentrations of histamine detected in samples of the grape must, at the end of AF and at the end MLF in **Figure 3A** Cabernet Sauvignon and **Figure 3B** Shiraz treatments.

SYNTHETIC MEDIUM FERMENTATIONS WITH COMPLEX NUTRIENTS

Figure 4 shows the concentrations of each biogenic amine detected in samples of the synthetic must, at the end of AF and at the end of the 14-day growth period of LAB in the synthetic wine.

Biogenic amines were absent or detected only at very low levels in the synthetic grape must with or without dissolved complex nutrients.

When no LAB were present (treatments 1 and 4), no significant production of any biogenic amines was measured in this study.

It seems as if though the *Lactobacillus* species produced much higher levels of histamine and tyramine when their growth was not preceded by the growth and fermentation of yeast (treatment 3). Also, the absence of any complex nutrients seemed to stimulate histamine and tyramine formation by *Lactobacillus* species (treatment 3). Similar concentrations (for both histamine and tyramine) were reached for all treatments in the presence of both yeast and bacteria (treatments 5–12) regardless of whether complex nutrients were absent from the medium (treatment 2) or present in any combination or alone.

More putrescine was produced in treatments that were supplemented with complex nutrients than in control treatments 2 and 3 containing *Lactobacillus* species but no added nutrients. No significant amounts of putrescine were produced in the absence of LAB (treatments 1 and 4). Therefore, all of the tested nutrient combinations had an influence on putrescine production, with bacterial nutrients added alone (treatments 11 and 12) leading to a slightly higher production when compared to any combination of yeast and bacterial nutrients. It appears that the yeast NT202 produced low levels of cadaverine during AF (treatments 2 and 4), more so in the presence of complex yeast nutrients (treatments 5–10). After the introduction of *Lactobacillus* species an increase of cadaverine

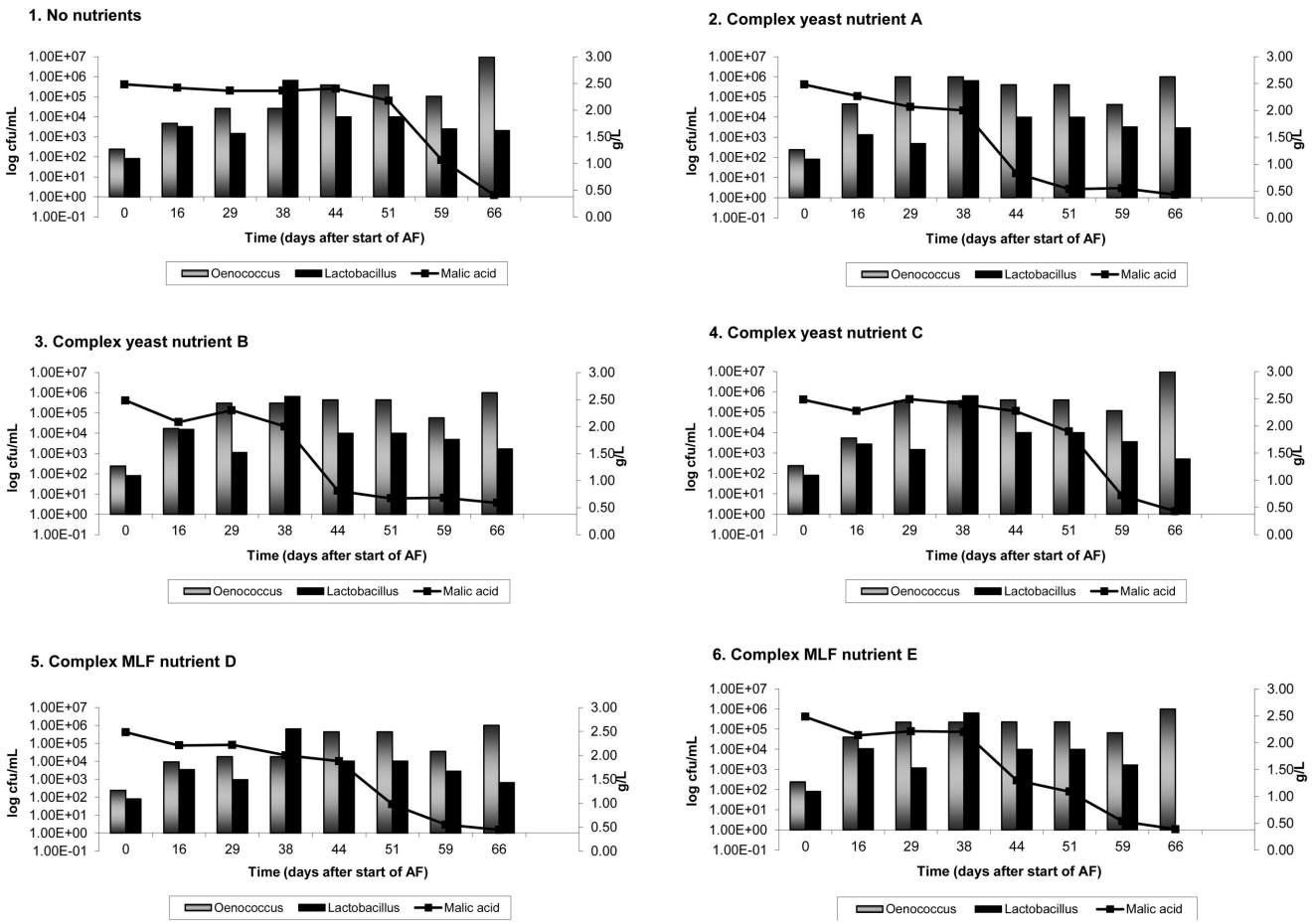

FIGURE 1 | Malolactic fermentation (MLF) (malic acid degradation) and growth of lactic acid bacteria in Cabernet Sauvignon wines. Results are the average of duplicate treatments. AF = alcoholic fermentation.

for all treatments where *Lactobacillus* species were present could be observed. As with histamine and tyramine, it appears that the *Lactobacillus* species produced much higher levels of cadaverine when their growth was not preceded by the growth of yeast. When comparing treatment 2 (no complex nutrients in the presence of yeast) and treatments 11 and 12, it is seen that the nutrients could have influenced cadaverine production by LAB when yeast was also present.

INFLUENCE OF MLF INOCULATION TIME ON BIOGENIC AMINES

In the Pinotage the total concentration of biogenic amines was higher in the co-inoculation treatments than in the sequential treatments (**Figure 5**). Precisely the opposite was seen in the Shiraz, where the total biogenic amine concentration was higher in the sequential treatments (**Figure 6**).

All four biogenic amines that were measured could be detected in one or more of the wines. For both inoculation scenarios in the Pinotage and Shiraz, histamine did not increase significantly from before to after MLF in any of the treatments (**Figures 5** and **6**). No significant production of tyramine occurred in any of the treatments, except for the treatments inoculated with *L. hilgardii* 1 (**Figures 5** and **6**). This is not a surprising result. All the strains used in these fermentations were analyzed for the presence of histidine, tyrosine, and ornithine decarboxylases by a multiplex PCR assay (Marcobal et al., 2005) and only this *L. hilgardii* strain gave a positive result for the tyrosine decarboxylase gene (data not shown). The concentration of tyramine produced by this strain during the sequential inoculation was also higher than what was produced when co-inoculated. In all the other treatments the concentration of tyramine was between 0 and 0.007 mg/L.

Putrescine was the most abundant amine in the analyzed wines. In the Pinotage co-inoculation treatments putrescine increased significantly from before MLF to after AF and MLF (**Figure 5**). However, in the Shiraz co-inoculation treatments putrescine decreased during the course of these processes (**Figure 6**). The exact same incidence was seen for cadaverine (**Figures 5** and **6**). In the sequential treatments of both cultivars, putrescine concentrations were the same or less than before MLF. For cadaverine, there was also no significant difference in the concentrations before and after MLF in the sequential treatments, except for the uninoculated

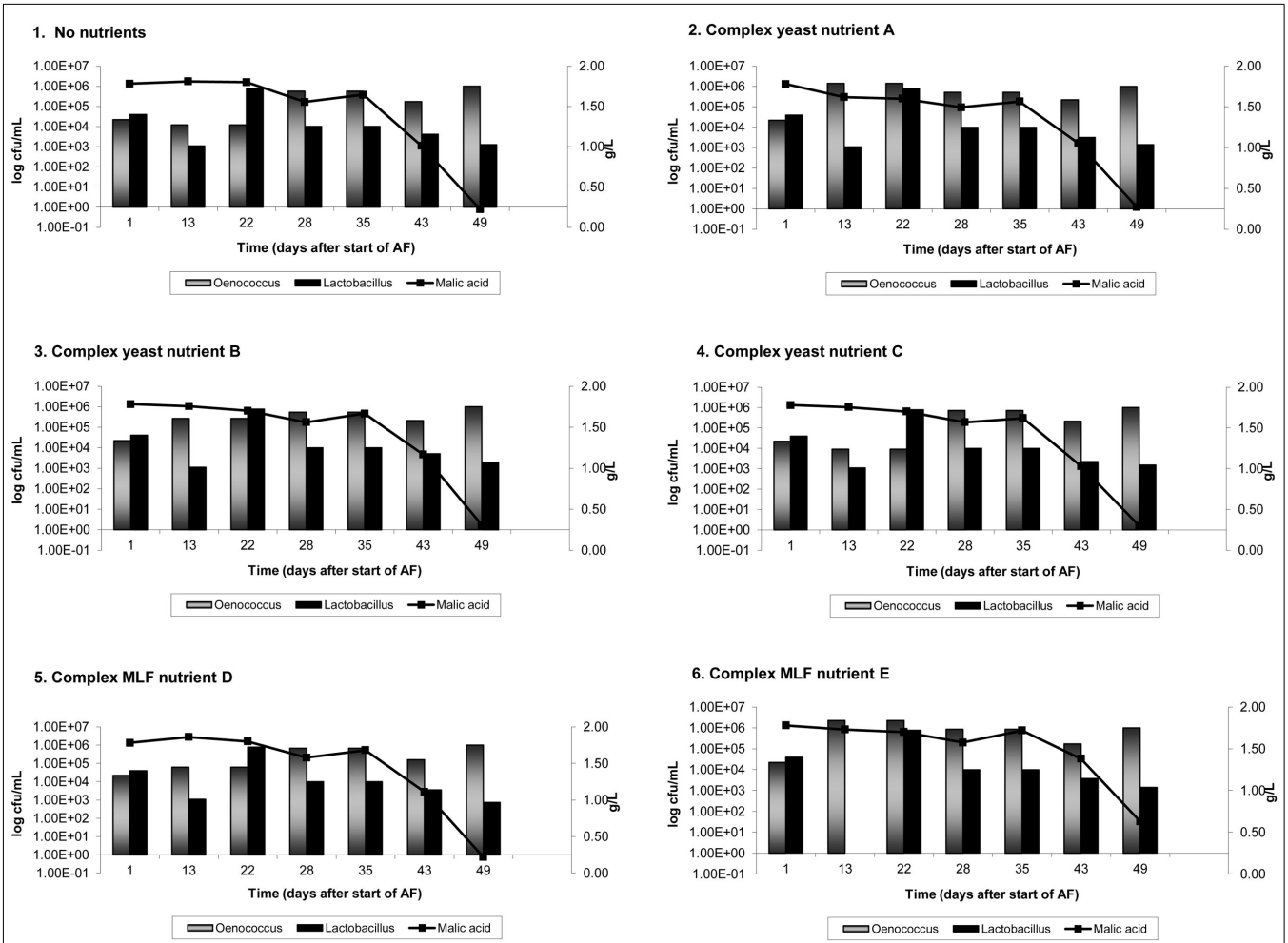

FIGURE 2 | Malolactic fermentation (MLF) (malic acid degradation) and growth of lactic acid bacteria in Shiraz wines. Results are the average of duplicate treatments. AF = alcoholic fermentation.

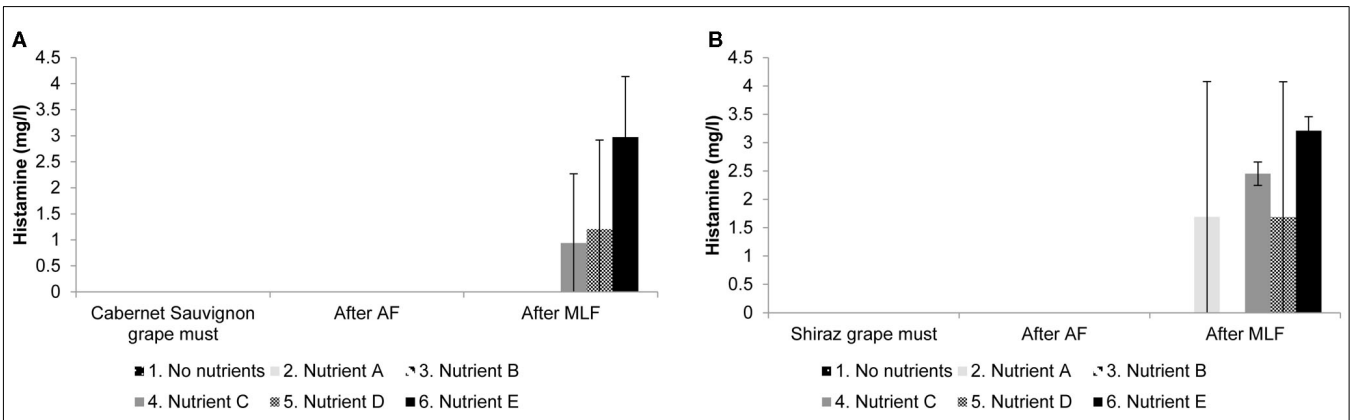

FIGURE 3 | Histamine production in (A) Cabernet Sauvignon and (B) Shiraz wines supplemented with complex yeast or bacterial nutrients. Results are the average of duplicate treatments.

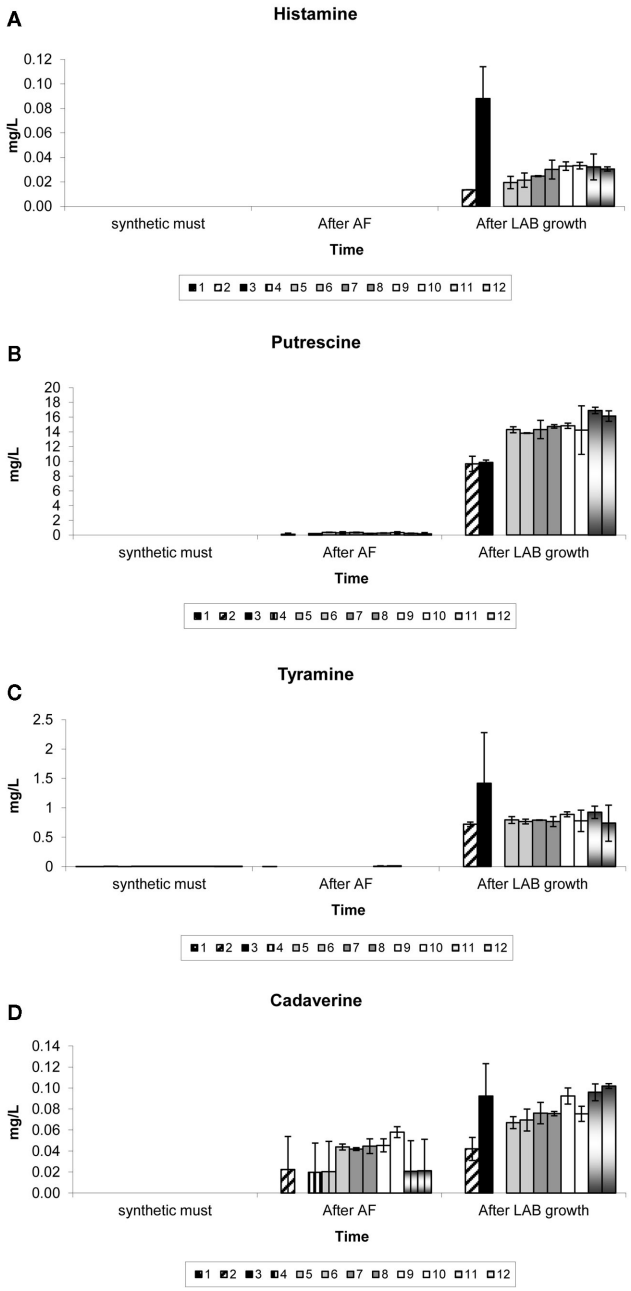

FIGURE 4 | Biogenic amines produced in synthetic medium supplemented with complex yeast or bacterial nutrients. Results are the average of duplicate treatments. The treatments consisted of (1) no nutrient addition, in the absence of yeast and LAB, (2) no nutrient addition, in the presence of yeast and LAB, (3) no nutrient addition, in the absence of yeast but presence of LAB, (4) no nutrient addition, in the absence of LAB but presence of yeast, (5) nutrients A and D with yeast and LAB, (6) nutrients A and E with yeast and LAB, (7) nutrients B and D with yeast and LAB, (8) nutrients B and E with yeast and LAB, (9) nutrients C and D with yeast and LAB, and (10) nutrients C and E with yeast and LAB, (11) nutrient D with yeast and LAB (12) nutrient E with yeast and LAB.

control in the Shiraz that only differed significantly from the concentration before MLF.

DISCUSSION

GRAPE MUST FERMENTATIONS WITH COMPLEX NUTRIENTS

Some biogenic amines, particularly polyamines such as putrescine, are often present in grape berries since they are produced by the metabolism of plants (Halász et al., 1994; Bover-Cid et al., 2006). In this study, putrescine was present in the grapes used to assess complex nutrients at relatively high concentrations, at 24.2 mg/L in Shiraz grape must and 35.7 mg/L in Cabernet Sauvignon grape must. Vine nutrition, such as potassium deficiencies in the soil, has been linked to elevated concentrations of putrescine (Adams, 1991). In addition, vine nutrition and grape variety are significant contributors to the concentration and composition of grape amino acids, which could determine the final biogenic amine concentration that is found or formed by microorganisms in wine (Soufleros et al., 1998; Herbert et al., 2005).

Histamine was the only biogenic amine showing treatment differences potentially attributable to the presence of complex nutrients. In some treatments we observed large variation in histamine levels between the treatment replicates. Still, it seems possible that there are differences between treatments and that nutrients C, D, and E (Cabernet Sauvignon and Shiraz) and nutrient A (Shiraz only) influenced the final histamine concentration in the wine (**Figure 3**). Due to the small volumes used in small scale winemaking, it is possible that the inherent heterogeneity in a product such as complex nutrients could have caused the discrepancy in treatment duplicates. Since the increase in histamine occurred during MLF, the formation of this biogenic amine can most likely be attributed to the decarboxylation of histidine by LAB. Of the four biogenic amines analyzed in this study histamine is the most important to human physiology, since it is one of the most biologically active amines (Halász et al., 1994). Histamine can cause hypotension, flushing, and headache (Silla Santos, 1996) as well as abdominal cramps, diarrhea, and vomiting (Taylor, 1986). The levels of histamine produced in some treatment replicates in both cultivars during grape must fermentations supplemented with complex nutrients are above the upper limits suggested for histamine in wine in Germany (2 mg/L) and Holland (3 mg/L; Lehtonen, 1996).

During this study, a few trends regarding biogenic amine production in relation to complex yeast and bacterial nutrients could be observed. The production of relatively high levels of histamine in wine during or after MLF (by decarboxylase positive LAB) as a result of complex nutrient addition could significantly impact the wholesomeness of the wine and present negative trade implications.

These results obtained in wine are in accordance with a similar study reported in the literature which examined the impact of yeast autolyzate, often a component of complex yeast nutrients, on biogenic amine production during winemaking. The enrichment of Chardonnay must by yeast autolyzate did not lead to an increase of biogenic amines during AF. However, the concentration of biogenic amines, particularly tyramine and cadaverine, were higher after MLF (González-Marco et al., 2006).

Another study that yielded similar results with regards to yeast nutrients was performed by Marques et al. (2008), who tested two commercial fermentation activators. A nutritive factor used for AF was added to the must, and a nutritive factor used for MLF was

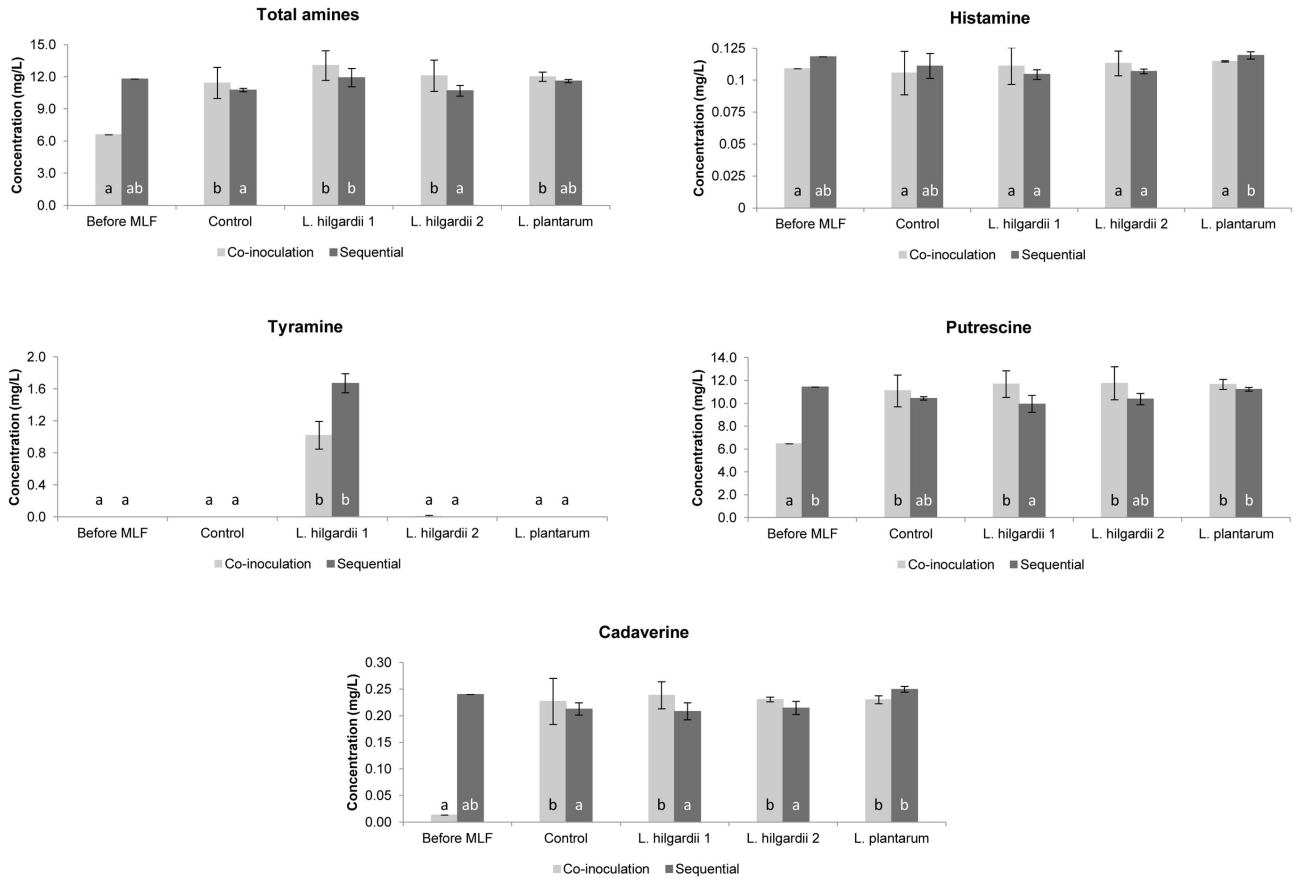

FIGURE 5 | The individual and total biogenic amine content of all treatments in the Pinotage wine fermentations before and after MLF. Each bar indicates the mean and the SEM. Different letters indicate significant differences ($p < 0.05$) between treatments of the same MLF inoculation time.

added at the end of AF to the respective treatments. In their study, a slight increase in biogenic amines (particularly isoamylamine and tyramine) resulted from the addition of the AF activator. This increase was especially noticeable at the end of MLF. No significant differences could be observed between wines to which the MLF activator were added and the control wines.

Histamine was produced at higher levels in Shiraz than in Cabernet Sauvignon treatments. This seems to be particularly true for nutrients A and C (**Figure 3**); however, due to the large variability between replicates this result is probably not significant. Still, it is clear that grape cultivar is a variable that may significantly influence the production of biogenic amines. It is possible that the amino acid composition of the cultivar could influence the amount of available precursors for biogenic amine production, or that the phenolic composition of the grape cultivar could play a role. In another study, using the same grape cultivars but from different vintages and locations, we also found significantly higher histamine production in Shiraz compared to Cabernet Sauvignon (Smit et al., submitted).

SYNTHETIC MEDIUM FERMENTATIONS WITH COMPLEX NUTRIENTS

From the results presented in **Figure 4**, it can be concluded that any major contribution to the increase of biogenic amines was contributed by the *Lactobacillus* species and that they likely produce more histamine, tyramine, putrescine, and cadaverine in an environment that lacks complex nutrients. An explanation for this observation could be that LAB produce biogenic amines in order to generate metabolic energy or regulate (increase) the pH of the growth medium (Molenaar et al., 1993). When AF preceded LAB inoculation, biogenic amine production was reduced. It can be proposed that yeast potentially eliminate precursor amino acids from the medium, leading to a reduction of subsequent biogenic amine production. Co-inoculation was not tested in this study.

In synthetic wine medium, it was confirmed that biogenic amines are produced by LAB and not by yeast. However, the impact complex nutrients appear to have on histamine production in grape must was not confirmed in synthetic medium. In the synthetic grape must experiment, the absence of any complex nutrients seemed to stimulate histamine and tyramine formation by *Lactobacillus* species (treatment 3). This is contradictory to our result observed in two cultivars of grape must, where histamine production corresponded to the addition of certain nutrients. A relationship seems to exist between yeast and bacterial growth and biogenic amine concentrations produced in the synthetic medium. It appears from this experiment in synthetic medium that the absence of complex nutrients (the medium composition) has a

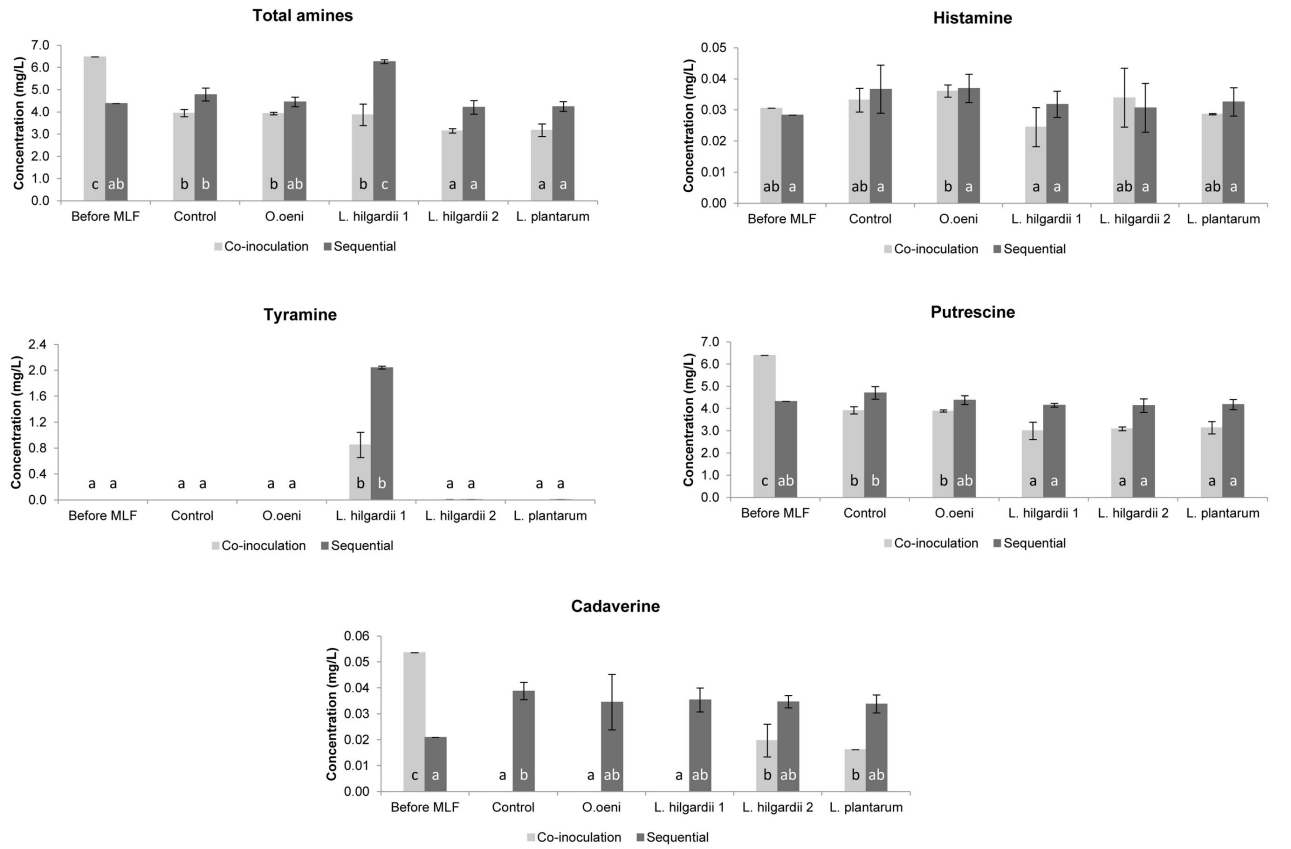

FIGURE 6 | The individual and total biogenic amine content of all treatments in the Shiraz wine fermentations before and after MLF. Each bar indicates the mean and the SEM. Different letters indicate significant differences ($p < 0.05$) between treatments of the same MLF inoculation time.

more pronounced impact on biogenic amine production than the presence of any complex fermentation nutrients.

Wine is a complex medium in which many compounds and microorganisms can inhibit the growth and metabolisms of one another or produce synergistic effects that are not observable in a simple chemically defined synthetic medium. It has been observed in a number of studies that biogenic amine production by wine microorganisms in various synthetic media can show a different set of results when evaluated in wine (Guerrini et al., 2002; Granchi et al., 2005; Landete et al., 2007). The synthetic matrix used was perhaps not an ideal model for wine, since it did not promote MLF in the 14-days of the experiment, even though LAB growth occurred.

INFLUENCE OF MLF INOCULATION TIME ON BIOGENIC AMINES

The evolution of biogenic amines during MLF was monitored to evaluate the impact of different MLF inoculation scenarios on the biogenic amine concentration. Inoculation for MLF simultaneously with AF has been shown to reduce the development of biogenic amines in wine compared to the traditional sequential inoculation (van der Merwe, 2007; Smit and Du Toit, 2011). This seems to be a realistic tool to limit biogenic amine contamination. The inoculated culture may be able to dominate and inhibit the growth of the natural LAB flora and thus the chances of unwanted activities by these bacteria are lessened during the course of AF. Our results support and contradict the findings of van der Merwe (2007) and Smit and Du Toit (2011) where co-inoculation resulted in lower biogenic amine concentrations. The total biogenic amine concentration after MLF in the co-inoculated treatments was lower than before MLF in the Shiraz, but higher in the Pinotage. This contradictory result can possibly be explained by the large number of factors (such as the vintage, geographical region, grape variety, vinification methods, availability of amino acid precursors, etc.) that can influence the formation of biogenic amines in wine (Smit et al., 2008).

Histamine did not increase significantly from before MLF to after MLF in any of the treatments. Thus, the contribution of the malolactic bacteria together with the yeast during co-inoculation and the malolactic bacteria alone in the sequential treatments did not affect the formation of histamine at all.

Significant tyramine formation was only observed in the treatments inoculated with *L. hilgardii* strain 1. A PCR assay confirmed that this strain is indeed a tyramine producing strain. It is also important to note that this strain produced more tyramine when it was inoculated sequentially compared to the co-inoculation in both cultivars. Thus, by applying co-inoculation with a LAB strain that is able to produce biogenic amines, could lower the risk of biogenic amine contamination. Moreover, this result highlights

the importance of inoculating with a starter culture unable to produce biogenic amines.

Of all the amines measured, putrescine was present in the highest concentration. The significant increase in the putrescine and cadaverine concentration in the Pinotage co-inoculated treatments, which was even seen in the uninoculated control treatment, suggests that the yeast had the largest contribution. Exactly the opposite was seen in the Shiraz co-inoculated treatments. A decrease in the amount of these two amines was observed. Several authors reported that yeast can produce biogenic amines during AF (Buteau et al., 1984; Ancín-Azpilicueta et al., 2008; Smit et al., 2008), but the prevailing opinion is that biogenic amines are produced by LAB during MLF (Soufleros et al., 1998; Lonvaud-Funel, 1999). From these results it appears that the inoculated bacteria did not affect the biogenic amine content of the wine during MLF. In the sequential inoculated treatments, where the involvement of the LAB in biogenic amine formation alone can be seen, all amines were present in the same or significantly lower concentration as before inoculation for MLF, except for cadaverine in the uninoculated control treatment in the Shiraz and tyramine in both cultivars. Thus, the relationship between biogenic amines and MLF is debatable in this dataset. In some previous studies biogenic amines increased during MLF (Marcobal et al., 2006; Izquierdo Cañas et al., 2008), while it decreased in others (Buteau et al., 1984). This inconsistency in results can be ascribed to the wide variety of factors that can influence biogenic amine production in wine, especially the fact that different species of yeast and LAB as well as strains of the same species differ in their ability to produce biogenic amines (Ancín-Azpilicueta et al., 2008; Marques et al., 2008; Smit et al., 2008).

In conclusion, when comparing the observations made in real grape must and synthetic grape must supplemented with complex nutrients, it is clear that two different results were obtained. From the real grape must experiment we could conclude that complex nutrient additions have the potential to increase histamine. A similar result was observed for amines putrescine and cadaverine in synthetic grape must. However, from the synthetic grape must experiment, it appears that biogenic amine production by LAB is influenced largely by the combination or succession of microorganisms and not always stimulated by the presence of complex nutrients, as noted for histamine and tyramine. Importantly, it is clearly observed that the presence of decarboxylase positive LAB is associated with biogenic amine increase in both the synthetic and real grape must experiments. It is therefore recommended to inoculate for MLF with commercial starter cultures that do not contain harmful decarboxylase activities. From our results in real grape must it appears that complex nutrients could influence wine wholesomeness negatively by stimulating biogenic amine production. However, from our results in synthetic must it seems that a lack of nutrients could be equally or more harmful in this regard. Thus, the judicious use of complex yeast and bacterial nutrients, used in combination with a decarboxylase negative LAB strain is recommended.

By inoculating for MLF simultaneously with AF has shown to reduce the incidence of biogenic amines in previous studies. Our results agree and disagree with these findings. However, by applying co-inoculation with a strain (*L. hilgardii* 1) that is capable of producing tyramine resulted in a lower concentration of the specific amine compared to when it was sequentially inoculated. These results also emphasize the importance of using decarboxylase negative LAB strains for MLF.

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Biogenic amines degradation by *Lactobacillus plantarum*: toward a potential application in wine

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Biogenic amines (BA) in wine represent a toxicological risk for the health of the consumer, with several trade implications. In this study 26 strains of *Lactobacillus plantarum* were analyzed for their ability to degrade BA commonly found during wine fermentation. Two strains of *L. plantarum* were selected in reason of their ability to degrade putrescine and tyramine. The degradation was assessed *in vitro*, both in presence of the BA and in presence of the specific chemical precursor and of producer bacteria. The two *L. plantarum* biotypes were found capable to work synergically. In addition, the survival in wine-like medium and the aptitude to degrade malic acid after alcoholic fermentation of the selected *L. plantarum* strains was analyzed. Our results suggest the potential application of wine *L. plantarum* strains to design malolactic starter cultures able to degrade BA in wine.

Keywords: lactic acid bacteria, amine degradation, biogenic amines, malolactic fermentation, wine, *Lactobacillus plantarum*, putrescine, tyramine

INTRODUCTION

Biogenic amines (BA) are low molecular weight organic bases with aliphatic, aromatic, and heterocyclic structures commonly found in fish, fish products, meat, dairy products, vegetables, wine, cider, and beer (Suzzi and Gardini, 2003; Spano et al., 2010). In general, foods likely to contain high levels of BA are fermented foods or foodstuff exposed to microbial contamination during food process or storage (Ali, 1996). Histamine, tyramine, cadaverine, 2-phenylethylamine, spermine, spermidine, putrescine, tryptamine, and agmatine are considered to be the most important BA occurring in foods. The main bacteria responsible for BA production in fermented food matrices are the lactic acid bacteria (LAB; Lonvaud-Funel, 2001). In fact, LAB can catabolize (principally decarboxylate) amino acids into amine-containing compounds. The physiological role of BA synthesis mainly seems to be related to defense mechanisms used by bacteria to withstand acidic environments (Spano et al., 2010).

Indigenous BA compounds are also naturally produced in different human tissues because of their biological role in processes such as synaptic transmission, blood pressure control, allergic response, and cellular growth control (Russo et al., 2010). The gastro-intestinal tract, as a function of dietary intake of food containing BA, therefore represents an exogenous source of these molecules for humans. This exogenous source of BA can provoke high levels in the human organism, and in reason of their importance in physiological processes, with negative consequences to human health (Ladero et al., 2010; Russo et al., 2010). Human sensitivity fluctuates according to the correct functioning of the detoxification systems since BA are generally metabolized in the human gut through the action of amine oxidases (Spano et al., 2010). Under physiological conditions this system has a protective role against the absorption of dietary BA. For this reason, in individuals with a pathological deficiency of amine oxidases activities, the ingestion of food products containing excess of BA may lead to high levels of BA in the organism (Kuefner et al., 2004). Additionally, the ethanol and some drugs have been found to inhibit the detoxification systems (Zimatkin and Anichtchik, 1999; Horton et al., 2005). In the wine industry, the occurrence of BA has been receiving increasingly attention in reason of the potential threats of toxicity to humans, connected with alcohol content (Smit et al., 2008). There are trade implications due to the recommended or suggested existing limits for histamine in wine in some European countries such as Germany (2 mg/l), Holland (3 mg/l), Finland (5 mg/l), Belgium (5–6 mg/l), France (8 mg/l), Switzerland, and Austria (10 mg/l; Smit et al., 2008). In addition, last year, the Panel on Biological Hazards of the European Food Safety Authority release a Scientific Opinion on risk based control of BA formation in fermented foods 9 [EFSA Panel on Biological Hazards (BIOHAZ), 2011], testifying the attention of European institutions to the subject.

In reason of the toxicity and of the existing legislative limits, several technological strategies have been proposed in order to control BA in foods (Naila et al., 2010). The main measure to prevent BA formation is the microbial control through cold treatments. However, it is difficult to operate with low temperature in the case of fermented foods, where a microbial growth is crucial to obtain the final products. Secondary control measures to prevent BA formation in foods or to reduce their levels once formed need to be considered as alternatives. Methods to limit microbial growth may include hydrostatic pressures, irradiation, controlled atmosphere

packaging, or the use of food additives (Naila et al., 2010). Furthermore, BA could be controlled, especially in fermented foods, modulating microbial resources. Indeed, fermented foods producers commonly utilize a specific starter culture, that is "a microbial preparation of large numbers of cells of at least one microbial species to be added to a raw material to produce a fermented food by accelerating and steering its fermentation process" (Leroy and De Vuyst, 2004). In general, the choice of starter cultures is crucial to guarantee the quality of the final products and microbial starter unable to produce BA might dominates the autochthonous microbiota, reducing the occurrence of BA producers (Dapkevicius et al., 2000; Latorre-Moratalla et al., 2007; Naila et al., 2010; Spano et al., 2010). Additionally, starter may control BA in food via their amine oxidase activity because oxidizes BA into aldehyde, hydrogen peroxide, and ammonia (Suzzi and Gardini, 2003; Naila et al., 2010; Spano et al., 2010). In red wine, the most abundant amines are usually putrescine, histamine, tyramine, and cadaverine (Beneduce et al., 2010), the last being considered the most important reason for wine intolerance (Konakovsky et al., 2011). Inoculation with starter cultures that are unable to produce BA is a feasible option for the control of these chemical substances in wine (Martín-Álvarez et al., 2005; Spano et al., 2010). However, it does not represent the definitive solution when the dominance of starter cultures on the indigenous BA-producer microflora is not sufficient. Recently, García-Ruiz et al. (2011) isolated some LAB from wine and other oenological source and tested their ability to degrade BA. Whereas a negative influence of the wine matrix was observed, their findings indicated a potential application of wine LAB in order to reduce BA in wine (García-Ruiz et al., 2011).

In this paper we report the selection of autochthonous strains of *Lactobacillus plantarum* able to degrade BA and their suitability to be used as malolactic starter in wine production.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

The LAB strains included in this study were routinely cultured on MRS (de Man-Rogosa–Sharp, Oxoid, Milan, Italy) medium. The pH of the medium was adjusted to pH 5.8 with KOH before sterilization for 15 min at 121˚C. Samples from 10 natural vinifications of "Nero di Troia" wines were aseptically collected at the early stages of malolactic fermentation from winery located in Apulia region (Italy). For microbiological analyses, 10 ml of each sample were homogenized with 90 ml of a saline–peptone water, after which serial dilutions were prepared. For the isolation of LAB, MRS agar containing 100 mg/l of cycloheximide was used, and the plates were incubated under anaerobic conditions (BBL, GasPack-System) at 30˚C for 72 h. Isolates were identified as bacilli and putative LAB by positive Gram staining and negative catalase assay. All strains were stored at −80˚C in MRS supplemented with glycerol (20% v/v).

STRAINS IDENTIFICATION

Genomic DNA of putative LAB was isolated using the Microbial DNA extraction kit (Cabru, Milan, Italy) according to manufacturer's procedure.

Preliminary identification of *L. plantarum* strains was performed by amplification of the *recA* gene. Primers planF and pREV were used in the PCR tests (Torriani et al., 2001). For PCR amplification about 50 ng of genomic DNA were added to a 50 μl PCR mixture and amplified with Taq DNA Polymerase (Qiagen, Milan, Italy). The temperature profile was as follows: 94˚C for 5 min; 30 cycles of 94˚C for 0.5 min, 46˚C for 0.5 min, 72˚C for 0.5 min; 72˚C for 5 min. Furthermore, the isolated strains were identify by partially sequencing the 16S rRNA-encoding gene. Most of the gene encoding the 16S ribosomal RNA was amplified by PCR using primers pA and pH according to Edwards et al. (1989). The amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Milan, Italy) and sequenced. The resulting sequences were compared with sequences available at NCBI database (GenBank) using the standard nucleotide–nucleotide homology search basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

In order to verify if the bacteria analyzed in this study harbor genes encoding enzymes involved in production of BA such as tyramine, histamine, and putrescine, a multiplex-PCR-based assay was carried out to detect the presence of tyrosine decarboxylase (*tdc*), histidine decarboxylase (*hdc*), and ornithine decarboxylase (*odc*) genes (Coton et al., 2010). About 50 ng of genomic DNA was added to a 50 μl PCR mixture and amplified with Taq DNA Polymerase (Qiagen, Milan, Italy). The reaction mix was cycled through the following temperature profile: 94˚C for 5 min; 15 cycles of 94˚C for 1 min, 55˚C for 1.3 min, 72˚C for 1 min; 12 cycles of 94˚C for 1 min, 58˚C for 1.3 min, 72˚C for 1 min; 72˚C for 5 min.

PCR fragments were analyzed by agarose gel electrophoresis carried out by loading 10 μl of sample on 1.2% agarose gels.

ANALYSIS OF BA BY HPLC AND PRELIMINARY SCREENING

In order to test the individual degrading capacity of isolated strains, BA concentration was quantified in MRS broth samples supplemented with putrescine (1 mM), tyramine (1 mM), cadaverine (1 mM), or histamine (2.5 mM) after 24 h with or without strain inoculation (2×10^6 CFU ml^{-1}; total volume 20 ml). One milliliter of each sample was centrifuged and the supernatant was filtered through 0.45 μm PTFE filters (VWR, USA). Five microliters were derivatized using dansyl chloride, separated, and analyzed using the conditions described by Ladero et al. (2011).

VALUATION OF DEGRADING BACTERIA *IN VITRO*

MRS broth samples (1 ml) supplemented with putrescine (1 mmol l^{-1}), tyramine (1 mmol l^{-1}), cadaverine (1 mmol l^{-1}), and histamine (2.5 mmol l^{-1}) were collected after 24 h with and without degrader strain inoculation (starting from an OD_{600} of about 0.05; total volume 1 l). In a separate trial the two strains were co-inoculated (starting from an OD_{600} of about 0.05 for each strain; total volume 1 l). MRS broth samples (1 ml) supplemented with tyrosine (15 mmol l^{-1}) or agmatine (4.38 mmol l^{-1}; Arena et al., 2011) were collected after 24 h from inoculation with degrader strain (starting from an OD_{600} of about 0.05) and producer strain (starting from an OD_{600} of about 0.05; total volume 1 l). BA content was determined as previously indicated and degradation activity was expressed as a percentage of the control without degrader strain. The producer strains were *Lactobacillus brevis* IOEB 9809 (Lucas et al., 2007; Arena et al., 2010) and *Enterococcus*

faecium OT23 (Capozzi et al., 2011), respectively for putrescine and tyramine.

SURVIVAL IN WINE-LIKE MEDIUM AND MALOLACTIC FERMENTATION

The composition of the synthetic must and winemaking conditions were based on the protocol described by Capozzi et al. (2010). All components were purchased from Sigma, Milan, Italy. The synthetic musts contained glucose (96.5 g l^{-1}), fructose (108.5 g l^{-1}), DL-malic acid (10 g l^{-1}), L-tartaric acid (2 g l^{-1}), ammonium chloride (0.2 g l^{-1}), and yeast carbon base (11.7 g l^{-1}); pH was adjusted to 3.5 with NaOH. The must was clarified and sterilize by filtration through a 0.22 μm pore size filter (Millipore, Italy). The must was inoculated at the concentration of 10^6 colony-forming unit (CFU) ml^{-1} with a commercial yeast culture (*Saccharomyces cerevisiae* strain AWRI R2, Maurivin, Australia), and alcoholic fermentation was performed at 22°C for 22–30 days. Then, the wine was clarified by filtration through a 0.22 μm pore size filter (Millipore, Italy) to remove yeasts. After filtration, the physico-chemical properties of the wine-like medium were: 12.1% (v/v) ethanol, 4.9 g of L-malic acid per liter, pH 3.5. Wine was stored at 4°C until required for further experiments. MLF was initiated by direct inoculation with *L. plantarum* strains grown at pH 3.5 for 16 h ($OD_{600\,nm} = 0.6$) to a final concentration of 2×10^6 CFU ml^{-1} in 50 ml of wine. A control without inoculation was performed to verify any spontaneous MLF. Thereafter, the containers were incubated at 18°C. Bacterial numeration was performed by counting cells (CFU ml^{-1}) spread on agar plates of MRS, pH 5.8, and incubated under anaerobic conditions (BBL, GasPack-System) at 30°C for 72 h. The concentration of L-malic acid was determined with the Boehringer enzymatic kit (Mannheim, Germany) according to the manufacturer.

RESULTS

IDENTIFICATION OF *L. PLANTARUM* WINE ISOLATES AND SELECTION OF THOSE WITH ABILITY TO DEGRADE BA

Samples from 10 natural vinifications of "Nero di Troia" wines were collected in Apulian wineries. A total of 100 microbial isolates were randomly recovered from the analyzed samples using MRS agar containing cycloheximide in order to inhibit yeasts growth. Following a preliminary Gram stain and catalase reaction, 63 isolates considered as presumptive LAB (i.e., being Gram positive and catalase negative) were selected. These strains were screened by PCR with primers planF and pREV, using *L. plantarum* WCFS1 as positive control. The amplicon, of approximately 318 bp, was present in both the DNA extracted from the positive control and the DNA extracted from 26 of the 63 strains tested. 16S rDNA sequence analysis confirmed that all 26 isolated strains belong to *L. plantarum* species.

Furthermore, the *L. plantarum* strains, named NDT with numbers from 01 to 26, were examined for their ability to degrade histamine, tyramine, cadaverine, and putrescine, the main BA present in wines. Of these, 30.8% were able to degrade putrescine, 26.9% tyramine, 19.2% histamine, and 19.2% cadaverine. The five strains demonstrating a percentage of BA degradation >5% are reported in **Table 1**. These strains belong to five different vinifications of three diverse wineries. In reason of entity of degradation, *L. plantarum* NDT 09 (22.12% of tyramine degraded) and *L. plantarum* NDT 16 (31.09% of putrescine degraded) strains were selected as good candidates for further characterization. The presence of the main genes involved in BA production in these two strains was also evaluated. However, none of the *L. plantarum* strains bacteria analyzed harbor genes involved in BA production (data not shown).

ABILITY OF *L. PLANTARUM* NDT 09 AND *L. PLANTARUM* NDT 16 TO DEGRADE TYRAMINE AND PUTRESCINE IN CULTURE MEDIA

The ability of *L. plantarum* NDT 09 and *L. plantarum* NDT 16 to degrade tyramine and putrescine was analyzed in 11 of culture medium, also in simultaneous inoculation (**Figure 1**). After 24 h of incubation, *L. plantarum* NDT 09, *L. plantarum* NDT 16, and NTD 09-NTD 16 co-inoculation showed 22.58, 2.83, and 26.74% of tyramine degradation respectively (**Figure 1**). In the case of putrescine the breakdown was 3.53% for *L. plantarum* NDT 09, 31.43% for *L. plantarum* NDT 16, and 35.28% for the simultaneous inoculation trial (**Figure 1**).

ABILITY OF *L. PLANTARUM* NDT 09 AND *L. PLANTARUM* NDT 16 TO DEGRADE TYRAMINE AND PUTRESCINE IN PRESENCE OF BA PRODUCERS

Finally, in order to mimic the real dynamics of production/degradation of tyramine and putrescine, we used the producer strains *L. brevis* IOEB 9809 (putrescine) and *E. faecium* OT23 (tyramine), respectively in presence of tyrosine

Table 1 | Degradation (in percentage) of putrescine, tyramine, and histamine by *Lactobacillus plantarum* strains isolated from wine after 24 h of culture in MRS broth (20 ml) supplemented with putrescine (1 mM), tyramine (1 mM), histamine (2.5 mM), and cadaverine (1 mM).

Strain	Degradation percentage			
	Putrescine	Tyramine	Histamine	Cadaverine
L. plantarum NDT 03	7.15	n.d.	8.63	n.d.
L. plantarum NDT 09	3.47	22.12	2.21	1.83
L. plantarum NDT 10	11.05	n.d.	3.66	n.d.
L. plantarum NDT 16	31.09	2.75	7.49	3.41
L. plantarum NDT 21	8.72	13.33	7.19	6.68

Degradation activity is expressed as a percentage of control without strain. The data presented are mean values from three separate experiments; n.d., no detected effect.

(15 mmol l^{-1}) and agmatine (4.38 mmol l^{-1}; **Figure 2**). Twenty-four hours after inoculation with degrader and producer strains, tyramine and putrescine concentration were lowered to 29.62 and 38.17% respectively. Moreover, an increase capability to eliminate the BA analyzed was noted when co-inoculation experiments (producers vs. degraders) were performed. The increase degradation of BA observed, could be due to the degradation activity of the *L. plantarum* strains analyzed, but could also be attributable to inhibition phenomena (for example by production of bacteriocins) of the degrader on the producer strains.

SURVIVAL IN WINE-LIKE MEDIUM AND MALOLACTIC FERMENTATION OF *L. PLANTARUM* NDT 09 AND *L. PLANTARUM* NDT 16

In order to analyze the survival and fermentation suitability of *L. plantarum* strains able to degrade BA, the strains were inoculated in a wine-like medium. The synthetic must was subjected to fermentation by a commercial oenological yeast strain to achieve 12.1% v/v of ethanol content. In three independent experiments, MLF was induced with *L. plantarum* strains NDT 09 and NDT 16 previously adapted at pH 3.5. The results obtained by the quantification of bacterial biomass and L-malic acid degradation are reported in **Figure 3**. The sampling times were 0, 1 h, 1, 2, 3, 7, 14, and 21 days after strain inoculation. Neither degradation of L-malic acid nor spontaneous growth was observed within the control wine (uninoculated wine). The *L. plantarum* NDT 09 and *L. plantarum* NDT 16 strains were able to survive in the wine-like medium (**Figure 3**). An initial decrease in the survival with respect of expected population (2×10^6 CFU ml^{-1}) was noticed after the inoculation in wine, probably due to the harsh conditions imposed to bacterial cells. Bacterial population remained stable after 1 h. Subsequently, LAB biomass concentration diminished progressively up to the seventh day post inoculation (**Figure 3**). The strain *L. plantarum* NDT 16 better survive in wine-like medium than *L. plantarum* NDT 09 (**Figure 3**). At the 14th day after inoculation, no viable cell was detected. Both strains were found able to degrade L-malic acid. The efficient consumption of this dicarboxylic acid was conducted up to the 14th day after inoculation (**Figure 3**). *L. plantarum* NDT 16 was found to better degrade L-malic acid than *L. plantarum* NDT 09. After the 14th day, consistently to the findings in the survival, also L-malic acid degradation stopped. The growth level and the L-malic acid consumption rates showed to be strain-dependent. Overall, the results reported demonstrated that the analyzed strains could be tested as malolactic strains for wine with low L-malic acid contents.

DISCUSSION

The biotechnological approach reported in this paper, aimed to conciliate malolactic fermentation and BA degradation within the same biotechnological resources: the malolactic starter cultures. The main species involved in malolactic fermentation in wine is *Oenococcus oeni*. However, as reported by García-Ruiz et al. (2011), in the wine environment, the highest potential for BA degradation among LAB seem to be related to *Lactobacillus* and *Pediococcus* species, while *O. oeni* demonstrated low and rare degradation characteristics (García-Ruiz et al., 2011). In the last years, *Lactobacillus* spp. received increasing attention as an important resource for the design of a new generation of malolactic fermentation starter cultures (du Toit et al., 2010), insomuch as we found in commerce malolactic starter formulate using *L. plantarum* V22 strain released in 2010 by Lallemand (Cho et al., 2011; Miller et al., 2011). Other than malic acid decarboxylation, the main advantages of *L. plantarum* employed were: resistance to the stressing wine conditions, production of wine aroma compounds, production of plantaricins, all important factors to compete with spoilage LAB (du Toit et al., 2010).

In this work, we selected two *L. plantarum* strains (named NDT 09 and NDT 16) from a pool of *L. plantarum* strains isolated from red wine undergoing malolactic fermentation able to degrade BA such as putrescine and tyramine. The degradation rates observed was higher than that previously reported for *L. plantarum* strains (Leuschner et al., 1998; García-Ruiz et al., 2011). Furthermore, the strains were evaluated in wine-like medium for

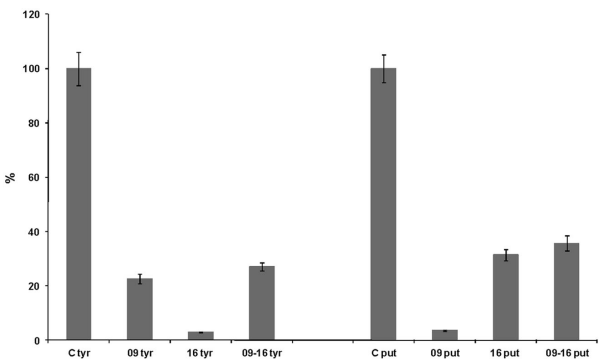

FIGURE 1 | Degradation (in percentage) of putrescine and tyramine by *Lactobacillus plantarum* strains NDT 09 and NDT 16 isolated from wine after 24 h of culture in MRS broth (1 l) supplemented with putrescine (1 mM) and tyramine (1 mM). A trial with the co-inoculation of both strains was performed. Degradation activity is expressed as a percentage of control without strain(s). Data are the mean ± SD, for $n = 3$.

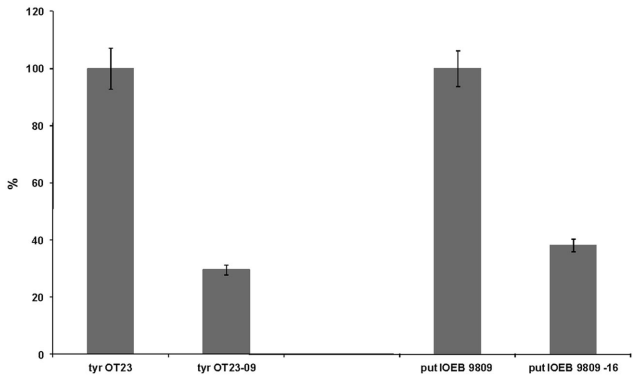

FIGURE 2 | Degradation (in percentage) of putrescine and tyramine by *Lactobacillus plantarum* strains NDT 09 and NDT 16 in presence of producer strains *Lactobacillus brevis* IOEB 9809 (putrescine) and *Enterococcus faecium* OT23 (tyramine), respectively in MRS added with tyrosine (15 mmol l^{-1}) and agmatine (4.38 mmol l^{-1}). BA were detected after 24 h of culture. Degradation activity is expressed as a percentage of control without degrader strain. Data are the mean ± SD, for $n = 3$.

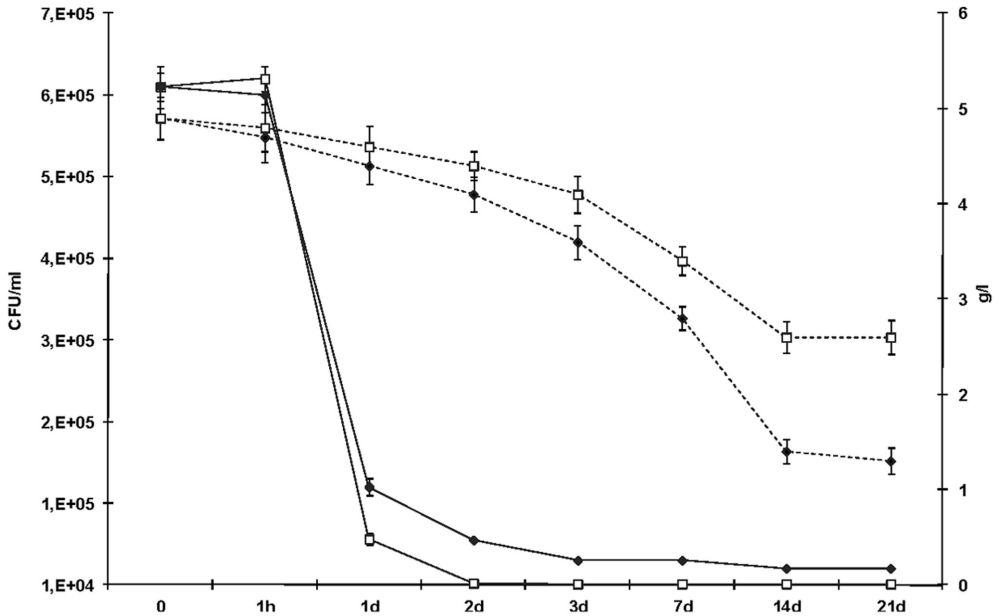

FIGURE 3 | Evolution of the bacterial population during MLF in wine-like medium separately carried out by the two selected *Lactobacillus plantarum* strains. Samples were taken at the time of inoculation (time zero) and after 1 h, 1, 2, 3, 7, 14, and 21 days. *L. plantarum* NDT 09 (open squares, continuous line) and *L. plantarum* NDT 16 (filled rhombi, continuous line) counts on de Man Rogosa Sharpe agar were reported in figure. Also the evolution of l-malic acid consumption during MLF was monitored (*L. plantarum* NDT 09, open squares, dotted line; *L. plantarum* NDT 16, filled rhombi, dotted line). Data are the mean ± SD, for $n = 3$, **Figure 1**.

their suitability to degrade malic acid. Although MLF was incompletely performed, the two biotypes showed a respectable aptitude to degrade malic acid, indicating a possible application in reason of wine/must malic acid content. As BA production ability was found to be both species- and strain-dependent (Bover-Cid and Holzapfel, 1999; Russo et al., 2010; Ladero et al., 2012), our work confirmed the scientific evidence indicating a species- and strain dependence also in BA degradation by LAB. Our findings provided the basis for an innovative approach that might be of particular interest for hot climate regions where grapes are harvested at high maturity usually resulting in wine with pH higher than pH 3.5, high ethanol concentration, low malic acid content, and with considerable spoilage-associated risks. Further studies are ongoing in order to select new *L. plantarum* strains able to degrade histamine, and to analyze the influence of wine conditions on BA degradation and malic acid consumption by *L. plantarum* NDT 09 and *L. plantarum* NDT 16. Indeed, as indicated by García-Ruiz et al. (2011), wine chemicals such as ethanol (12%) and polyphenols (75 mg/l), and additives like SO2 (30 mg/l) may reduce the degrading ability of LAB strains.

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Strains of *Staphylococcus* and *Bacillus* isolated from traditional sausages as producers of biogenic amines

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Histidine, lysine, ornithine, and tyrosine decarboxylase activities were tested in 38 strains of *Staphylococcus* (15 of *S. equorum*, 11 of *S. epidermidis*, 7 of *S. saprophyticus*, and 5 of *S. pasteuri*) and 19 strains of *Bacillus* (13 of *B. subtilis* and 6 of *B. amyloliquefaciens*) isolated from two Spanish traditional sausage varieties. The four decarboxylase activities were present in most of the strains studied, but some variability was observed between strains within each microbial species. Accumulation of putrescine and cadaverine was assessed in the culture media of the strains that displayed ornithine and lysine decarboxylase activities. The aminogenic potential of the strains was low, with amounts accumulated lower than 25 mg/L for the putrescine and than 5 mg/L for the cadaverine, with the exception of a strain of *S. equorum* that produced 1415 mg/L of putrescine, and of a strain of *S. epidermidis* that accumulated 977 mg/L of putrescine and 36 mg/L of cadaverine.

Keywords: decarboxylase activities, biogenic amines, *Staphylococcus, Bacillus*, putrescine, cadaverine, traditional sausages

INTRODUCTION

Biogenic amines are basic nitrogen compounds usually formed by decarboxylation of precursor amino acids (Janz et al., 1983; Halász et al., 1994; Silla Santos, 1996).

Formation of biogenic amines in foods is important for health and also for unfavorable effects on flavor (Suzzi and Gardini, 2003). Biogenic amines affect blood pressure, and excessive quantities in food can trigger migraines, gastric and intestinal problems, and allergic responses in sensitive people (Smith, 1980; Taylor, 1985; Stratton et al., 1991). These substances are especially dangerous in people being treated with monoaminooxidase enzyme inhibitors (Stratton et al., 1991).

During ripening of meat products, the proteins undergo degradation processes; large peptides are first generated and then degraded into oligopeptides, and these are in turn degraded to free amino acids. The free amino acids are then catabolized, giving rise to different compounds such as ammonia, α-ketoacids, methylketones, and amines.

In meat products, formation of biogenic amines is largely associated with the activity of microorganisms present in meat (Ten Brink et al., 1990; Shalaby, 1996; Paulsen and Bauer, 1997). Ripening of sausages provides conditions that are very favorable for the production of biogenic amines, due to the active growth of microbial populations, acidification, and proteolysis.

Different measures have been taken with the aim of preventing or minimizing formation of biogenic amines during the manufacture of raw-cured sausages, such as improved hygiene in production plants, the use of starter cultures formed by lactic acid bacteria with acidifying capacity, and the use of certain preservatives (Buncic et al., 1993; Maijala et al., 1993; Bover-Cid et al., 2000a,b; Suzzi and Gardini, 2003; Komprda et al., 2004; Lu et al., 2010). Although such practices usually reduce the production of biogenic amines, they do not totally prevent the production, and moreover, the increased proteolysis that results from the use of starter cultures may actually increase the availability of amino acids precursors.

Complete inhibition of biogenic amine formation during production of sausages, without any adverse effects, is desirable. However, production of biogenic amines is an extremely complex phenomenon that depends on several variables such as the growth kinetics of the microorganisms and their proteolytic and decarboxylase activities. In order to design strategies for specific inhibition of the production of these compounds, it is essential to obtain information about the potential production of biogenic amines by the microorganisms present in fermented meat products.

Of all the microbial groups present during the fermentation/ripening of the raw-cured sausages, the Enterobacteriaceae and the lactic acid bacteria have been abundantly studied as producers of biogenic amines. Information in the literature about the ability of the *Staphylococcus* species to produce biogenic amines is more reduced (Masson et al., 1996; Silla Santos, 1998; Martín et al., 2006; Drosinos et al., 2007; Bonomo et al., 2009; Even et al., 2010), and studies on the production of biogenic amines by *Bacillus* species are practically inexistent.

The objective of this research was to investigate the decarboxylase activity and the ability to produce biogenic amines "*in vitro*" by the species of *Staphylococcus* and *Bacillus* isolated from two Spanish traditional sausage varieties, with the aim of to elucidate the role of the microorganisms belonging to these two genera in the production of biogenic amines during the manufacture of the fermented and ripened sausages.

MATERIALS AND METHODS

BACTERIAL STRAIN IDENTIFICATION AND MOLECULAR TYPING

In this study, 38 strains of *Staphylococcus* (15 of *S. equorum*, 11 of *S. epidermidis*, 7 of *S. saprophyticus*, and 5 of *S. pasteuri*) and

19 strains of *Bacillus* (13 of *B. subtilis* and 6 of *B. amyloliquefaciens*) were used. The strains were isolated from 20 units of Androlla sausage and from 15 units of Botillo sausage (two Spanish traditional sausage varieties) at the end of the manufacturing process. Manufacture process and features of these two sausages have been previously described (Lorenzo et al., 2000). The strains were initially identified by classical methods in previous researches (García Fontán et al., 2007a,b) and their identity was confirmed prior carrying out the present work by sequentiation of the 16S rRNA gene, comparing the obtained sequences with those available in the database GenBank (National Center for Biotechnology Information, Bethesda, MD, USA).

Strains were molecular typed by $(GTG)_5$-PCR fingerprinting techniques. Genomic DNA extracted from each strain was subjected to rep-PCR analysis using the single oligonucleotide primer $(GTG)_5$ (Iacumin et al., 2006). Reactions were carried out in a final volume of 25 μL containing 12.5 μL of 2× ReddyMix 1.5 mM $MgCl_2$ (ABgene, Epsom, UK), 2 μL of extracted DNA, and 1 μM of $(GTG)_5$ primer. Amplifications were performed in a MyCycler thermal cycler (Bio-Rad, Hercules, USA). Initial denaturation (95°C, 2 min) was followed by 31 cycles of denaturation at 94°C for 3 s, a step at 92°C for 30 s, primer annealing at 40°C for 1 min and extension at 65°C for 8 min. The last cycle was followed by the final single extension step (65°C, 8 min).

Amplicons were separated by electrophoresis in a 1.5% agarose gel in buffer TBE 1× at 75 V for 2 h. After the run, gels were stained with ethidium bromide 1 μg/mL (Sigma-Aldrich, St. Louis, USA) for 30 min. The resulting fingerprints were visualized under UV light and digitally captured using the imaging system Gel Doc XR+ (Bio-Rad, Hercules, USA) and analyzed with the Quantity One software (Bio-Rad, Hercules, USA).

Strains were stored at −80°C in BHI broth (Oxoid Ltd., Basingstoke, Hampshire, UK), with 20% glycerol as a cryoprotective agent. Before use, the strains were reactivated by incubation in BHI broth at 37°C.

PREPARATION OF INOCULA

In order to prepare the inocula used in the quantitative analysis, firstly a correlation between the log CFU/mL and the Optical Density (at 650 nm) of the cultures was established for each strain by determining throughout the growth the O.D. and the log CFU/mL by plate counting in BHI agar (OXOID).

Samples of BHI broth cultures were collected after 24 h of incubation, the O.D. was measured (in order to calculate the number of CFU/mL), the cultures were centrifuged at 12000 × *g* and the cells were washed by resuspension in a solution of 0.85% NaCl and centrifugation at 12000 × *g* (three times). Finally, the cells were suspended in the 0.85% NaCl solution to provide inocula containing 10^9 CFU/mL.

PRELIMINARY QUALITATIVE TESTS FOR BIOGENIC AMINE PRODUCTION

As a preliminary test of the capacity of the bacterial strains to produce biogenic amines, the method described by Joosten and Northolt (1987) was used. The culture medium used contained tryptone (0.5%), yeast extract (0.5%), NaCl (0.5%), glucose (0.1%), Tween 80 (0.05%), $MgSO_4$ $7H_2O$ (0.02%), $CaCO_3$ (0.01%), $MnSO_4$ $4H_2O$ (0.005%), $FeSO_4$ $7H_2O$ (0.004%), bacteriological agar (2%), and purple bromocresol (0.006%) as pH indicator. The precursor amino acids of each biogenic amine (histidine, lysine, ornithine, and tyrosine) were added individually to the culture medium to a final concentration of 2%. The final pH was adjusted to 5.5 ± 0.1, the medium was sterilized and distributed in Petri dishes. Plates of the culture medium containing each one of the precursor amino acids were streaked, in order to obtain individual colonies, with each bacterial strain. The plates were incubated at 37°C and examined after 12, 24, 48, 72, and 120 h of incubation; a positive result was manifested by the appearance of a purple halo around the colonies.

QUANTITATIVE ANALYSIS OF THE BIOGENIC AMINES PRODUCED BY THE BACTERIAL STRAINS

In a previous study (Lorenzo et al., 2008), the different biogenic amines were quantified in the sausage units from which the microbial strains tested in the present work were isolated. We observed that in these sausages the putrescine and cadaverine were by far the major biogenic amines. In order to quantify the production of each biogenic amine (putrescine and cadaverine) by the different bacterial strains, in each bacterial strain, and for each individual precursor amino acid (ornithine and lysine), 2 tubes (5 mL each) of the culture medium (Joosten and Northolt, 1987) containing 2% of the corresponding individual precursor amino acid were each inoculated with 0.1 mL of a solution (0.85 g NaCl/L), containing 10^8 CFU. The tubes, with a final concentration of 2×10^7 CFU/mL, were incubated at 37°C for 72 h (previously, quantification of the biogenic amines was performed along 96 h of growth, showing that for most strains maximum accumulation took place after 72 h of incubation). After incubation, the O.D. was measured in one tube, and the corresponding biogenic amine was determined in the other. Firstly, 1 mL of 2 N HCl was added to the tube in order to stop microbial growth and decarboxylation. The content of the tube was then placed in a 25 mL volumetric flask, 1 mL of 1,7-diaminoheptane (internal standard) was added, and the final volume was made up with a 0.6 N $HClO_4$ solution. An aliquot (0.5 mL) of the mixture was then immediately placed in a tube, and 100 μL of 2 N NaOH (to make the solution more alkaline), 150 μL of a saturated solution of $NaHCO_3$, and 1 mL of dansyl chloride, were added consecutively. The tube was shaken gently, and placed in a water bath at 40°C for 45 min. In order to remove residues of dansyl chloride, 50 μL of ammonia were then added and the mixture was left to stand for 30 min. Finally, the volume was made up to 2.5 mL with acetonitrile and the mixture was filtered (0.25 μm).

Separation, identification, and quantification of the biogenic amines were carried out by HPLC, following the procedure described by Eerola et al. (1993), using the equipment and chromatographic conditions reported by Lorenzo et al. (2010).

A standard solution containing appropriate amounts of agmatine, tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, spermine, and 1,7-diaminoheptane (as internal standard) was used to quantify the biogenic amines present in the samples.

All the samples and standards were injected at least in duplicate in different days. Repeatability tests were performed by injecting

a standard and a sample consecutively six times in a day. Reproducibility tests were also carried out by injecting the standard and the sample twice a day for 3 days, under the same experimental conditions. There were no significant differences ($P < 0.05$) in the results obtained in these tests.

The quantity of each biogenic amine was expressed in milligram per liter.

RESULTS

MOLECULAR TYPING OF THE *STAPHYLOCOCCUS* AND *BACILLUS* STRAINS ISOLATED FROM TRADITIONAL SAUSAGES

The obtained resulting fingerprints of the strains of *Staphylococcus* and *Bacillus* used in the present study demonstrated that the strains belonging to the same species were different. This is important, because some strains belonging to the same species came from the same sausage unit and could be the same.

DECARBOXYLASE ACTIVITY OF THE *STAPHYLOCOCCUS* AND *BACILLUS* STRAINS ISOLATED FROM TRADITIONAL SAUSAGES

The decarboxylase activity of the *Staphylococcus* strains tested is shown in **Table 1**. In this table, results reported in the literature for the same species were summarized. The *Staphylococcus* strains displayed a great variability in the histidine decarboxylase activity, with 7 of the 15 strains of *S. equorum* (46.67%), 9 of the 11 strains of *S. epidermidis* (81.82%), 6 of the 7 strains of *S. saprophyticus* (85.71%), and 4 of the 5 strains of *S. pasteuri* (80%) displaying this activity. The tyrosine decarboxylase, ornithine decarboxylase, and lysine decarboxylase activities were less variable. All strains of *S. saprophyticus* displayed tyrosine, ornithine, and lysine decarboxylase activities, whereas the frequency of the presence of these activities was slightly lower in strains of *S. equorum*, *S. epidermidis*, and *S. pasteuri*. Lysine was the amino acid decarboxylated with a higher frequency; the 100% of the strains of *S. equorum* and *S. saprophyticus*, the 91% of the strains of *S. epidermidis* and the 80% of the strains of *S. pasteuri* were able to decarboxylate this amino acid.

The decarboxylase activity observed in the *Bacillus* strains tested is shown in **Table 2**. Of the 13 strains of *B. subtilis* studied, 10 displayed histidine decarboxylase activity (76.92%), 12 tyrosine decarboxylase activity (92.31%), 8 ornithine decarboxylase activity (61.54%), and 8 lysine decarboxylase activity (61.54%). The strains of *B. amyloliquefaciens* showed less variable behavior; of the six strains studied, four were able to decarboxylate histidine (66.72%), and six were able to decarboxylate tyrosine, ornithine, and lysine (100%).

BIOGENIC AMINE ACCUMULATION AFTER 72 h OF GROWTH

The values of putrescine and cadaverine accumulated after 72 h of growth by the strains of *Staphylococcus* and *Bacillus*, respectively are shown in **Tables 3** and **4**.

The quantities of putrescine produced by the *Staphylococcus* strains tested ranged from 1.46 to 1415.05 mg/L in *S. equorum* (although one strain accumulated 1415.05 mg/L, the rest accumulated less than 25 mg/L) and from 0.37 to 977.13 mg/L in *S. epidermidis* (although one strain accumulated 977.13 mg/L, the rest accumulated less than 15 mg/L). The strains of *S. pasteuri*

Table 1 | Decarboxylase activity of the *Staphylococcus* strains isolated from traditional sausages in this work and from different sausages and other sources in previous studies.

Species	No. of strains	No. of strains positive	%	Bonomo et al. (2009)	Drosinos et al. (2007)	Even et al. (2010)	Martín et al. (2006)	Masson et al. (1996)	Silla Santos (1998)
HISTIDINE DECARBOXYLASE ACTIVITY									
Staphylococcus equorum	15	7	47	0	0	0	NT	NT	NT
S. epidermidis	11	9	82	NT	NT	3	14	NT	NT
S. saprophyticus	7	6	86	0	7	0	NT	0	<70
S. pasteuri	5	4	80	0	NT	NT	NT	NT	NT
TYROSINE DECARBOXYLASE ACTIVITY									
S. equorum	15	14	93	0	0	nt	NT	NT	NT
S. epidermidis	11	8	73	NT	NT	nt	0	NT	NT
S. saprophyticus	7	7	100	0	50	nt	NT	100	100
S. pasteuri	5	5	100	0	NT	nt	NT	NT	NT
ORNITHINE DECARBOXYLASE ACTIVITY									
S. equorum	15	14	93	0	0	0	NT	NT	NT
S. epidermidis	11	10	91	NT	NT	3	21	NT	NT
S. saprophyticus	7	7	100	0	16	3	NT	NT	<70
S. pasteuri	5	4	80	0	NT	NT	NT	NT	NT
LYSINE DECARBOXYLASE ACTIVITY									
S. equorum	15	15	100	86	0	0	NT	NT	NT
S. epidermidis	11	10	91	NT	NT	3	21	NT	NT
S. saprophyticus	7	7	100	50	50	3	NT	NT	<70
S. pasteuri	5	4	80	100	NT	NT	NT	NT	NT

(NT) Species not tested in the corresponding study. (nt) Decarboxylase activity not tested in the corresponding study.

also produced variable quantities of putrescine, with concentrations ranging between 1.19 and 12.39 mg/L. The strains of *S. saprophyticus* generally produced less putrescine, at less variable concentrations ranging from 0.43 to 1.91 mg/L.

Regarding the production of cadaverine, the quantities accumulated were again very variable within the strains of *S. equorum* (from 0.25 to 5.31 mg/L) and *S. epidermidis* (from 0.46 and 36.52 mg/L). A considerable variability was also registered within the strains of *S. saprophyticus* (from 0.58 to 4.79 mg/L) and *S. pasteuri* (from 0.40 to 4.33 mg/L).

The species that accumulated the highest amounts of putrescine and cadaverine were *S. equorum* and *S. epidermidis*. One of the strains of *S. equorum* accumulated 1415.05 mg/L of putrescine and one of the strains of *S. epidermidis* displayed a high aminogenic ability, accumulating 977.13 mg/L of putrescine or 36.52 mg/L of cadaverine after 72 h of growth in the culture medium.

The *Bacillus* strains in the present study also displayed a high degree of variability in their ability to produce putrescine and cadaverine. In the strains of *B. subtilis*, the quantities accumulated ranged from 0.39 to 18.43 mg/L for putrescine and from 0.43 to 4.29 mg/L for cadaverine. In the strains of *B. amyloliquefaciens*, the quantities ranged from 0.76 to 3.27 mg/L for putrescine, and from 0.53 to 3.07 mg/L for cadaverine.

Table 2 | Decarboxylase activity of the *Bacillus* strains isolated from traditional sausages in this work.

Species	No. of strains	No. of strains positive	%
HISTIDINE DECARBOXYLASE ACTIVITY			
Bacillus subtilis	13	10	77
B. amyloliquefaciens	6	4	67
TYROSINE DECARBOXYLASE ACTIVITY			
B. subtilis	13	12	92
B. amyloliquefaciens	6	6	100
ORNITHINE DECARBOXYLASE ACTIVITY			
B. subtilis	13	8	62
B. amyloliquefaciens	6	6	100
LYSINE DECARBOXYLASE ACTIVITY			
B. subtilis	13	8	62
B. amyloliquefaciens	6	6	100

DISCUSSION

Information in the literature concerning the amino acid decarboxylase activity in strains of the genus *Staphylococcus* is scarce and shows in general that microorganisms belonging to this genus are not significant possessors of these activities. Nonetheless, Silla Santos (1998) reported a high frequency of histidine, tyrosine, ornithine, and lysine decarboxylase activity in strains of *S. xylosus* and *S. saprophyticus* isolated from Spanish fermented sausages, which is in agreement with our results. Furthermore, Martín et al. (2007) observed ornithine and lysine decarboxylase activities in 57% of the strains of *S. xylosus* isolated from Iberian dry-cured sausages.

However, Drosinos et al. (2007) analyzed 300 staphylococci strains isolated from traditional fermented Greek sausages and observed that only a low proportion of strains displayed amino acid decarboxylase activity. The species with the highest proportion of strains that displayed histidine, tyrosine, ornithine, or lysine decarboxylase activity were *S. saprophyticus*, *S. simulans*, and *S. xylosus*, but within each species the proportion of strains that were positive for a specific amino acid decarboxylase activity was never

Table 3 | Values of accumulation of putrescine (mg/L) and cadaverine (mg/L) in the culture medium after 72 h of growth of the *Staphylococcus* strains isolated from traditional sausages.

Species	Putrescine				Cadaverine			
	No. of strains	Range of values*	Average	SD	No. of strains	Range of values*	Average	SD
Staphylococcus equorum	14	1.46–1415.05	112.67	374.92	15	0.25–5.31	1.85	1.59
S. epidermidis	10	0.37–977.13	100.58	308.01	10	0.46–36.52	5.83	10.91
S. saprophyticus	7	0.43–1.91	1.13	0.49	7	0.58–4.79	2.11	1.88
S. pasteuri	4	1.19–12.39	4.47	5.29	4	0.40–4.33	2.35	1.76

**Range of values of the strains.*

Table 4 | Values of accumulation of putrescine (mg/L) and cadaverine (mg/L) in the culture medium after 72 h of growth of the *Bacillus* strains isolated from traditional sausages.

Species	Putrescine				Cadaverine			
	No. of strains	Range of values*	Average	SD	No. of strains	Range of values*	Average	SD
Bacillus subtilis	8	0.39–18.43	3.14	6.19	8	0.43–4.29	1.30	1.50
B. amyloliquefaciens	6	0.76–3.27	1.89	0.94	6	0.53–3.07	1.40	1.11

**Range of values of the strains.*

greater than 50%. Martín et al. (2006) studying 239 *Staphylococcus* strains isolated from fermented sausages reported that only the 14.6% (35 strains) were able to decarboxylate one or more amino acids. In this same way, Even et al. (2010) working with 129 strains of coagulase-negative staphylococci isolated from various environments including cheeses and fermented sausages, observed that only 5 strains (∼6%) were able to produce detectable amounts of biogenic amines.

Bonomo et al. (2009) did not find any tyrosine or ornithine decarboxylase activity in any of the 37 staphylococci strains tested, and only observed histidine decarboxylase activity in two strains of *S. warneri*. As observed in the present study, lysine was the amino acid most frequent decarboxylated, and 62% of strains, belonging mainly to the *S. equorum* and *S. xylosus* species, were able to decarboxylate this amino acid. In the latter study the highest proportion of lysine-decarboxylating strains were in the *S. pasteuri* and *S. succinus* species.

Masson et al. (1996) did not observe histidine decarboxylase activity in any of the tested strains of *S. carnosus*, *S. xylosus*, *S. warneri*, and *S. saprophyticus* isolated from sausages. They observed tyrosine decarboxylase activity in all these strains, but the amounts of tyramine produced never achieved 40 μg/mL. Bover-Cid et al. (2001) did not observe any decarboxylase activity in any of the staphylococci strains tested.

Information regarding the amino acid decarboxylase activity of *Bacillus* strains isolated from meat products is very scarce. Roig-Sagués et al. (1996) analyzed four strains of *Bacillus* spp. isolated from *salchichón* (a Spanish traditional sausage) and found that some of the strains displayed histidine decarboxylase activity and were able to produce histamine, although in very low quantities (about 0.5 μg/mL).

There is some information about *Bacillus* strains isolated from salted and ripened Spanish anchovies. Hernández-Herrero et al. (1999) reported that *B. pumilus* was able to produce histamine, but in low quantities (12–17 μg/mL) and at low environmental NaCl concentrations (0.5–3% NaCl); this ability disappeared at higher concentrations of NaCl (10 and 20%).

Rodríguez-Jerez et al. (1994) analyzed 16 strains of *Bacillus* spp. isolated from Spanish salted semi-preserved anchovies and observed that none of the strains displayed ornithine or lysine decarboxylase activity; the percentage of strains that displayed histidine decarboxylase activity ranged from 75 to 81.25% depending on the culture medium (Niven or modified Niven) used in the test. The quantities of histamine produced ranged from 0 to 10.54 μg/mL.

The aminogenic potential of the *Staphylococcus* and *Bacillus* strains analyzed in the present study was generally low (quantities produced lower than 25 mg/L for putrescine and lower than 5 mg/L for cadaverine), with the exception of one strain of *S. epidermidis* and one of *S. equorum*, which produced higher quantities. These results therefore confirm that the amino acid decarboxylase activities are not particularly high in species of the genera *Staphylococcus* and *Bacillus*, especially when compared with other microbial groups such as Enterobacteriaceae or lactic acid bacteria, present in the fermented meat products (Bover-Cid et al., 2001; Lorenzo et al., 2010).

In the literature, there is little available information on the production of putrescine or cadaverine by *Staphylococcus* species. Martín et al. (2006) observed the production of variable quantities of putrescine (from 25 to >1000 mg/L) and cadaverine (from 25 to 1000 mg/L), being the strains of *S. epidermidis* the main producers. Even et al. (2010) also reported the production of variable quantities of putrescine (from 7 to 1499 mg/L) and cadaverine (from 3 to 140 mg/L).

In the literature, there is no information about putrescine and cadaverine production by species of the genus *Bacillus*, either from meat products or other different origin.

The high variability in biogenic amine production within strains belonging to the same species confirms previous findings (Bover-Cid and Holzapfel, 1999; Martín et al., 2006; Even et al., 2010) and again shows that the amino acid decarboxylase activity is a strain-dependant property. Although the aminogenic capacity of these two bacterial genera is not usually very high, since they can reach high counts in the sausages it is important to reduce their counts by the implementation of rigorous hygienic measures, in order to reduce the risk of accumulation of biogenic amines in the final products.

CONCLUSION

(a) Histidine, lysine, ornithine, and tyrosine decarboxylase activities were present in most of the strains of *Staphylococcus* and *Bacillus* isolated from Spanish traditional sausages. However, some variability was observed between strains, even within the same species.

(b) The production of putrescine and cadaverine by the species of *Staphylococcus* and *Bacillus* isolated from Spanish traditional sausages was in general low, with amounts accumulated lower than 25 mg/L for putrescine and lower than 5 mg/L for cadaverine. Only a strain of *S. equorum* produced 1415 mg/L of putrescine and a strain of *S. epidermidis* accumulated 977 mg/L of putrescine and 36 mg/L of cadaverine.

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Control of biogenic amines in fermented sausages: role of starter cultures

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Biogenic amines show biological activity and exert undesirable physiological effects when absorbed at high concentrations. Biogenic amines are mainly formed by microbial decarboxylation of amino acids and thus are usually present in a wide range of foods, fermented sausages being one of the major biogenic amine sources. The use of selected starter cultures is one of the best technological measures to control aminogenesis during meat fermentation. Although with variable effectiveness, several works show the ability of some starters to render biogenic amine-free sausages. In this paper, the effect of different starter culture is reviewed and the factors determining their performance discussed.

Keywords: starter cultures, biogenic amines, amino acid decarboxylase, fermented sausages, amino oxidase, autochthonous

INTRODUCTION

Biologically active amines are nitrogenous low-molecular-weight substances with biological functions in animals, plants, and microorganisms. The biologically active amines known as biogenic amines include tyramine, histamine, phenylethylamine, tryptamine, putrescine, and cadaverine. They are mainly derived from the bacterial decarboxylation of precursor amino acids and can be found in nearly all types of foods in a wide and variable range of concentrations (Halász et al., 1994; Vidal-Carou et al., 2007).

Histamine, tyramine, and to lesser extent phenylethylamine are the main dietary biogenic amines associated with certain health disruptions, mainly involving vasoactive and psychoactive reactions: histaminic intoxication, food intolerance due to enteral histaminosis, food-induced migraines, and interaction between tyramine and MAOI drugs (Mariné-Font et al., 1995; Spano et al., 2010; Linares et al., 2011). Additionally, high contents of biogenic amines in foods have traditionally been used as an index of undesired microbial activity as a result of hygienically defective manufacturing or storage practices (Mariné-Font et al., 1995; Suzzi and Gardini, 2003; Ruiz-Capillas and Jiménez-Colmenero, 2004). However, the manufacture of fermented foods involve the activity of a variety of microorganisms, not only associated with the desired technological fermentative properties but also undesired contaminants. Both types of microbial activities entail noticeable risk of biogenic amine production. In this respect, the control of biogenic amine accumulation in fermented products is one of the present challenges of the food industry (Vidal-Carou et al., 2007). In this review, the influence of starter cultures as a technological measure to control aminogenesis during meat fermentation is reviewed and the factors determining their performance discussed.

BIOGENIC AMINE CONTENTS IN FERMENTED SAUSAGES

Fermented meat sausages, together with other fermented foods or beverages, constitute one of the food products that can accumulate higher biogenic amine contents (Suzzi and Gardini, 2003; Spano et al., 2010; EFSA Panel on Biological Hazards (BIOHAZ), 2011). **Table 1** summarizes the occurrence of biogenic amines in retail fermented meat products of different countries. Tyramine is usually the most frequent and abundant biogenic amine found in fermented sausages. In terms of average values, it has been reported that fermented sausages show the highest tyramine content among fermented products (EFSA Panel on Biological Hazards (BIOHAZ), 2011). In fermented sausages, tyramine is produced by fermentative microbial population, mainly lactic acid bacteria (LAB, including lactobacilli and enterococci) and more rarely coagulase negative staphylococci (CNS; Straub et al., 1994; Masson et al., 1996; Montel et al., 1999; Bover-Cid et al., 2001a; Aymerich et al., 2006; Martín et al., 2006; Latorre-Moratalla et al., 2010a; Talon and Leroy, 2011). Latorre-Moratalla et al. (2010a) reported that 48% of LAB and 13% of staphylococci isolated from spontaneously fermented sausage are able to decarboxylate one or more amino acids.

The occurrence of putrescine and cadaverine is also quite common but more variable than tyramine. Although the contents of diamines in fermented sausages are relatively low, in a few cases their levels are extremely high, exceeding those of tyramine. The production of diamines is usually attributed to contaminant Gram-negative bacteria, such as enterobacteria and pseudomonas (Bover-Cid et al., 2001a; Durlu-Özkaya et al., 2001; Suzzi and Gardini, 2003). However, a number of publications show several LAB and CNS strains with a powerful capability of producing putrescine and/or cadaverine (Straub et al., 1995; Bover-Cid et al., 2001a; Martín et al., 2006; Latorre-Moratalla et al., 2010a).

Table 1 | Biogenic amine contents (mg/kg fresh matter) in fermented sausages of the retail market from several countries.

	Reference	Product	*n*	Tyramine	Histamine	Phenylethylamine	Tryptamine	Cadaverine	Putrescine
Spanish sausage	Vidal-Carou et al. (1990)	Chorizo	11	176 ± 149[a] (2–509)[b]	76 ± 80 (2–249)	–[c]	–	–	–
	Salchichón	19	133 ± 62 (35–270)	18 ± 27 (1–103)	–	–	–	–	
	Salami	5	6 ± 3 (3–12)	66 ± 39 (2–102)	–	–	–	–	
	Sobrasada	3	8 ± 6 (3–14)	55 ± 36 (14–78)	–	–	–	–	
	Hernández-Jover et al. (1997a)	Chorizo	20	282 ± 129 (30–627)	18 ± 27 (0–314)	1 ± 3 (0–52)	16 ± 20 (0–88)	20 ± 16 (0–658)	60 ± 141 (3–416)
	Salchichón	22	281 ± 109 (53–513)	7 ± 14 (0–151)	7 ± 6 (0–35)	9 ± 11 (0–65)	12 ± 23 (0–342)	103 ± 76 (6–400)	
	Fuet	11	191 ± 73 (32–743)	2 ± 40 (0–358)	2 ± 4 (0–34)	9 ± 8 (0–68)	19 ± 18 (5–51)	72 ± 41 (2–222)	
	Sobrasada	7	332 ± 131 (58–501)	9 ± 17 (3–143)	2 ± 6 (0–39)	12 ± 23 (0–65)	13 ± 14 (3–42)	65 ± 50 (2–501)	
	Bover-Cid et al. (1999a)	Secallona	15	92 ± 72 (1–218)	1 ± 2 (0–5)	4 ± 8 (0–29)	5 ± 11 (0–39)	43 ± 48 (1–115)	80 ± 152 (1–513)
	Fuet delgado	23	119 ± 64 (22–272)	12 ± 34 (0–158)	8 ± 13 (0–47)	8 ± 11 (0–36)	28 ± 42 (2–156)	49 ± 43 (1–169)	
	Salchichón	19	141 ± 124 (3–490)	14 ± 20 (0–59)	12 ± 28 (0–126)	15 ± 33 (0–142)	18 ± 30 (0–127)	99 ± 96 (0–325)	
	Ruiz-Capillas and Jiménez-Colmenero (2004)	Chorizo	3	129 ± 100 (19–214)	6 ± 9 (1–16)	nd	nd	103 ± 113 (9–229)	92 ± 92 (0.8–185)
French sausage	Montel et al. (1999)	Saucisson (industrial)	5	220 (172–268)	71 (16–151)	4 (0–8)	4 (0–9)	103 (31–192)	279 (195–410)
	Saucisson (traditional)	3	164 (84–217)	15 (15–16)	1 (0–4)	nd	71 (39–110)	223 (61–317)	
Italian sausage	Parente et al. (2001)	Soppressata	9	178 (0–557)	22 (0–101)	3 (0–20)	–	61 (0–271)	99 (0–416)
	Salsiccia	10	77 (0–339)	nd	nd	–	7 (0–39)	20 (0–78)	
	Coïsson et al. (2004)	Salamini Italiani	10	205 ± 105 (60–372)	46 ± 54 (8–165)	14 ± 20 (nd–53)	20 ± 25 (nd–69)	–	–
Finnish sausage	Eerola et al. (1998)	Finnish sausage	11	88 (4–200)	54 (0–180)	13 (2–248)	14 (0–43)	50 (0–270)	79 (0–230)
	Russian sausage	4	110 (6–240)	89 (0–200)	11 (1–33)	22 (0–43)	10 (3–18)	93 (3–310)	
	Danish sausage	8	54 (5–110)	9 (1–56)	2 (0–4)	27 (0–91)	180 (0–790)	130 (0–450)	
	Meatwurst	12	72 (5–320)	21 (0–170)	3 (0–5)	18 (0–54)	6 (0–16)	77 (2–580)	
	Lubeck	9	73 (9–150)	6 (0–40)	4 (0–7)	10 (0–20)	3 (0–8)	49 (0–220)	
	Salami	13	93 (3–200)	3 (0–9)	5 (0–8)	20 (0–51)	14 (0–71)	54 (0–210)	
	Pepperoni	11	94 (5–190)	21 (0–200)	6 (0–48)	18 (0–42)	82 (0–390)	61 (0–230)	

Dutch sausage	Brink et al. (1990)		14	110 (40–310)	11 (1–63)	14 (5–45)	–	63 (1–150)	52 (1–190)
Egyptian sausage	Shalaby (1993)	Egyptian sausages	50	14 (10–53)	5 (7–41)	10 (2–81)	13 (3–34)	19 (6–39)	39 (12–100)
Thai sausage	Riebroy et al. (2004)	Som-fug	7	87 ± 72 (19–228)	120 ± 82 (55–291)	–	49 ± 25 (19–86)	161 ± 111 (20–328)	127 ± 90 (17–275)
Turkish sausage	Ekici et al. (2004)	Turkish dry sausage	46	–	32 ± 17 (20–87)	–	–	–	–
	Erkmen and Bozkurt (2004)	Sucuk–factory	19	62 ± 69 (1–189)	69 ± 83 (4–255)	9 ± 20 (0–87)	11 ± 14 (0–47)	–	75 ± 123 (0–383)
		Sucuk–butcher	31	77 ± 92 (2–316)	94 ± 151 (2–478)	6 ± 9 (0–32)	25 ± 31 (0–7)	1 ± 2 (0–7)	121 ± 239 (0–919)

[a] *Mean ± standard deviation when available;* [b] *range (minimum–maximum);* [c] *–not reported; nd, not detected.*

Therefore, fermentative activities can also result in a considerable diamine accumulation.

In contrast, histamine is usually more scarcely found in fermented sausages. However, in some particular samples it may reach quite high levels, usually accompanied by high amounts of other biogenic amines. Histamine production seems to be delimited to some strains of a reduced number of isolates of enterobacteria or LAB, which are not commonly found unless specific contaminations occur (Maijala and Eerola, 1993; Roig-Sagués et al., 1996; Silla-Santos, 1998; Bover-Cid et al., 2001a).

Phenylethylamine and tryptamine could be considered minor amines occurring in fermented sausages. Their accumulation seems dependent on the occurrence of high contents of tyramine associated with some LAB or CNS (Vidal-Carou et al., 2007).

Levels of biogenic amines in fermented sausages show a great variation among different types of products, manufacturers and products from the same manufacturer. The influence of the microbiological quality of raw materials, which varies in each production batch, is a key parameter to explain this variability. Additionally, other factors such as ingredients and additives (sugar, curing agents, spices, etc), diameter of sausage and technological ripening conditions (temperature and relative humidity) can also influence the phenomena associated with aminogenesis, including microbial growth, acidification, proteolysis, and activity of decarboxylases (Maijala et al., 1995; Bover-Cid et al., 1999a, 2001b; Parente et al., 2001; González-Fernández et al., 2003; Bozkurt and Erkmen, 2004; Komprda et al., 2004; Latorre-Moratalla et al., 2012).

STARTER CULTURES FOR AMINOGENESIS CONTROL IN FERMENTED SAUSAGE

TECHNOLOGICAL ROLE OF STARTER CULTURES IN RELATION TO AMINOGENESIS

The hygienic quality of meat raw materials and ingredients is crucial to minimize the occurrence of microbial contaminants, and it thus constitutes a key point in controlling aminogenesis in fermented meat products (Maijala et al., 1995; Bover-Cid et al., 2000a,b; Naila et al., 2010). However, hygiene is a necessary, though not sufficient condition and additional technological measures focused on the control of aminogenic activity of endogenous microbiota are usually needed. Among the possible technological strategies, the use of starter cultures is one of the most important factors quantitatively affecting the accumulation of biogenic amines during sausage fermentation (Bover-Cid et al., 2000c; Suzzi and Gardini, 2003; Naila et al., 2010; EFSA Panel on Biological Hazards (BIOHAZ), 2011; Talon and Leroy, 2011). Indeed, the mechanism of starter cultures is based on preventing the outgrowth of the potential aminogenic endogenous bacteria together with their own inability to produce biogenic amines (Lonvaud-Funel, 2001; Suzzi and Gardini, 2003).

Strains of LAB and CNS specifically selected as starter cultures have to comply with some technological criteria, among which the adaptation to meat fermentation, the ability to compete with the natural (endogenous) microbiota of raw materials and the lack of amino acid decarboxylase capability are the most relevant for the control of biogenic amine production (Buckenhüskes, 1993; EFSA Panel on Biological Hazards (BIOHAZ), 2011; Talon and Leroy, 2011). Some LAB and CNS species usually

used as meat starters (i.e., *L. curvatus* and *S. carnosus*) have been reported as strong biogenic amine producers, mainly of tyramine (Latorre-Moratalla et al., 2010a; Talon and Leroy, 2011). In contrast, species such as *L. sakei*, *L. plantarum*, and *S. xylosus* are usually described as weak or non-aminogenic microorganisms (Bover-Cid et al., 2001a; Aymerich et al., 2006; Latorre-Moratalla et al., 2010a; Linares et al., 2011). However, though some genera or species are more frequently reported than others to be able to produce specific biogenic amines, the ability to decarboxylate amino acids is a strain-dependent property (Bover-Cid and Holzapfel, 1999; Lonvaud-Funel, 2001; Linares et al., 2011). Therefore, it is necessary to carry out a case-by-case evaluation of the aminogenic activity of the strains to be selected as amine negative starter culture. For this purpose, several procedures have been reported (Marcobal et al., 2006; Landete et al., 2007; EFSA Panel on Biological Hazards (BIOHAZ), 2011). Amino acid decarboxylase potential might be tested by molecular techniques detecting specific genes coding for amino-acid decarboxylase. Nevertheless, the aminogenic potential of a given strain should be confirmed through the study of the phenotypic expression of this activity, both *in vitro* (as a screening procedure) and finally in real fermentation and ripening conditions (EFSA Panel on Biological Hazards (BIOHAZ), 2011).

EFFECTIVENESS OF AMINE-NEGATIVE DECARBOXYLASE STARTER CULTURES IN BIOGENIC AMINE REDUCTION

Several studies have evaluated the use of commercial and experimental starter cultures in order to reduce aminogenesis during the fermentation of sausages. Although a number of studies have demonstrated the beneficial effect of starter cultures in reducing biogenic amine accumulation (Maijala et al., 1995; Hernández-Jover et al., 1997b; Bover-Cid et al., 1999b; González-Fernández et al., 2003; Gençcelep et al., 2007; Gücükoglu and Küplülü, 2010; Lu et al., 2010; Baka et al., 2011), other studies failed to demonstrate the efficiency of starter cultures to reduce the presence of biogenic amines in some fermented meat products (Rice and Koehler, 1976; Buncic et al., 1993; Bauer et al., 1994; Paulsen and Bauer, 1997; Roig-Sagués et al., 1997; Parente et al., 2001; Bozkurt and Erkmen, 2002).

Table 2 summarizes the relative reduction of biogenic amines by the use of negative amine producer starter cultures obtained from different experiments. In these studies, different percentages of biogenic amine reduction are observed depending on the bacterial species inoculated, varying from 9% to practically 100%. Moreover, some starters showed an ability to reduce the production of all amines and in other cases only reduce certain amines (Ayhan et al., 1999; Bover-Cid et al., 1999b; Gençcelep et al., 2007; Coloretti et al., 2008; Baka et al., 2011; Casquete et al., 2011).

In general, starters containing LAB species showed a higher effectiveness in biogenic amine reduction than starters including only CNS (Bover-Cid et al., 1999b, 2001b). LAB starter cultures could exert a more efficient replacement of endogenous microbiota with potential aminogenic ability, usually consisting of lactobacilli and enterococci. In particular within starters including LAB species, those containing *L. sakei* or *L. plantarum* are reported to significantly inhibit amine accumulation, though with different intensity depending on the strain and product (Hernández-Jover et al., 1997b; Coloretti et al., 2008; Latorre-Moratalla et al., 2010b; Baka et al., 2011; Tosukhowong et al., 2011). Within CNS, although their proteolytic activity could stimulate the aminogenesis by means of providing the amino acid precursors, Bover-Cid et al. (1999b) showed the potential of proteolytic staphylococci to inhibit biogenic amine production.

Several studies support a greater efficiency in biogenic amine reduction when *L. sakei* was used as the starter in comparison with other species (González-Fernández et al., 2003; Gençcelep et al., 2007; Latorre-Moratalla et al., 2010b; Baka et al., 2011). *L. sakei* are usually well adapted to the ecology of meat fermentation and are competitive between the temperatures of 15 and 25°C, which is the temperature range for sausages manufacture in European Countries (Hugas and Monfort, 1997; Bover-Cid et al., 2001b). In the study carried out by González-Fernández et al. (2003), among all the decarboxylase negative strains tested, *L. sakei* K29 showed the most efficiency in reducing amine production probably because this strain caused a rapid pH drop during sausage fermentation. Bover-Cid et al. (2001b) also described that the amino acid decarboxylase negative strain *L. sakei* CTC494 showed a strong ability to reduce biogenic amine formation in Spanish fermented sausage. However, when this same strain (*L. sakei* CTC494) was combined with *S. carnosus* LHT 2102, *S. xylosus* CTC3037 or *S. xylosus* CTC3050 an even more effective reduction of amine accumulation was achieved compared with the effect of each strain used alone (Bover-Cid et al., 1999b, 2000c). Similarly, Latorre-Moratalla et al. (2010b) described that the rate of reduction was improved when a mixed starter was used. Thus, after the addition of a single strain of *S. equorum,* the contents of cadaverine were reduced by 45% and the single strain of *L. sakei* inoculated in the same product was far more effective, reducing cadaverine by 75%. However, both strains (*L. sakei* and *S. equorum*) used together as a mixed starter reduced cadaverine by 89%. In fact, mixed starters may perform better than single starters being able to control the growth of different bacterial groups (Bover-Cid et al., 2000c; Latorre-Moratalla et al., 2010b; Naila et al., 2010).

Commercial starters usually used in industrial manufacture may not be fully adapted to the meat fermentation environment or more specifically to traditional fermenting conditions. Nowadays, for the fermentation of artisanal sausages the addition of the so-called autochthonous starter cultures consisting of selected strains originating from each specific fermented meat product is recommended (Benito et al., 2007; Talon et al., 2007; Casquete et al., 2011). Thus, an autochthonous starter helps to maintain the typical unique characteristics of artisanal products (Talon et al., 2007). This better adaptation and competitiveness of autochthonous starters compared with commercial ones could explain the greater reduction of biogenic amine contents in artisanal meat fermented products.

In this line, commercial mixed starters combining different LAB and staphylococci species (Hernández-Jover et al., 1997b; Ayhan et al., 1999; Gücükoglu and Küplülü, 2010) showed poorer amine reductions than those provided by mixed autochthonous starter cultures (Talon et al., 2008; Latorre-Moratalla et al., 2010b; Casquete et al., 2011). Talon et al. (2008) and Latorre-Moratalla et al. (2010b) evaluated the effect of autochthonous starter cultures without amino decarboxylase activity on biogenic amine

Table 2 | Different studies on the effect of amine-negative starter cultures on biogenic amine reduction during the manufacture of fermented sausages.

Product	Starter culture	% Of reduction	Reference
Fuet	*Micrococcus carnosus* + *Lactobacillus plantarum* (Texel, France)	25% of TI, 61% of CA, and 25% of PU	Hernández-Jover et al. (1997b)
	M. carnosus + *Pediococcus pentosaceus (Texel, France)*	34% of TI, 50% of CA, and 56% of PU	
Fuet	*Staphylococcus carnosus* LTH 2102	25% of TY, 23% of CA, and 17% of PU	Bover-Cid et al. (1999b)
	S. xylosus CTC3037	69% of TY, 66% of CA, and no effect on PU	
	S. xylosus CTC3050	69% of TY, 17% of CA, and 28% of PU	
Fuet	*L. sakei* CTC494 + *S. carnosus* LTH2102	90% of TY, 87% of CA, and 37% of PU	Bover-Cid et al. (2000c)
	L. sakei CTC494 + *S. xylosus* CTC3037	87% of TY, 87% of CA, and 37% of PU	
	L. sakei CTC494 + *S. xylosus* CTC3050	90% of TY, 87% of CA, and 37% of PU	
Fuet	*L. sakei* CTC494 (high quality raw material)	87% of TY, 38% of HI, 41% of CA, and 67% of PU	Bover-Cid et al. (2001b)
	L. sakei CTC494 (poor-quality raw material)	39% of TY, 29% of HI, 14% of CA ,and 57% of PU	
Fuet	*L. sakei* CTC6626 and *S. xylosus* CTC6013	19% of TY and 46% of PU	Latorre-Moratalla et al. (2010b)
	L. sakei CTC494 and *S. xylosus* CTC6013	45% of TY and 50% of PU	
Salchichón	*P. acidilactici* MS200 + *S. vitulus* RS34*	38% of TY, 74% of HI, and 77% of CA. No effect on PU	Casquete et al. (2011)
		70% of TY, 82% of HI, 64% of CA, and 89% of PU	
	P. acidilactici MS198 + *S. vitulus* RS34*	65% of CA. No effect on HI, TY, and PU	
		58% of HI, 71% of CA, and 72% of PU. No effect on TY	
Chorizo	*L. sakei* K29	98% of TY, 100% of CA and 98% of PU	González-Fernández et al. (2003)
	Pediococcus sp. P22	92% of TY, 67% of CA, and 93% of PU	
	Pediococcus sp. P208 (Rhodia Food, France)	81% of TY, 100% of CA, and 89% of PU	
Chorizo	*L. sakei* CTC6469 + *L. sakei* CTC6626 + *S. xylosus* CTC6013 + *S. xylosus* CTC6169	76% of TY, 97% of CA, and 90% of PU	Garriga et al. (2005)
Xouriço	*L. sakei*	17% of TY and 75% of CA. No effect on PU	Latorre-Moratalla et al. (2010b)
	S. equorum	45% of CA. No effect on TY and PU	
	L. sakei + *S. equorum*	15% of TY and 89% of CA. No effect on PU	
French fermented sausage	*L. sakei* + *S. succinus* + *S. equorum*	87% of TY, 35% of CA, and 38% of PU	Talon et al. (2008)
Salami	*L. plantarum* VLT 73 + *Kokuria varians* MIAL	26% of CA and 27% of PU. No effect on TY and HI	Coloretti et al. (2008)
	L. plantarum VLT 73	47% of CA. No effect on TY, HI, and PU	
Greek fermented sausage	*L. sakei* (+ 0.5% of satureja tymbra extract oil)	62% of TY, 71% of HI, and 100% of PU	Latorre-Moratalla et al. (2010b)
Greek fermented sausage	*L. sakei* 4413	13% of TY and 72% of PU. No effects on CA	Baka et al. (2011)
	L. sakei 8426	25% of CA. No effect on TY and PU	
	L. plantarum 7423	26% of CA. No effect on TY and PU	
	L. curvatus 8427	9% of TY and 29% on PU. No effect on CA	
Finnish fermented sausage	*P. pentosaceus* + *S. carnosus* (Rudolf Müller and Co, Germany)*	41% of TY, 28% of HI, and 86% of CA. No effect on PU	Maijala et al. (1995)
		79% of TY, 62% of HI, and 70% of CA. No effect on PU	
		67% of TY, 77% of HI. No effect on PU and CA	

(Continued)

Table 2 | Continued

Product	Starter culture	% Of reduction	Reference
Turkish Soudjoucks	*L. sakei* + *P. pentosaceous* + *S. carnosus* + *S. xylosus* (Bioback-K, Wiberg, Germany)	100% of PU. No effect on TY	Ayhan et al. (1999)
Turkish Sucuk	*L. sakei* + *S. carnosus F-SC-111(CHR-HANSEN, Germany)*	88% of TY, 54% of CA, and 63% of PU. No effect on HI	Gençcelep et al. (2007)
	P. acidilactici + *L. curvatus* + *S. xylosus F-LC (CHR-HANSEN, Germany)*	86% of TY, 27% of HI, 62% of CA, and 60% of PU	
Turkish fermented sausage	*L. sakei* + *S. xylosus* B-FM (CHR-HANSEN, Germany)	54% of TY and 62% of PU	Gücükoglu and Küplülü (2010)
	L. plantarum + *S. carnosus* TD-66 (CHR-HANSEN, Germany)	52% of TY and 61% of PU	
	L. curvatus + *S. carnosus* + *S. xylosus* RM-10 (CHR-HANSEN, Germany)	55% of TY and 63% of PU	
Xinese fermented sausage	*P. pentosaceus* + *S. xylosus*	66% of TY, 49% of CA, and 30% of PU. No effect on HI	Lu et al. (2010)
	L. farciminis + *S. saprophyticus*	83% of TY, 99% of HI, 99% of CA, and 66% of PU	
Thai fermented sausage	*L. plantarum* BCC9546 (BIOTEC, Thailand)	94% of TY, 75% of CA, and 97% of PU	Tosukhowong et al. (2011)
	L. plantarum BCC9546 + *L. brevis* BCC26756 (BIOTEC, Thailand)	37% of TY, 75% of CA, and 99% of PU	

**Starter has been tested in different technological conditions. It is showed the different% of reduction for each technological condition.*

reduction in different artisanal European fermented sausages. The work demonstrated the importance of case-by-case basis strain selection to obtain a good adaptation to the meat fermentation environment and in turn a good biogenic amine prevention.

It has been suggested that the use of bacterial strains with amine oxidase activity might enhance the reduction of biogenic amine accumulation by metabolizing the amines formed during the fermentation. Amine oxidase activity, metabolizing tyramine and/or histamine under *in vitro* conditions, has been described in microorganisms involved in sausage fermentation, such as specific strains of LAB (*L. sakei* or *L. plantarum*) and CNS (*S. xylosus*; Leuschner and Hammes, 1998; Martuscelli et al., 2000; Fadda et al., 2001; Gardini et al., 2002). However, under real conditions of sausage fermentation, amine-oxidizing microorganisms have shown a limited effect on tyramine and histamine levels, likely due to a low oxygen availability inside the sausage and/or an insufficient number of amine-oxidizing bacteria, e.g., below the minimum 10^7 cfu/g, required for amine degradation (Leuschner and Hammes, 1998; Gardini et al., 2002).

FACTORS THAT INFLUENCE THE PERFORMANCE OF STARTER CULTURES IN REDUCING BIOGENIC AMINE ACCUMULATION

The magnitude of reduction of biogenic amine accumulation achieved by a starter culture depends on the factors that determine the presence of endogenous microbiota as well as the competitiveness and implantation of the added starter culture.

It is clearly demonstrated that poor hygienic quality of raw materials and ingredients diminished the protective effect of the amine negative starter culture. Bover-Cid et al. (2001b) reported a significant reduction in the effectiveness of the amino acid decarboxylase negative strain *L. sakei* CTC494 when sausages were made from raw materials of relatively poor hygienic quality, since high bacterial loads (both of Gram-negative and Gram-positive) increase the number of potentially aminogenic microorganisms and also hamper the implantation and competitiveness of amine negative technological microbiota.

Different processing environment (e.g., pilot plant versus traditional processing plant) and the different type of formulation (e.g., *chorizo* or *fuet*) can also significantly influence the performance of the amine negative starter culture. The addition of a mixed starter culture including *L. sakei* (CTC6469 + CTC6626) and *S. xylosus* (CTC6013 + CTC6169) with no amino acid decarboxylase activity was successfully used in a fermentation study carried out in pilot plants by Garriga et al. (2005). The starter prevented the accumulation of tyramine, putrescine, and cadaverine by up to 90% of total amines. However, when the same strains (*L. sakei* CTC6626 + *S. xylosus* CTC6013) were inoculated into products produced in a traditional real processing plant, the starter culture showed a weaker effect, only slightly reducing the contents of tyramine and cadaverine, by 19 and 46%, respectively (Latorre-Moratalla et al., 2010b).

In the literature, several studies have evaluated the effect of factors that influence starter culture on biogenic amine reduction, such as the type and quantity of sugar added (González-Fernández et al., 2003) or the addition of some additives (Bozkurt and Erkmen, 2007; Coloretti et al., 2008). According to the results reported by González-Fernández et al. (2003), when the starter culture, especially *L. sakei* K29, was used with a sugar concentration of

0.5 or 1%, the presence of biogenic amines decreased considerably in comparison with the control and low sugar concentration sausage. Bozkurt and Erkmen (2007) described a higher decrease of tyramine, histamine and putrescine contents when a mixture of antimicrobials (nitrite and nitrate), antioxidants, and flavoring and coloring compounds were added with the starter culture mixture of *Pediococcus acidilactici, L. plantarum*, and *S. carnosus*.

The influence of processing temperatures on the reducing effect of starter cultures has been fully studied (Maijala et al., 1995; Komprda et al., 2001; Gücükoglu and Küplülü, 2010; Casquete et al., 2011). Gücükoglu and Küplülü (2010) reported that higher ripening temperatures (26°C versus 22°C) potentiated the reduction of putrescine by different starter cultures (*L. sakei* + *S. xylosus* B-FM, *L. plantarum* + *S. carnosus* TD66, and *L. curvatus* + *S. carnosus* + *S. xylosus* RM-10) in experimental Turkish fermented sausages. However, the fermentation temperatures did not have any significant effect on tyramine reduction. Similarly, Casquete et al. (2011) described an influence of temperatures on the development and activity of autochthonous starter cultures consisting of *P. acidilactici* MS200 + *S. vitulus* RS34 and *P. acidilactici* MS198 + *S. vitulus* RS34. In this case, higher fermentation temperature (12°C versus 7°C) also obtained the highest reduction of amines, probably due to a better adaptation of the starters to the meat fermentation environment.

In conclusion, the absence of decarboxylase activity should be a criterion for the selection of strains intended for use as starter cultures to obtain fermented sausages free of biogenic amines. Mixed starter cultures consisting of amine negative strains of LAB and CNS species, well adapted to the meat fermentation environment, seem the best alternative. Moreover, the use of autochthonous strains is presented as a promising control measure especially for the manufacture of traditional fermented sausages. High quality raw materials and optimal technological conditions are crucial factors to ensure a proper performance of starter cultures for the reduction of biogenic amine accumulation in fermented sausages.

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Biogenic amines in Italian Pecorino cheese

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The quality of distinctive artisanal cheeses is closely associated with the territory of production and its traditions. Pedoclimatic characteristics, genetic autochthonous variations, and anthropic components create an environment so specific that it would be extremely difficult to reproduce elsewhere. Pecorino cheese is included in this sector of the market and is widely diffused in Italy (~62.000t of production in 2010). Pecorino is a common name given to indicate Italian cheeses made exclusively from pure ewes' milk characterized by a high content of fat matter and it is mainly produced in the middle and south of Italy by traditional procedures from raw or pasteurized milk. The microbiota plays a major role in the development of the organoleptic characteristics of the cheese but it can also be responsible for the accumulation of undesirable substances, such as biogenic amines (BA). Bacterial amino acid decarboxylase activity and BA content have to be investigated within the complex microbial community of raw milk cheese for different cheese technologies. The results emphasize the necessity of controlling the indigenous bacterial population responsible for high production of BA and the use of competitive adjunct cultures could be suggested. Several factors can contribute to the qualitative and quantitative profiles of BA's in Pecorino cheese such as environmental hygienic conditions, pH, salt concentration, water activity, fat content, pasteurization of milk, decarboxylase microorganisms, starter cultures, temperature and time of ripening, storage, part of the cheese (core, edge), and the presence of cofactor (pyridoxal phosphate, availability of aminases and deaminases). In fact physico-chemical parameters seem to favor biogenic amine-positive microbiota; both of these environmental factors can easily be modulated, in order to control growth of undesirable microorganisms. Generally, the total content of BA's in Pecorino cheeses can range from about 100–2400 mg/kg, with a prevalence of toxicologically important BA's, tyramine and histamine. The presence of BA is becoming increasingly important to consumers and cheese-maker alike, due to the potential threats of toxicity to humans and consequent trade implications.

Keywords: Italian ewe cheese, microbial groups, biogenic amines

INTRODUCTION

The name Pecorino is commonly given to Italian cheeses made exclusively from pure ewes' milk and it has in most cases a protected designation of origin or PDO status. Generally this type of cheese is produced in the middle and south of Italy by a traditional procedure, characterized by a different ripening time ranging between 8 and 12 months (Di Cagno et al., 2003). The most popular Pecorino cheeses, such as Pecorino Romano, Fiore Sardo, Canestrato Pugliese, Pecorino Abruzzese, are usually made from raw or pasteurized milk which is inoculated with natural cultures, "scotta fermento," which is produced by acidifying the "scotta," the whey obtained from the manufacture of Ricotta (Di Cagno et al., 2003; **Figure 1**). The natural whey cultures are composed mainly of thermophilic lactic acid bacteria (LAB), such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Lactobacillus helveticus*. The milk is coagulated at 34–39°C adding liquid or powder calf rennet, lamb paste rennet, or pig rennet only for Pecorino di Farindola. After cutting the coagulum, the curds are cooked at 45°C for 5–10 min. The curds removed from the vats are placed in traditional and different molds, pressed manually, and ripened from 4 to 18 months.

However, in spite of these varieties there are many other typologies of Pecorino (**Table 1**) produced throughout the Italian territory according to local or regional costumes, also characterized by a shorter ripening time (1–2 months), semi-hard consistency, but low flavor and aroma (Caridi et al., 2003).

For this reason, a differentiation as well as the reduction of the ripening time can positively influence the consumption of these products. In fact the consumer generally prefers sweet flavor which is the result of short ripening (Gobbetti, 2004). More specifically, traditional products can take advantage from variants form specific attributes.

The principal technological features of more representative Pecorino cheeses are shown in **Table 2**.

Each unique combination of ingredients and processing parameters leads to a specific type of cheese with unique properties. In fact, the use of raw milk and natural thermophilic starters, cooking of the curd to a high temperature, long ripening, and generally, an ancient tradition are common features to most of Pecorino cheeses.

The particular flavor and typical organoleptic properties of Pecorino cheeses are associated with specific attributes of milk and

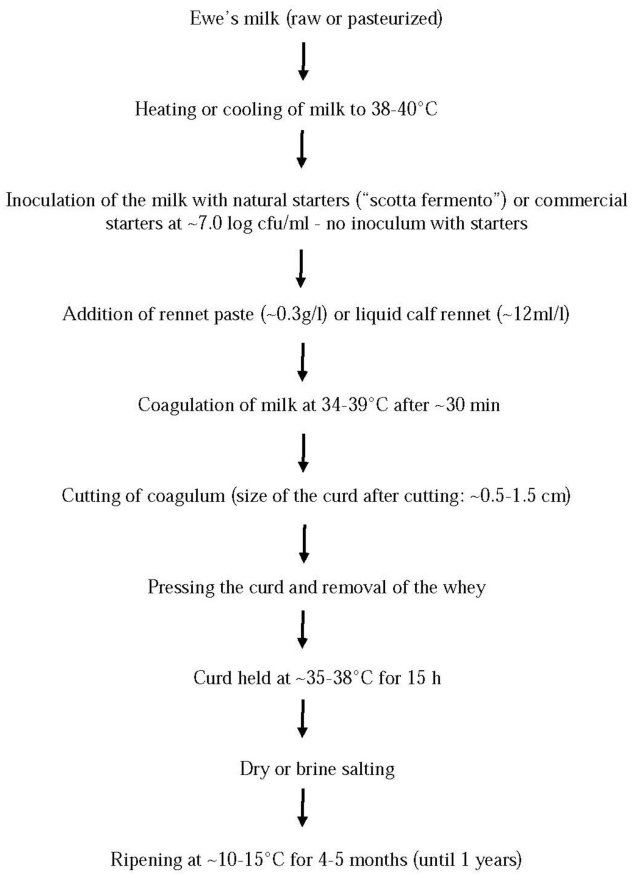

FIGURE 1 | Protocols for the manufacture of some Italian Pecorino cheeses.

Table 1 | List of some Italian Pecorino cheeses.

Production region	Variety
Abruzzo	Pecorino Abruzzese, Pecorino di Farindola, Cacio marcetto, Canestrato di Castel del Monte
Molise	Pecorino di Capracotta, Pecorino del Sannio
Calabria	Pecorino del Monte Poro, Pecorino Calabrese, Pecorino di Vezzano
Basilicata	Pecorino del Pollino
Campania	Pecorino Bagnolese, Pecorino di Carmasciano, Pecorino di Laticauda, Casu ri pecora, Pecorino Campano
Sicily	Pecorino Pepato, Primusali
Sardinia	Pecorino di Nule, Pecorino di Osilo
Umbria	Pecorino di Norcia, Pecorino Umbro
Tuscany	Pecorino Senese, Pecorino di Pistoia, Pecorino del Casentino, Pecorino del Parco di Migliarino-San Rossore, Pecorino Massese, Pecorino Baccellone, Pecorino Pisano, Pecorino di Pienza, Abbucciato Aretino
Apulia	Pecorino di Maglie, Pecorino Brindisino
Emilia-Romagna	Pecorino del Pastore, Pecorino dell'Appennino Reggiano, Pecorino di Palesio, Pecorino di Vergato
Piedmont	Pecorino di Bagnolo
Liguria	Pecorino di Malga
Marche	Pecorino in Botte
Veneto	Pecorino dei Berici, Pecorino Veneto
Lazio	Pecorino di Picinisco, Pecorino della Sabina, Pecorino di Amatrice, Pecorino del Viterbese

the natural microbiota responsible for fermentation and ripening processes (Corroler et al., 1998; Beresford et al., 2001). The artisanal cheeses have different and typical microbial population dynamics related to the production technology and geographic area of origin, with a microbiota quite heterogeneous (Poznanski et al., 2004).

The formation of large amounts of biogenic amines (BA) on this kind of cheese during the ripening process is allowed by some conditions, such as the availability of free amino acids produced as an outcome of proteolysis and viability of microorganisms possessing amino acid decarboxylating activity. Many genera of food bacteria are responsible of this characteristic (Suzzi and Gardini, 2003). Lactobacilli are considered producer of histamine, tyramine, and putrescine, whereas enterococci are considered tyramine formers and enterobacteria cadaverine and putrescine producers.

THE MICROBIAL GROUPS OF PECORINO

The microbial composition of raw milk of Pecorino cheeses is generally within the limits reported by different authors (Clementi et al., 1994; Ricci et al., 1995; Gobbetti et al., 1997). Total mesophilic bacteria can range from 10^6 to 10^7 cfu/g, micrococci and coagulase negative staphylococci are present at 10^3 to 10^4 cfu/g, such as *Enterobacteriaceae* and yeasts. Enterococci, representing a typical microbiota of ewes' raw milk, are present from 10^2 to 10^3 cfu/g. As expected LAB are detected from 10^2 to 10^5 cfu/g. A high level of biodiversity characterizes the natural microbiota of traditional Pecorino, in particular the indigenous microorganisms present in milk and coming from the environment during milking and cheesemaking, have a role in the cheese ripening. **Figure 2** shows the concentration range among the prevalent microbial groups (Di Cagno et al., 2003; Gardini et al., 2006; Lanciotti et al., 2007; Schirone et al., 2011). Generally high numbers of total mesophilic bacteria have been reported in Pecorino cheeses.

LACTIC ACID BACTERIA

The main groups detected are LAB, lactococci, and enterococci that are associated with streptococci, mesophilic and thermophilic lactobacilli (represented by *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus rhamnosus*, *Lactobacillus curvatus*), and other microbial groups (Bizzarro et al., 2000; Randazzo et al., 2006; Vernile et al., 2007). In traditional cheeses *S. thermophilus* has been isolated up to 9 log cfu/g at 1 month of ripening, in particular from Pecorino Sardo (Pisano et al., 2006; Comunian et al., 2010). Recently a strain of *S. thermophilus* has been found to have a tyrosine decarboxylase gene (*tdcA*), that appear to be a horizontal gene transfer event from *tdcA* of *L. curvatus* (La Gioia et al., 2011).

Table 2 | Main characteristics of some Italian Pecorino cheeses (modified by Gobbetti, 2004).

Italian Pecorino cheeses	Type of milk	Starter	Type of rennet	Cooking (°C)	Salting	Ripening, months	Dimension		
							Weight (kg)	Diameter (cm)	Height (cm)
Canestrato pugliese*	Sheep, raw, whole	None or natural culture in whey or milk	Calf, powder or liquid	No heat treatment or 45	Dry salting for 4–6 days	4–12, at 11–14°C	7–15	25–30	10–14
Fiore Sardo*	Sheep, raw, whole	Natural whey culture	Lamb or goat, paste	No heat treatment	Saturated brine and/or dry salting	3–6, at 12–16°C	1.5–4	12–20	12–15
Pecorino di Bagnolo	Sheep, raw, whole	None or natural culture in whey or milk	Lamb, paste or calf, liquid	45–48	Dry salting for 2–6 days	6–12, at 15°C	1.5–2	14–16	8–10
Pecorino di Farindola	Sheep, raw, whole	None or natural culture in whey or milk	Pig, liquid	No heat treatment	Dry salting for 20–40 days	2–12, at 14–15°C	1–3	14–22	4–8
Pecorino Romano*	Sheep, raw or thermized, whole	Natural culture in "scotta"	Lamb, paste	45–48	Dry salting for 30–60 days	8–12, at 10–14°C	18–30	25–30	25–30
Pecorino Sardo*	Sheep, raw or thermized, whole	Natural whey culture	Calf, paste	40–45	Saturated brine and/or dry salting	3–12, at 12–16°C	1–4	15–20	6–15
Pecorino Siciliano*	Sheep, raw or thermized, whole	None or natural culture in whey or milk	Lamb, paste	40–45	Dry salting for 10–20 days	4–18, at 12–15°C	4–15	40–45	10–18
Pecorino Toscano*	Sheep, thermized, whole	Natural culture in "scotta"	Calf, paste	40–42	Saturated brine or dry salting		1–3	15–22	7–11
Pecorino Veneto	Sheep, thermized, whole	Natural culture in "scotta"	Lamb, paste	40–42	Dry salting for 12 days	3–12, at 10–14°C	2–2.5	15–20	6–13
Pecorino di Palesio	Sheep, thermized, whole	Natural culture in "scotta"	Lamb, paste or calf, liquid	40–42	Dry salting for 10–20 days	2–12, at 10–15°C	0.8–2	12–14	5–8
Pecorino di Norcia	Sheep, raw, whole	None or natural culture in whey or milk	Lamb, paste	45–50	Dry salting for 4–6 days	2–12, at 14–15°C	3–5	15–24	8–14
Pecorino di Carmasciano	Sheep, raw, whole	None or natural culture in whey or milk	Lamb or goat, paste	No heat treatment	Dry salting for 10 days	2–6, at 10°C	1–2	16–20	8–10

**Protected designation of origin (PDO).*

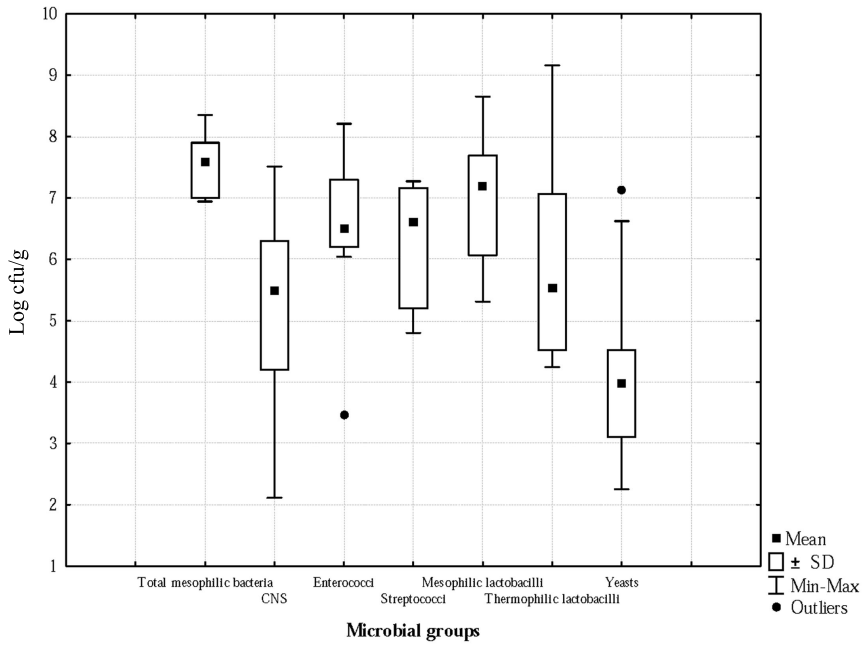

FIGURE 2 | Principal microbial groups in some Italian Pecorino cheeses.

Enterococci represent a typical microbiota of ewe's milk, with *Enterococcus faecium* and *Enterococcus faecalis* being the most prevalent ones (Gonzàlez De Llano et al., 1992; Centeno et al., 1995; Giraffa et al., 1997; Martín-Platero et al., 2009). Generally their numbers increase during ripening from 10^6 to 10^8 cfu/g in the final product. In the 24-h-old cheese, LAB belonging to *Lactococcus lactis* are the major components and reached 10^9 cfu/g, while the enterococci represent 1–10% of the total microbiota (Bizzarro et al., 2000). During ripening, the level of lactococci decrease, while the enterococci maintain a constant number, so that the enterococci may become the most important group. They constitute part of the normal food microbiota and play an important role in manufacture of cheeses typical from mediterranean countries (Ledda et al., 1994; Arizcun et al., 1997; Franz et al., 1999, 2003; Mannu et al., 1999; Gomez et al., 2000; Prodromou et al., 2001; Mannu and Paba, 2002; Foulquié Moreno et al., 2006; Pisano et al., 2007; Ogier and Serror, 2008). In Pecorino cheese enterococci constitute a relevant part of the cheese microbiota (**Figure 2**). Gelsomino et al. (2002) have demonstrated that enterococci survive and grow in the hidden corners of the milking machine and the bulk tank, thus contaminating the milk directly. From the milk, the enterococci are transferred into the cheese. Regarding BA production, Suzzi et al. (2000) found that great part of enterococci isolated from Semicotto caprino cheese produce tyramine but not histamine in synthetic medium. These data are in agreement with those of Giraffa et al. (1995) and Celano et al. (1992) who found that tyramine was the only BA produced by enterococci in milk.

NON-STARTER LACTIC ACID BACTERIA

Non-starter lactic acid bacteria (NSLAB) dominate the microbiota of Pecorino cheeses made from raw milk than in those made from pasteurized milk (Fox, 1998). In the early phase of manufacture NSLAB are present at very low numbers, but they increase throughput ripening and the major part of Pecorino cheeses has >6.0 log cfu/g at the end of the process (Coda et al., 2006). In fact indigenous NSLAB have the unique ability to grow under highly selective conditions prevailing in a cheese ripening and have been shown to contribute to the formation of small peptides and amino acids that are precursors for the flavor components but also for BA formation (Wouters et al., 2002).

ENTEROBACTERIACEAE

Enterobacteriaceae are not commonly detected in the final product due to their gradual decrease during cheese ripening (Medina et al., 1991; Hatzikamari et al., 1999; Dahl et al., 2000). However, high numbers of *Enterobacteriaceae* have been reported in different mediterranean cheeses made from ewes' and goats' raw milk after 30 days of ripening (Sánchez-Rey et al., 1993; Freitas et al., 1995, 1996; Freitas and Malcata, 2000; Psoni et al., 2003; Macedo et al., 2004). In Pecorino Abruzzese Enterobacteriaceae can be detected after 15 days of ripening at levels of 10^3 and 10^5 cfu/g in spring and summer, respectively, and are still present (10^2 cfu/g) after 60 days (Chaves-López et al., 2006). According to previous data obtained from *Enterobacteriaceae* inoculated in UHT milk (Chaves-López et al., 2006), the strains from Pecorino Abruzzese produced high quantities of putrescine but only small or undetectable quantities of cadaverine. Moreover, histamine was produced by all the strains. Many *Enterobacteriaceae* can produce considerable levels of histamine in fermented foods and particularly *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Klebsiella oxytoca*, as well as *Escherichia coli* and *Morganella morganii* (Suzzi and Gardini, 2003). Although these microorganisms are usually present in low numbers in the

final product, *Enterobacteriaceae* can release their decarboxylases in the early steps of cheesemaking (Bover-Cid et al., 2001).

OTHER MICROORGANISMS

In some cases, psychrotrophic microorganisms and *Staphylococcus aureus* have been reported (Palmas et al., 1999), indicating a possible contamination and a poor hygiene during manufacturing. Generally coagulase negative staphylococci (CNS) are represented by non-pathogenous species, saprophytic, or useful for conferring typical characteristics to the cheese, such as *Staphylococcus xylosus* (Palmas et al., 1999). The secondary adventitious microbiota belong to the CNS ranging from about 10^2 to 10^8 cfu/g and yeasts ranging from about 10^2 to 10^7 cfu/g. In Pecorino Crotonese *Kluyveromyces lactis* and *Debaryomyces hansenii* strains were found to dominate during the later stages of maturation. Some strains of *Yarrowia lipolytica* resulted in the highest aminobiogenic potential decarboxylating ornithine, phenylalanine, tyrosine, and lysine (Gardini et al., 2006).

PHYSICO-CHEMICAL FACTORS ON BA FORMATION

Other extrinsic factors may play an important role in the accumulation of BA in cheese, e.g., pH, salt concentration, water activity, and redox potential (Pinho et al., 2001). In **Table 3** the physico-chemical characteristics of Pecorino cheese are reported. The range values for some parameters are very large, indicating important differences during the manufacturing and the ripening conditions of Pecorino cheeses. For example NaCl concentration ranges from 1 to 9%, because some cheeses are salted by immersion in NaCl brine or by application of salt crystals at different times. This parameter could influence water activity, enzyme activity, and microbial dynamics (Fox and Guinee, 1987; Van der Berg and Exterkate, 1993). The NaCl concentration in milk slows down the formation of different amines such as agmatine, spermine, spermidine, putrescine, histamine, tyramine, and tryptamine (Santos et al., 2003).

Then, the characteristics of a Pecorino cheese depend on microbiota dynamics. Both starter and secondary microbiota modify the physico-chemical properties of cheese. Each cheese type should be studied particularly, given the high variability in physico-chemical composition. Then, the quality of raw milk, the environmental conditions, and the traditional manufacture play an important role in determining the characteristics of an artisanal Pecorino cheese. Characterizing cheese microbial population may contribute to understand the ecological processes that drive microbial interaction in cheese and their technological relevance. Knowledge of the structure and dynamics of the whole microbial community of cheese would promote better understanding of BA's formation with respect to microbial growth and metabolism.

Table 3 | Range of the gross chemical composition in Italian Pecorino cheeses (modified by Gobbetti, 2004).

Parameters	
Moisture (% w/w)	30–34
Fat (% w/w)	27–32
NaCl (% w/w)	1–9
Protein (% w/w)	25–32
Ash (%)	1–3
Soluble N/Total N (%)	20–28
pH	5.1–5.5

BIOGENIC AMINES IN PECORINO CHEESES

The presence of relevant amounts of BA in cheeses has been recently documented for a few types of Pecorino cheeses (Martuscelli et al., 2005; Lanciotti et al., 2007; Del Signore and Di Giacomo, 2008; Mascaro et al., 2010; Mercogliano et al., 2010; Bavazzano et al., 2011; Forzale et al., 2011; Schirone et al., 2011). In these studies the quantitative and qualitative accumulation of such compounds was extremely variable (**Table 4**). The diamines, putrescine and cadaverine, were found in higher concentrations in Pecorino di Fossa and Pecorino di Farindola. In particular, Mascaro et al. (2010) in Pecorino di Fossa found values of 579.60 and 1302.86 mg/kg for putrescine and cadaverine, respectively; while in samples of Pecorino di Farindola, putrescine ranged from 9.9 to 394 mg/kg and cadaverine from 26.8 to 276.1 mg/kg (Schirone et al., 2011). Tyramine was always present at high concentrations compared with the other amines of the same cheese. Among ten batches of Pecorino di Farindola (Schirone et al., 2011) tyramine resulted to be the BA present in the highest concentration in all cheeses examined, representing in six samples more than 40% of the total amines. A high relationship between tyramine and total BA content in Pecorino di Farindola cheese was found ($R^2 = 0.9869$). High values of this BA have been determined also in Pecorino di Fossa, Pecorino Abruzzese and Pecorino di Migliarino-San Rossore, particularly in the cheese core (1300 mg/kg). The relevant incidence of tyramine in cheese manufactured from raw ewes' milk has been reported (Roig-Saguès et al., 2002; Martuscelli et al., 2005; Pintado et al., 2008; Ladero et al., 2010).

Tyramine-producing bacterial strains are expected to be present in Pecorino especially within the groups of NSLAB, *Enterobacteriaceae* and enterococci, as indicated in the study of Martuscelli et al. (2005). The high level of tyramine in the Pecorino cheese could be due to the activity of thermoresistant enterococci, usual contaminants of raw milk. However, the heat resistance at more than 65°C of some *Lactobacillus* species, and in particular of *L. paracasei*, has been demonstrated (Jordan and Cogan, 1999). There is evidence that LAB are occasionally associated with tyramine formation, although they can also contribute to the accumulation of other BA such as putrescine. On the contrary, *Enterobacteriaceae* would be associated with cadaverine, putrescine, and histamine formation, mainly when a deterioration process occurs in either raw materials or end products (Marino et al., 2003). It is well known that BA accumulation in cheese can be influenced, firstly by the microbial quality of raw milk, the sanitation procedures adopted, the use of starter cultures, and the condition and time of the ripening process (Novella-Rodríguez et al., 2004; Pinho et al., 2004). The differences detected in BA accumulation could be attributable to the milk used, probably because of the heterogeneity of the cheese typology considered. An analogous heterogeneity in BA content was observed by Novella-Rodríguez et al. (2003) in cheeses from bovine milk. The microbial population of raw milk can influence BA presence in cheese, even when thermal treatment are applied such as for a Pecorino cheese (Lanciotti et al., 2007) or for Pecorino Abruzzese (Martuscelli et al., 2005). It can

Table 4 | Levels of Biogenic Amines in Italian Pecorino cheeses at different time of ripening.

Italian Pecorino cheeses	Biogenic amines (mg/kg)							References
	Ethylamine	Putrescine	Cadaverine	2-phenylethylamine	Tyramine	Histamine	Total biogenic amines	
Pecorino Abruzzese[1]								Martuscelli et al. (2005)
Batch A*	20.0	80.0	18.0	35.0	185.0	261.0	697.0	
Batch B**	180.0	163.0	75.0	305.0	230.0	76.0	1086.0	
Pecorino[2]								Lanciotti et al. (2007)
Milk HPH***		14.80 ± 4.46	20.3 ± 1.88	19.5 ± 5.24	62.8 ± 9.08	3.35 ± 1.32		
Thermized milk		70.92 ± 11.11	257.0 ± 11.7	155.0 ± 19.2	350.0 ± 21.6	23.9 ± 1.67		
Raw milk		29.3 ± 5.83	107.0 ± 6.20	63.3 ± 4.74	162.0 ± 12.2	6.32 ± 0.59		
Pecorino[3]								Del Signore and Di Giacomo (2008)
Ripening 5°C		2.0–5.80	2.20–4.20		28.70–51.70	7.70–14.20		
Ripening 10°C		8.20	6.90		43.70	23.0	114.0	
Ripening 15°C		6.00–6.20	6.80–7.10		29.10–33.10	20.20–25.30		
Pecorino Carmasciano[4]		90.0	120.0		136.41	65.5		Mercogliano et al. (2010)
Formaggio di Fossa[5]		579.60 ± 0.88	1302.86 ± 5.02	173.0 ± 39.13	461.62 ± 11.99	24.11 ± 10.06	2557.69 ± 39.13	Mascaro et al. (2010)
Pecorino di Farindola[6]	12.9–601.3	9.9–394.0	26.8–276.1	0–127.1	52.3–1171.3	0–21.8	209–2393	Schirone et al. (2011)
Pecorino Toscano[7]		3.0–84.2	7.9–25.6	1.3–21.8	6.9–122.5	0.8–52.6		Bavazzano et al. (2011)
Pecorino Del Parco Di Migliarino-San Rossore[8]								Forzale et al. (2011)
In the core		172.95 ± 15.56	22.38 ± 20.1		1300.05 ± 117.01	32.41 ± 2.91	1578.72 ± 142.08	
In the external part		84.96 ± 9.34	30.61 ± 3.37		527.85 ± 58.06	39.45 ± 5.44	721.81 ± 80.51	

*[1]After 60 days of ripening; *whole raw milk without starter culture; **whole thermized milk with starter culture; [2]after 21 days of ripening; ***milk high pressure homogenization; [3]after 4 months of ripening; [4]after 201 days of ripening; [5]after 90 days of ripening into pits; [6]after 90 days of ripening; [7]after 8 months of ripening; [8]after 153 days of ripening.*

be hypothesized that the mild thermal treatment applied selects a decarboxylating microbial population, which dominates during cheesemaking and, possibly, during ripening. Similar results have been reported by Marino et al. (2008) showing the highest BA contents in cheese produced using pasteurized milk and natural milk culture; therefore, the thermal treatment of milk seems to be not enough by itself to reduce the counts of decarboxylase-positive bacteria in cheese. However, other authors observed a higher BA accumulation in cheeses obtained from raw milk than in similar products from pasteurized milk (Schneller et al., 1997).

Cheese is a matrix with a high protein content in which their demolition to free amino acids (or short peptides) is guaranteed by proteases or peptidases produced by microorganisms (including starter cultures), present in milk (especially if not thermally treated), and/or in rennet. Given these activities, the availability of precursors is not a central problem in BA accumulation (Lanciotti et al., 2007). Fernández-García et al. (2000) studied the influence of addition of exogenous proteinases to milk and found that tyramine concentration was significantly ($P < 0.05$) influenced by this addition.

The high content of BA in many Pecorino cheeses could be due, in part, to proteinases, and peptidases released from rennet used, particularly artisanal lamb rennets, or pig rennet during cheesemaking of Pecorino di Farindola. In fact the traditional lamb paste preparation procedure has a significant impact on rennet composition, as has animal age, precedent diet, or slaughtering condition (Jacob et al., 2011). In artisanal lamb paste production, there is a substantial microbiota, dominated by LAB (Etayo et al., 2006; Gil et al., 2007). The use of artisanal rennets often entails problems concerning curd formation and final characteristics of the cheese. Vincente et al. (2001) reported that the release of free amino acids during ripening is strongly affected by starter added to the cheese, and that this effect varies markedly with the rennet used for cheesemaking. However the level of starter culture does not appears to affect the population of tyrosine decarboxylase microorganisms (Fernández-García et al., 2000). As a consequence of this, the natural whey starter used for Pecorino cheese manufacture and the rennet could affect the quality, the quantities, and variability of BA accumulation.

CONCLUSION

Pecorino cheeses, ewe's milk dairy products of many Italian regions, have an important link with their areas of production which confer on them specific microbiological, chemical, and sensorial characteristics (Pirisi et al., 2011). The spread use of raw milk creates a high microbiological biodiversity, such as the use of different traditional rennets contribute to typical aromas and flavors. The presence of high contents of BA in Pecorino cheeses could be related to the enzymatic activity of proteases derived from microorganisms, or from another origin (rennet) that is important from a qualitative point of view, i.e., in relation to the type of amino acids provided to the amino acid decarboxylating microbiota, in particular tyrosine. The bacteriological composition of milk could be critical to define the BA profile in this type of cheese, that is generally produced in little farms. Even though Pecorino cheese is very important from economical point of view in certain Italian areas, studies on BA content and their origin are still scarce. Therefore, large amounts of BA in cheese could indicate unsuitability, from a hygienic point of view, of the milk used for cheesemaking. The need to know in depth and control the indigenous bacterial population responsible for high production of BA and the use of competitive adjunct cultures could be useful to improve the characteristics of Pecorino cheese.

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Factors influencing biogenic amines accumulation in dairy products

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Fermented foods are among the food products more often complained of having caused episodes of biogenic amines (BA) poisoning. Concerning milk-based fermented foods, cheese is the main product likely to contain potentially harmful levels of BA, specially tyramine, histamine, and putrescine. Prompted by the increasing awareness of the risks related to dietary uptake of high biogenic amine loads, in this review we report all those elaboration and processing technological aspects affecting BA biosynthesis and accumulation in dairy foods. Improved knowledge of the factors involved in the synthesis and accumulation of BA should lead to a reduction in their incidence in milk products. Synthesis of BA is possible only when three conditions converge: (i) availability of the substrate amino acids; (ii) presence of microorganisms with the appropriate catabolic pathway activated; and (iii) environmental conditions favorable to the decarboxylation activity. These conditions depend on several factors such as milk treatment (pasteurization), use of starter cultures, NaCl concentration, time, and temperature of ripening and preservation, pH, temperature, or post-ripening technological processes, which will be discussed in this chapter.

Keywords: biogenic amines, cheese, producing microorganisms, pasteurization, starters, ripening, chemico-physical factors

INTRODUCTION

Fermented foods, and especially cheese, are within the food products more often related with biogenic amines (BA) poisoning (Sumner et al., 1985; EFSA Panel on Biological Hazards (BIOHAZ), 2011). Under normal conditions, exogenous BA ingested with food are rapidly detoxified by the action of amine oxidases, but whenever the detoxification process is disturbed, or the BA concentration in food is very high, BA become toxic metabolites responsible of serious human health problems (Repka-Ramírez and Baraniuk, 2002; Soufleros et al., 2007; Ladero et al., 2010a; Spano et al., 2010). In the 60s of last century, a close relation between migraine crisis and ingestion of tyramine-rich food (especially cheese) was observed (Blackwell, 1963; Hanington, 1967; Coutts et al., 1986). In fact, these effects of tyramine consumption were coined as *cheese-reaction*. Despite the toxicity of BA, their limit concentrations in fermented foodstuffs have not yet been adequately standardized by regulatory agencies.

The most important BA in dairy foods are histamine, tyramine (produced by enzymatic decarboxylation of histidine and tyrosine, respectively), putrescine (synthesized via ornithine decarboxylation or agmatine deamination), and to a minor extent, cadaverine (originated by lysine decarboxylation; Linares et al., 2011). These amino acid decarboxylating activities are mainly attributable to the microbial groups that participate in the fermentation process.

Many bacteria of different genera and species have the capacity to produce BA. Gram negative bacteria (mainly *Enterobacteriaceae*) that can be present in milk are able to produce histamine, putrescine, and cadaverine (ten Brink et al., 1990; Marino et al., 2000; Pircher et al., 2007). However, the main BA producers in cheese are mostly lactic acid bacteria (LAB) included in the genera *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus* (Fernández et al., 2004; Martín et al., 2005; Bonetta et al., 2008; Calles-Enríquez et al., 2010; Ladero et al., 2011a). These Gram positive bacteria can be present: (i) in the milk, (ii) introduced by contamination throughout the entire process of cheese production, (iii) and may even be part of starter or adjunct cultures. Several authors have reported the presence of tyrosine and histamine decarboxylase activity in strains from various starter cultures (Burdychova and Komprda, 2007; Calles-Enríquez et al., 2010; La Gioia et al., 2011).

Consequently, formation of BA can occur during food processing and storage as a result of bacteria that have amino acid decarboxylase activities. The manufacture of dairy foods is not a sterile process, and BA producers are likely to enter the food chain as non-starter LAB that are indigenous to the raw material. Besides the presence of microorganisms with the appropriate metabolic pathway, BA formation is only possible if there is availability of the free substrate amino acids and the environment conditions are favorable to the decarboxylation activity (Russo et al., 2010). There are different factors related with such conditions: the milk treatments, the use of starter cultures and enzymes, the duration and the temperature of ripening, the level of proteolysis, the pH, the NaCl concentration, the presence of oxygen, the activity of water and the relative humidity, the bacterial density and synergistic effect between microorganisms are the most important (Gardini et al., 2001). In spite of great technological advantages, much of the dairy fermentations are still based on traditional, experienced-based techniques that are strongly rooted in specific geographic

regions. As a result, these factors can not always be modified in order to reduce the accumulation of BA without modifying the organoleptic and sensory properties of the final product.

Cheese represents an ideal matrix for production of BA because it is not sterile and the casein proteolysis ensures the availability of free substrate amino acids. Therefore, BA can reach concentrations in cheese up to 2000 mg kg^{-1} (Roig-Sagués et al., 2002; Fernández et al., 2007a). Ingestion of cheese containing such a high concentration of BA is a direct threat to the consumer, especially in places like Europe where cheese is one of the major foods in the diet. The consumption of cheese in Europe in 2009 was 16.6 kg per capita [International Dairy Federation (IDF) Bulletin 446/2010, Statistics Canada]. However, BA concentration differs widely between different cheeses because depends – as indicated previously – on several factors.

Beside cheese, kefir is another fermented dairy product where BA may be present. Recently, Özdestan and Üren (2010) reported total BA contents in kefir samples between 2.4 and 35.2 mg l^{-1}, being tyramine the prevailing BA. Similarly, Chaves-López et al. (2011) reported a total BA content of 15.31 mg l^{-1} in kumis, a traditional Colombian fermented cow milk. Nevertheless, these levels are far below the recommended limits. Other milk-based fermented products including buttermilk or yogurt are not likely to contain significant levels of BA (Novella-Rodríguez et al., 2000; Souci et al., 2000). In the milk itself, the polyamines spermidine and spermine are the prevalent accumulated BA (Spano et al., 2010), however it remains unclear whether they are synthesized by microorganisms or they have an endogen origin.

Prompted by the increasing awareness of the risks related to dietary uptake of BA and the general agreement that they should not be allowed to accumulate in food, in this chapter we report about technological aspects affecting BA levels during cheese elaboration and processing. It should be noted that the control of these technological factors can be useful for the reduction of the BA accumulation on the final products.

OCCURRENCE OF BIOGENIC AMINES IN DAIRY PRODUCTS

Milk-based fermented foods, especially cheese, provide an adequate environment for BA production and accumulation (Roig-Sagués et al., 2002; Fernández et al., 2006a). However, big differences have been noted from different types of cheeses, which has been related to bacterial counts in the milk, thermal milk treatment intensity or duration and the length and conditions of the ripening process. The use of starter cultures could also influence the production of BA either directly or indirectly through the interaction with the wild cheese microbiota (Ordóñez et al., 1997). Beside this, a different distribution of BA through the cheese has been observed (Novella-Rodríguez et al., 2003). All these variables contribute to the enormous differences observed between different types of cheeses or even in the same type of cheese depending on the part analyzed or the ripening period. In order to offer an overview of BA content in dairy products, the data available in the literature have been summarized in **Table 1**.

BA-PRODUCING MICROORGANISMS IN DAIRY PRODUCTS

One of the indispensable conditions for the presence and further accumulation of BA in fermented dairy products is the presence of microorganisms with the capacity to synthesize these toxic compounds. Among those microorganisms that have been identified as usual microbiota of cheeses, some of them have been identified as BA producers including yeast, Gram positive and Gram negative bacteria (**Table 2**), although not all of them have an impact in the presence of high concentrations of BA in the final product.

Several yeasts species have been described as potential producers of aliphatic amines (putrescine and cadaverine; Suzzi et al., 2003). However, only a few strains of *Debaryomyces hansenii* and *Yarrowia lipolytica* isolated from cheese seem to be able to produce histamine and tyramine respectively (Gardini et al., 2006). The presence of *Y. lipolytica, Pichia jadinii*, or *Geotrichum candidum* has been related in some cases (Wyder et al., 1999; Roig-Sagués et al., 2002) with an increase in the concentration of histamine and putrescine.

Most of the Gram negative bacteria described as usual contaminants of milk are able to produce histamine, putrescine, or cadaverine, i.e., *Escherichia coli, Hafnia alvei, Klebsiella pneumoniae, Morganella morganii, Pseudomonas*, or *Serratia* spp (Marino et al., 2000; Roig-Sagués et al., 2002; Coton et al., 2011). However, the presence of elevated concentrations of Gram negative bacteria – especially *Enterobacteriaceae* – has been only correlated in some cases with an increase in the concentration of putrescine or cadaverine (ten Brink et al., 1990; Pircher et al., 2007; Delbès-Paus et al., 2012). In those cases, BA are result of bad manufacturing practices, poor quality or insufficient hygienic conditions.

Nevertheless, the main BA producers in cheese are Gram positive bacteria, being LAB the main histamine and tyramine producers (Linares et al., 2011). Moreover, recent discoveries pointed out the importance of this bacterial group as putrescine producers (Ladero et al., 2011a, 2012a). The genera *Enterococcus*, *Lactobacillus, Lactococcus*, *Leuconostoc*, and *Streptococcus* include some strains belonging to different species that have been described as BA producers (**Table 2**). Other LAB have been described as BA producers in some reports such as tyramine producing lactococci or lactobacilli (Fernández et al., 2004; Bunková et al., 2009; Nieto-Arribas et al., 2009). However, the identification of the species is not based on the sequencing of the 16S *rRNA* gene, or other gene with taxonomical relevance, which could lead to misclassification (Delgado and Mayo, 2004; Fernández et al., 2007b). All these BA-producing LAB belong to species that are normal microbiota of milk and cheese (Varnam and Sutherland, 1994) and can be potentially present during the elaboration of any fermented dairy product (Novella-Rodríguez et al., 2002; Ladero et al., 2009; Linares et al., 2011).

FACTORS INFLUENCING BIOGENIC AMINE PRODUCTION IN DAIRY PRODUCTS

The biosynthesis and accumulation of BA in dairy foods requires the presence of bacteria with decarboxylase activity, the appropriate environmental conditions for their growth and the activity of the decarboxylases, and the presence of the substrate amino acids. The influence of all these parameters in BA production is addressed in this section.

Table 1 | Biogenic amine content in dairy products (mg/kg).

Source		Type	Cad	His	Phe	Put	Tyr	Spn	Spd	Reference
Milk	Raw	Cow	nd	nd	nd	nd	nd	0.18	nd	Novella-Rodríguez et al. (2000)
			–	0.3	–	–	–	–	–	Bodmer et al. (1999)
		Ewe	–	–	–	–	–	nd	nd	Pinho et al. (2004)
	Pasteurized	Cow	–	0.7	–	–	–	–	–	Bodmer et al. (1999)
Yogurt			–	13	–	–	–	–	–	Bodmer et al. (1999)
			0.27	nd	nd	nd	nd	0.43	0.34	Novella-Rodríguez et al. (2000)
Cheese		Sheep	123.07	50	–	107.69	125	115.38	369.23	Mercogliano et al. (2010)
(raw milk)		Sheep	26.8	0	28.6	394.1	499.6	96.4		Schirone et al. (2011)
		Goat	349.72	15.6	9.39	217.84	216.28	nd	nd	Pinho et al. (2004)
		Goat	88.7	88.4	11.7	191.8	830.5	–	–	Novella-Rodríguez et al. (2002)
		Goat	94	117	160	941	258	70.5	23.5	Galgano et al. (2001)
		Cow	nd	nd	nd	nd	nd	nd	nd	Fernández et al. (2007b)
		Cow	nd	nd	nd	nd	nd	nd	nd	Fernández et al. (2007b)
	Parmigiano	Cow	3.2	10.9	–	1.8	6.4	4.4	–	Mayer et al. (2010)
	Parmigiano	Cow	15.56	28.55	9.51	75.87	29.89	7.71	4.46	Innocente and D'Agostin (2002)
	Extra hard grana	Cow		249	–		18	–	–	Mayer et al. (2010)
Cheese	Feta	Goat	82.8	84.6	4.94	193	246	nd	nd	Valsamaki et al. (2000)
(pasteurized	Emmental Hard	Cow	98.3	23.5	–	38	52.2	–	16.8	Mayer et al. (2010)
milk)	Cheddar	Cow	nd	25.4	–	4.8	–	8.5	18.2	Mayer et al. (2010)
	Semi hard	Cow	nd	–	–	–	24.3	5.0	17.3	Mayer et al. (2010)
	Gouda	Cow	nd	–	–	–	2.43	0.5	1.73	Mayer et al. (2010)
	Edam	Cow	nd	3.2	–	–	–	–	–	Mayer et al. (2010)
	Gorgonzola	Cow	nd	23.7	–	–	–	–	–	Mayer et al. (2010)
	Gorgonzola	Cow	33.7	255.3	–	3.2	13.2	10.8	17.2	Mayer et al. (2010)
	Smear	Cow	748.2	168.3	–	31.3	247.6	18.5	16.6	Mayer et al. (2010)
	Semiripenned	Cow	33.49	24.38	25.75	22.6	32.92	24.35	23.4	Latorre-Moratalla et al. (2009)
		Cow	nd	65.42	–	175.39	80.9	–	–	Fernández et al. (2007b)
		Goat	nd	27.68	–	18.12	30.48	–	–	Fernández et al. (2007b)
		Sheep	nd	nd	–	nd	301.6	–	–	Fernández et al. (2007b)
	Pecorino Abruzzese	Sheep	80	90	300	200	280	nd	20	Martuscelli et al. (2005)
Cheese	Roquefort		8.9	9.9	7.7	18.3		4.6	18.1	Mayer et al. (2010)
(blue)			nd	50	nd	25	2000	nd	18	Rabie et al. (2011)
			2101.4	376.6	39.7	257.2	1585.4	–	–	Novella-Rodríguez et al. (2003)
		Mixture	756.78	1041.81	875.8	1051.98	–	–	–	Fernández et al. (2007b)
			489.4	127.2	–	237.56	526.63	–	–	Fernández et al. (2007b)
Kefir			1.8	4.0	nd	12.1	12.8	nd	4.5	Özdestan and Üren (2010)
			2.2	1.6	nd	1.4	9.8	nd	1.5	Özdestan and Üren (2010)

Cad, cadaverine; His, histamine; Phe, 2-phenilethylamine; Put, putrescine; Tyr, tyramine; Spn, spermine; Spd, spermidine; nd, undetected; –, not determined.

ROLE OF BA-PRODUCING MICROORGANISMS

The presence of BA-producing microorganisms is an indispensable condition for the biosynthesis of these toxic compounds. However, their accumulation in high concentrations depends not only on the presence of these BA-producing microorganisms but also if they reach a minimum number, and the coincidence of different factors during the elaboration and storage of the dairy products (Joosten and Northolt, 1987; Ladero et al., 2008). In general, it was difficult to find a correlation between the presence of high concentrations of BA in cheeses with and increment of a specific group or population of LAB. This was due to two main causes: (i) the fact that the capability to produce BA is mostly related to strain rather than to specie (Novella-Rodríguez et al., 2002) and (ii) the use of non-specific methods for the identification of these bacteria.

Certainly, the presence of the capability to produce BA in LAB has been described as a characteristic acquired through horizontal gene transfer associated to plasmids, mobile elements, or acidic resistance islands (Lucas et al., 2005, 2007; Marcobal et al., 2006b). However, in certain cases this trait can be considered as species-characteristic, such as the production of tyramine in *Enterococcus* (Ladero et al., 2012a) or that of putrescine in *Lactococcus* (Ladero et al., 2011a). In this latter case, it seems that the adaptation to the

Table 2 | BA-producing microorganisms from dairy products.

Biogenic amine	Producer microorganisms		Reference
Histamine	Molds and yeast	*Debaryomyces hansenii, Geotrichum candidum*	Roig-Sagués et al. (2002), Gardini et al. (2006)
	Gram negative	*Enterobacteriaceae, Morganella morganii*	Marino et al. (2000), Coton et al. (2011)
	Gram positive	*Lactobacillus buchneri, Lactobacillus curvatus, Lactobacillus helveticus, Lactobacillus parabuchneri, Streptococcus thermophilus*	Martín et al. (2005), Burdychova and Komprda (2007), Calles-Enríquez et al. (2010)
Tyramine	Molds and yeast	*Yarrowia lipolytica*	Gardini et al. (2006)
	Gram negative	–	
	Gram positive	*Enterococcus casseliflavus, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Enterococcus hirae, Lactobacillus brevis, Lactobacillus curvatus, Streptococcus thermophilus.*	Fernández et al. (2007b), Lucas et al. (2007), Bonetta et al. (2008), Bunková et al. (2009), La Gioia et al. (2011), Ladero et al. (2012a)
Putrescine	Molds and yeast	*Debaryomyces hansenii, Yarrowia lipolytica*	Wyder et al. (1999)
	Gram negative	*Enterobacteriaceae, Proteus.*	ten Brink et al. (1990), Coton et al. (2011)
	Gram positive	*Enterococcus faecalis, Enterococcus hirae, Lactobacillus brevis, Lactobacillus curvatus, Lactococcus lactis*	Llacer et al. (2007), Lucas et al. (2007), Ladero et al. (2011a,b)
Cadaverine	Molds and yeast	*Yarrowia lipolytica*	Wyder et al. (1999)
	Gram negative	*Enterobacteriaceae, Halomonas* sp., *Morganella morganii, Pseudomonas putida*	Meng and Bennett (1992), Marino et al. (2000), Coton et al. (2011)
	Gram positive	–	
Tryptamine	Molds and yeast	–	
	Gram negative	*Morganella morganii, Proteus, Serratia*	Marino et al. (2000), Coton et al. (2011)
	Gram positive	–	
Phenylethylamine	Molds and yeast	*Yarrowia lipolytica*	Wyder et al. (1999)
	Gram negative	*Halomonas, Serratia*	Marino et al. (2000)
	Gram positive	*Enterococci*	Marcobal et al. (2006c)

In order to prevent misclassification, only those species which identification is based on the sequencing of 16S rRNA gene or other gene with taxonomical relevance have been included in the table.

milk environment and/or the empirical selective pressure exerted because of the use of the strains as fermentation starters resulted in the inactivation or even the loss of the capability to produce putrescine, a compound that confers undesirable flavor.

The development of molecular methods toward the specific detection and quantification of BA producers in dairy products (Fernández et al., 2006a; Ladero et al., 2010b,c, 2012b) allowed to establish a direct relationship between the number of BA-producing microorganisms and the final BA concentration (Ladero et al., 2008, 2010b, 2012b). A minimum number of these BA producers has been proposed to reach high concentrations of BA in the final product (Joosten and Northolt, 1987; Ladero et al., 2008, 2010b).

STARTER CULTURES

In most industrial dairy fermentations, starter cultures are used to ensure the standard quality of the final products. Some LAB generally used as starter cultures, may have specific amino acid decarboxylase activities and thus, the potential to synthesize BA that could be accumulated in the dairy products. Belonging to this group are lactococci, lactobacilli and streptococci, as mentioned in the previous section.

The risk of incorporating BA-producing strains can be minimized by using well-characterized starter cultures, excluding those strains with the undesirable potential to produce BA. Several strains of *Streptococcus thermophilus* (Calles-Enríquez et al., 2010; La Gioia et al., 2011), *Lactobacillus brevis* and *Lactobacillus curvatus* (Ladero et al., 2011b), and *Lactococcus lactis* (Ladero et al., 2011a) obtained from dairy sources have been identified as histamine, tyramine, or putrescine producers. This becomes particularly relevant if we consider that strains belonging to those species are frequently used as starter cultures for cheese production. Burdychova and Komprda (2007) have isolated a histamine-producing *Lactobacillus helveticus* strain from a starter used for cheese production. Similarly, other *Lactobacillus* spp. isolated from ripened cheese, have been shown to produce BA such as tyramine (Straub et al., 1994; Arena et al., 2007; Ladero et al., 2011b) and also putrescine (Ladero et al., 2011b).

These facts led the recommendation to include the non-production of BA as a condition of strains intended to be used as starter cultures (Crow et al., 2001; Linares et al., 2011; EFSA Panel on Biological Hazards (BIOHAZ), 2011) or even raise the question about the addition of absence of BA production and BA

production associated genes as qualification criteria in the "*Qualified Presumption of Safety*" (QPS) assessment scheme introduced by the European Food Safety Agency (EFSA). For this assessment, molecular methods for the identification and quantification of BA producers are currently available (Fernández et al., 2006b; Torriani et al., 2008; Ladero et al., 2010b, 2012b).

Another approach to reduce the accumulation of BA in dairy products, could be the use of adjunct cultures that include bacteria capable to degrade BA (Leuschner and Hammes, 1998; Naila et al., 2010) as is proposed for fish sauce, sausage, or wine fermentation (Martuscelli et al., 2000; Fadda et al., 2001; Gardini et al., 2002; García-Ruiz et al., 2011; Zaman et al., 2011). In the case of cheeses it was described the use of *Brevibacterium linens* to catabolize histamine and tyramine during the elaboration of Munster cheese (Leuschner and Hammes, 1998; Leuschner et al., 1998).

PASTEURIZATION

Milk is a rich organic fluid that provides the necessary nutrients for the growth of different microorganisms. Indeed, microbial loads of up to 10^7 cfu ml^{-1} may be reached if storage conditions are not adequate (Varnam and Sutherland, 1994). The main microbiological groups present in raw milk are mesophilic LAB (enterococci, lactococci, lactobacilli, or leuconostoc), enterobacteriaceae, and psychotropic microorganisms such as *Pseudomonas* or *Acinetobacter* (Varnam and Sutherland, 1994; Muir and Banks, 2003; Martuscelli et al., 2005; Serio et al., 2007). Members of all these groups have been described as BA producers (Linares et al., 2011).

Pasteurization is a heat treatment that reduces the microbial load of raw milk. It has long been used by dairy industry to extend the shelf-life of the dairy products by reducing the presence of spoilage bacteria, pathogens, and those microorganisms with the capacity to produce toxic compounds that would render products unsafe for consumption (Lewis, 2003). The aim of the pasteurization is not the absolute elimination of the bacteria present on the milk, but the reduction of the bacterial load to levels safe for health and to ensure that the fermentative process is not on risk. The pasteurization reduces the bacterial load present in milk, including the BA producers. Thereby, cheeses elaborated with pasteurized milk use to have lower BA concentrations than those made with raw milk (Novella-Rodríguez et al., 2002; Fernández et al., 2007a; Naila et al., 2010). However, it is possible to detect BA in cheeses elaborated with pasteurized milk and sometimes at elevated concentrations (Fernández et al., 2007a; Pircher et al., 2007). The contamination with BA-producing microorganisms during the manufacture of cheese, due to deficient hygienic conditions, has been proposed as explanation (Pircher et al., 2007; Ladero et al., 2009). Moreover, some BA-producing *Lactobacillus* and *Enterococcus* are resistant to pasteurization and could develop as secondary microbiota after the thermal treatment, resulting in the apparition of BA in the final product (Ladero et al., 2011b). Both facts explain the existence of cheeses made with pasteurized milk and relatively high levels of BA. Consequently, pasteurization itself is not the ultimate solution to the problem of BA. Some authors have suggested that hurdle technology combining pasteurization with other treatments, such as high pressure, might reduce the presence of BA in cheeses, although no results were obtained yet (Novella-Rodríguez et al., 2002; Ladero et al., 2011b). Finally, it is important highlight that even using milk of good microbiological quality, if it is inoculated with BA-producing starters, the problem would persist. Therefore, a good starter selection – taking into account the ability to produce BA – is essential.

RIPENING PROCESS AND PROTEOLYSIS

Cheese ripening involves a complex variety of biochemical processes that include degradation of the lactose, lipolysis, and the most complex process, the catabolism of the proteins or proteolysis. The ripening process is carried out by starter LAB and the secondary microbiota, which comprises non-starter LAB, propionic acid bacteria, molds, and yeasts (Beresford and Williams, 2004). Starter bacteria contribute to the protein break down (Lane and Fox, 1996; Lynch et al., 1997) while the non-starter LAB are responsible of the peptidolysis and the release of free amino acids (Muehlenkamp-Ulate and Warthesen, 1999). Some blue cheeses elaborated with raw milk show high levels of proteolytic activity – due to the action of the fungi – that correlates with a high accumulation of BA (Fernández et al., 2007a).

Ripening and proteolysis are very important factors affecting the accumulation of BA in cheeses (Fernández-García et al., 2000). The proteolysis rate increases with the ripening time, leading the accumulation of free amino acids that serve as substrate of decarboxylic activities, which ultimately derives in BA accumulation (Fernández et al., 2007a). In general, the longer the aging process, the higher the content of BA (Fernández et al., 2007a). There are several studies showing that long-ripened cheeses have high proteolysis rate, which contributes to the high level of BA found in these cheeses in comparison with short-ripened ones (Arlorio et al., 2003; Bunková et al., 2010; Ladero et al., 2010b). In the particular case of histamine, it is also common to find the highest concentrations in long-ripened cheeses, which suggest that proteolysis occurring during the ripening period increases the production and accumulation of histamine in cheeses (Fernández et al., 2006b; Ladero et al., 2008). The same trend was observed for tyramine accumulation (Fernández et al., 2007a; Ladero et al., 2010b).

The addition of proteinases to cheese milk or curd has shown to accelerate the cheese ripening process but also the availability of small peptides and amino acids (Núñez et al., 1991; Mohedano et al., 1998), which has a direct effect on the BA production (Fernández-García et al., 1999).

CHEMICO-PHYSICAL FACTORS

Many chemico-physical factors such as the pH, salt concentration, and temperature can affect the BA-producing microorganism growth and the decarboxylic activities during the production of fermented dairy products. Some studies have investigated the effect of these factors on BA production, but little is known about the consequences of their interaction.

pH

Dairy fermentations are intrinsically associated to a low pH environment caused by the fermentation of lactose to lactic acid. Although the physiological role of BA biosynthesis may differ depending of the BA and the producing microorganism, it has been proposed as a system for neutralization of low extracellular pH that increases survival under acidic stress condition (Meng

and Bennett, 1992; Rhee et al., 2002). In LAB, the theory most widely extended is that those strains with the specific amino acid decarboxylases, produce BA to counteract the acidic stress of some environments, as that of dairy fermentations (Bearson et al., 1997; Wolken et al., 2006). This theory is supported by the relation between the pH decrease and BA production observed in LAB (Marcobal et al., 2006a; Fernández et al., 2007b). Certainly, it is well known that low pH is a crucial factor for the activity of some amino acid decarboxylases (Chander et al., 1988; Teodorovic et al., 1994). There are in the literature different works describing an acid pH to be optimum for tyrosine decarboxylase (Moreno-Arribas and Lonvaud-Funel, 1999, 2001) and other bacterial amino acid decarboxylases (Gale, 1946; Schelp et al., 2001). Moreover, the genes encoding for the decarboxylases can be induced at low pH. Linares et al. (2009) revealed that the increased production of tyramine by *Enterococcus durans* under low pH conditions is prompted by a significant induction of the decarboxylase (*tdcA*) and transporter (*tyrP*) genes expression, whereas they are not expressed at neutral pH. Similarly, the *tdc* and *aguA1* genes (involved in tyramine and putrescine production, respectively) of *L. brevis* are transcriptionally induced by low pH (Arena et al., 2010).

Overall, although some authors indicate that rapid acidification could reduce the levels of BA production related to a decrease in the growth of the decarboxylating microorganisms (Gardini et al., 2001), low pH is a key parameter which represents a potential risk of BA accumulation in the final product. However, it is difficult to act on this parameter, since it is inherent to the fermentation process.

Temperature

Temperature is a key parameter in the elaboration of cheeses and affects the accumulation of BA particularly during maturation and storage steps. In general, the production and accumulation of BA increases with the temperature during production and storage of cheese. The storage of the product includes the period between the last step of production – the ripening – until consumption. A number of studies show that low ripening and storage temperatures (e.g., 5°C) decrease the accumulation of BA such as histamine, tyramine, putrescine, and cadaverine, while their content increases with higher temperatures (Stratton et al., 1991; Gardini et al., 2001; Pinho et al., 2001; Gennaro et al., 2003; Santos et al., 2003; Martuscelli et al., 2005; Bunková et al., 2010). A multifactorial study of factors influencing tyramine production by LAB revealed that high temperature play a role on tyramine production by *L. brevis* and *Enterococcus faecium* (Marcobal et al., 2006a). In addition, a histamine-producing *S. thermophilus* strain produces lower amounts of histamine when it is stored at low temperatures (4°C) after its growth in milk. This reduction was attributed to a reduction in the activity of the histidine decarboxylase itself rather than a reduction in gene expression or the presence of a lower cell number (Calles-Enríquez et al., 2010).

It has been described that even if the cheese is kept in refrigeration, the BA accumulation could rise above the safe limits for consumption (Bunková et al., 2010). A step further, frozen temperature (e.g., −18°C) impairs the increase on the BA accumulation, most likely because the microbial activity is stopped (Andiç et al., 2010). However, more affordable solutions have to be found.

Sodium chloride

Another factor that might have an effect on BA accumulation is the concentration of salt in the fermented product. Traditionally salt has been employed to control the growth of pathogens during the fermentation and ripening process of dairy products with the last aim of preventing the spoilage and food-poisoning. Another consequence of the reduction on the growing rate of bacteria – including BA producers – would be the decrease of the BA concentration in the final product.

Most of the cheeses made with raw milk reach high numbers of *Enterococcus* spp., which is one of the main important BA producers in artisanal and traditional cheeses. The addition of high concentration of sodium chloride (5%) to milk inoculated with *Enterococcus faecalis*, reduced to the minimum the amount of 2-phenylethylamine and tyramine production (Gardini et al., 2001). The decrease on the BA production could be explained by the inhibitory effect of high salt content on the growth rate of BA-producing bacteria (Gardini et al., 2001) and/or on the amino acid decarboxylation activities (Chander et al., 1989). A similar effect of sodium chloride concentration has been observed in cheeses made of milk inoculated with *Lactobacillus bulgaricus* (Chander et al., 1989) or *Lactobacillus buchneri* (Sumner et al., 1990).

POST-RIPENING TECHNOLOGICAL PROCESSES

In the last decades, the market has reacted to customer requirements and it has evolved to sell ready-to-eat products (cut, sliced, grated. . .) instead of traditional formats such as the entire cheese. This ulterior processing implies more manipulation and the consequent increase of risk for microbial contamination (Reij and Den Aantrekker, 2004). The post-ripening process appears to have a direct impact in the presence of BA and BA producers. This could be due to the development of BA-producing bacteria present on the raw milk or to the contamination during the technological process (Custódio et al., 2007; Ladero et al., 2009).

Ladero et al. (2009), reported the effect of post-ripening process in the histamine-producing microbiota and the histamine content on a variety of cheeses. In some cases, histamine-producing bacteria were not found in the entire piece, but an increase on the number of histamine producers and histamine content was detected after grating. This result suggests an external contamination due to the post-ripening process. Thus, cheese grating seems to facilitate microbiological contamination due to the manipulation and the increase in the surface/volume ratio of the cheese fragments, which facilitates the presence of BA-producing bacteria and the subsequent accumulation of BA.

CONCLUSION

Safety is a basic requirement that must be always satisfied in food production. BA levels are unevenly distributed within cheese, where they can reach concentrations that represent a health risk, at least for those consumers with diminished detoxification system. Currently, there is no legislation defining the limits of BA tolerance in fermented foodstuff. However, cheese is the food that reach the highest BA concentrations, and therefore, a more severe control should be exercised.

Greater knowledge of the factors involved in the synthesis and accumulation of BA should lead to a reduction in their incidence in dairy foods. The production of BA in cheese is a complex phenomenon that depends on several variables, such as the presence of BA-producing microorganisms, their proteolytic and decarboxylase activities, ripening time, ripening and storage temperature, etc. In this sense, acidic pH is described to enhance BA accumulation *in vitro*. However, similarly to other chemico-physical conditions (such as amino acid availability, temperature, or salt concentration), it becomes difficult to modify these parameters, since they are inherent to the fermentation process. This clearly indicates that key actions to prevent BA accumulation must be addressed toward a reduction of the numbers of BA-producer microorganisms during cheesemaking. So far, control of BA-producing microorganisms by adequate thermal treatment of milk is one of the most important factors for reducing BA accumulation in dairy products. Moreover, encouraging a more responsible production by improving hygienic conditions, selection of starters without BA synthesis capability, and low storage temperatures, would contribute to reduce BA accumulation and to produce healthier cheeses. For this purpose, a number of quantitative PCR-based methods allowing detection and quantification BA-producer microorganisms either in starters, raw material, or even during cheesemaking and ripening processes have been described (Ladero et al., 2010b,c, 2012b).

In addition, more research is needed to increase our knowledge in two directions: (i) the effects of BA on the health of the consumers and (ii) the factors involved in the synthesis of BA in order to develop new strategies to prevent their accumulation.

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Biogenic amines in raw and processed seafood

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The presence of biogenic amines (BAs) in raw and processed seafood, associated with either time/temperature conditions or food technologies is discussed in the present paper from a safety and prevention point of view. In particular, storage temperature, handling practices, presence of microbial populations with decarboxylase activity and availability of free amino acids are considered the most important factors affecting the production of BAs in raw seafood. On the other hand, some food technological treatments such as salting, ripening, fermentation, or marination can increase the levels of BAs in processed seafood. The consumption of high amount of BAs, above all histamine, can result in food borne poisoning which is a worldwide problem. The European Regulation established as maximum limits for histamine, in fishery products from fish species associated with high histidine amounts, values ranging from 100 to 200 mg/kg, while for products which have undergone enzyme maturation treatment in brine, the aforementioned limits rise to 200 and 400 mg/kg. Preventive measures and emerging methods aiming at controlling the production of BAs are also reported for potential application in seafood industries.

Keywords: fish, histamine, bacteria, raw and processed seafood

INTRODUCTION

Seafood may harbor a number of biological, chemical, and physical hazards, the most prevalent of which are biogenic amines (BAs) and biotoxins (chemical), pathogenic bacteria and viruses (biological), and metal inclusion (physical). BAs are low molecular weight organic bases with biological activity that are formed in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases (Zhai et al., 2012). The most important BAs, histamine, tyramine, tryptamine, putrescine, and cadaverine, are formed from free amino acids namely histidine, tyrosine, tryptophane, ornithine and lysine, respectively. Spermidine and spermine arise from putrescine (Zarei et al., 2011). Putrescine, cadaverine, spermidine, and spermine have an aliphatic structure; histamine, and tryptamine have a heterocyclic structure and tyramine and phenylethylamine have an aromatic structure (Mohamed et al., 2009). The free amino acids either occur as such in foods or may be liberated through proteolysis. In addition to the availability of precursors (amino acids), BAs accumulation in foods requires the presence of microorganisms with amino acid decarboxylases and favorable conditions for their growth and decarboxylation activity (Zarei et al., 2011). Storage temperature is the most important factor contributing to BAs formation (Chong et al., 2011). Other parameters (i.e., pH, water activity, NaCl concentration, additives) may influence the variation of microbiota composition and lead to the differences in BAs content (Suzzi and Gardini, 2003). In addition, modified atmosphere packaging and vacuum packaging represent popular preservation methods which may inhibit the growth and increase the lag phase of microorganisms with amino acid decarboxylase activity (Chong et al., 2011).

The toxicological level of BAs is very difficult to establish because it depends on individual characteristics and the presence of other amines. However, a maximum total BAs level of 750–900 mg/kg has been proposed (Ladero et al., 2010).

The microbiological complexity of seafood is linked to the specific as well as non-specific microbial contaminants originating from the natural environment or being acquired during processing. The wide range of environmental habitats (freshwater to saltwater, tropical waters to arctic waters, pelagic swimmers to bottom dwellers, and degree of pollution) and the variety of processing practices (iced fish products to canned products) are all important factors in determining the initial contamination of fish and fish products (Gram and Huss, 1996). The types of bacteria that are associated with histamine production are commonly present in the saltwater environment. They naturally exist on the gills, on external surfaces, and in the gut of live, saltwater fish, with no harm to the fish. Upon death, the defense mechanisms of the fish no longer inhibit bacterial growth in the muscle tissue, and histamine-forming bacteria may start to grow, resulting in the production of BAs [Food and Drug Administration (FDA), 2011]. Evisceration and removal of the gills may reduce, but not eliminate, the number of histamine-forming bacteria. Packing of the visceral cavity with ice may aid in chilling large fish in which internal muscle temperatures are not easily reduced. However, when done improperly, these steps may accelerate the process of histamine development in the edible portions of the fish by spreading the bacteria from the visceral cavity to the flesh of the fish. With some harvesting practices, such as long-lining and gill-netting, death may occur many hours before the fish is removed from the water. Under the worst conditions, histamine formation can already be under way before the fish is brought onboard the vessel. This condition can be further aggravated with certain tuna species that generate heat, resulting in internal temperatures that may exceed environmental temperatures and increasing the

likelihood of conditions favorable to growth of enzyme-forming bacteria (FDA, 2011).

Some technological processes such as salting, ripening, fermentation or marination can increase the possibility of formation of BAs. A low pH (4.0–5.5), which can be achieved in salted anchovies, for instance, is favorable for enhanced amino acid decarboxylase activity (Pons-Sánchez-Cascado et al., 2005a). Moreover, important proteolysis is observed during ripening of salted anchovies, resulting in the liberation of peptides and free amino acids including histidine (Hernández-Herrero et al., 2002). The association of salted fish with histamine formation is probably due to the presence of halophilic or halotolerant microorganisms. For instance, Hernández-Herrero et al. (1999) reported that *Staphylococcus epidermidis* and *Staphylococcus capitis*, isolated from salted anchovies, showed a powerful histamine-forming activity, producing 1000 and 400 μg/ml, respectively. They assumed that the presence of these bacteria could be the result of contamination of fish during capture and subsequent unhygienic handling. BAs can also be produced throughout the manufacturing process, as well as during storage of the end product if improper holding temperatures are employed (Periago et al., 2003; Yongsawatdigul et al., 2004).

BACTERIA IN FISH

The microorganisms of fish intended for human consumption depend on the environmental conditions of its natural habitat. In particular, the microflora of fish from temperate water consists primarily psychrotrophic Gram-negative bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella*, and *Flavobacterium*. Members of the *Vibrionaceae* (*Vibrio* and *Photobacterium*) and the *Aeromonadaceae* (*Aeromonas* spp.) families are also common aquatic bacteria and typical of the fish flora [Food and Agriculture Organization (FAO), 1995]. Although Gram-negative bacteria are the predominant microorganisms in fish, Gram-positive bacteria such as *Bacillus, Micrococcus, Clostridium, Lactobacillus*, and coryneforms can also be found at various levels. *Aeromonas* spp. are typical of freshwater fish, whereas a number of bacterial genera such as *Vibrio*, *Photobacterium*, and *Shewanella* require sodium for growth and are, thus, typical of marine waters (Gram et al., 1990). In polluted waters, high numbers of *Enterobacteriaceae* may be found. In clean temperate waters, these organisms disappear rapidly, but it has been shown that *Escherichia coli* and *Salmonella* can survive for very long periods in tropical waters, and that once introduced, they may become indigenous to the environment (Fujioka et al., 1988).

The composition of fish microbiota changes quite dramatically during spoilage. *Shewanella putrefaciens* and *Pseudomonas aeruginosa* have been identified as the prominent spoilage bacteria of fresh fish (Gram and Huss, 1996). At ambient temperature (25°C), the microbiota at the point of spoilage is dominated by mesophilic *Vibrionaceae* and, particularly if the fish is caught in polluted waters, *Enterobacteriaceae*. Some *Pseudomonas* spp. are the specific spoilers of iced stored tropical freshwater fish and are also spoilers of marine tropical fish stored in ice (Gram and Huss, 1996). Many different bacterial species of the *Enterobacteriaceae* family are known to possess histidine decarboxylase activity and have the ability to produce histamine, including the species *Morganella morganii*, *Klebsiella pneumoniae*, *Hafnia alvei*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *Serratia liquefaciens*, *Raoultella* (formerly *Klebsiella*) *planticola*, *Raoultella ornithinolytica*, *Providencia stuartii*, and *Citrobacter freundii* (Kim et al., 2003). In addition to the enteric bacteria, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lowffi*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Aeromonas* spp., and *Photobacterium* spp. have also been reported as histamine formers (Chen et al., 2010). Emborg et al. (2006) identified *Morganella psychrotolerans*, a strong histamine-former, as a novel psychrotolerant bacterium, whereas the study of Kanki et al. (2004) revealed that these low temperature-adapted bacteria could play a role in scombroid poisoning.

BIOGENIC AMINES PRODUCTION IN RAW SEAFOOD

Histamine levels in freshly caught fish are generally low, usually below 0.1 mg/100 g (Auerswald et al., 2006). At any time, exposure of certain fish to elevated temperatures after the catch and before consumption can cause formation of histamine from histidine by bacterial histidine decarboxylases. While most studies agree that histamine formation is negligible in fish stored at 0°C or below, data concerning storage conditions at higher temperatures are variable and do not allow for the establishment of standard procedures for avoiding potential negative effects of transport/storage conditions on fish safety (Rossano et al., 2006). However, fish is more likely to form BAs when decomposition occurs at harvest or in the first stages of handling on the fishing vessels, rather than later in the distribution chain (Staruszkiewicz et al., 2004).

The term "scombroid" is derived from the name of the family *Scombridae* which includes the fish species that were first implicated in histamine intoxication (i.e., tuna and mackerel). These species of fish share in common high levels of free histidine in their muscle tissues. It is known that other non-scombroid fish species are also implicated in scombroid poisoning, such as mahi-mahi (*Coryphaena* spp.), sardines (*Sardinella* spp.), pilchards (*Sardina pilchardus*), anchovies (*Engraulis* spp.), herring (*Clupea* spp.), marlin (*Makaira* spp.), bluefish (*Pomatomus* spp.), Western Australian salmon (*Arripis truttaceus*), sockeye salmon (*Oncorhynchus nerka*), amberjack (*Seriola* spp.), Cape yellowtail (*Seriola lalandii*), and swordfish (*Xiphias gladius*). With the exception of salmon and swordfish, most of these fish species are rich in free histidine (Hungerford, 2010). However, it has been found that histamine poisoning may not be caused to all the people consuming contaminated fish. Given that histamine-forming bacteria may be diversely distributed in fish, the diffusion of produced histamine may also vary widely in different parts of the animal. For instance, while 50 mg/kg of histamine may be found in one fish section, its level may exceed 500 mg/kg in another (FDA, 2011). Thus, even if the same histamine-containing fish is ingested, some consumers may be poisoned and some may not (Tao et al., 2002, 2009).

Histamine is produced in raw fish from the action of bacterial histidine decarboxylase following temperature/time abuse. Production of histamine is greater, however, at high abusive temperatures (21.1°C or higher) than at moderate abusive temperatures (7.2°C), while its generation is particularly rapid at temperatures near 32.2°C (FDA, 2011). According to European Regulation fresh fishery products, thawed unprocessed fishery products, and

cooked and chilled products from crustaceans and mollusks must be maintained at a temperature approaching that of melting ice (European Commission, 2004). Rapid chilling of scombrotoxin-forming fish immediately after death is the most important element in any strategy for preventing the formation of histamine, especially for fish that is exposed to warm waters or air, and for tunas which generate heat in their tissues. Failure to chill onboard may permit bacteria and enzymes, including those that form histamine, to increase to high levels (FDA, 2011). Even if ice storage is recommended, temperature/time abuse conditions often occur in the fish merchandising chain. Delays in removing fish from the water after capture, such as those captured by a longline, may significantly limit the amount of time left for chilling and may allow some fish to heat up. Moreover, mishandling coupled with high temperature abuse are likely when handling fish and may significantly enhance histamine formation. The amount of post-harvest time at elevated temperatures (after proper chilling onboard the harvest vessel) to which a fish can be exposed (e.g., during processing, storage, and distribution) without adverse effects depends primarily on whether the fish was previously frozen (e.g., in the harvest vessel) or heat-processed sufficiently to destroy histamine-forming bacteria (FDA, 2011). Rossano et al. (2006) studied the influence of storage temperature and time of freezing on histamine formation in anchovies, showing the ability of freezing to inhibit or slow down its formation.

Many scientists have studied the effects of storage temperatures on histamine formation in fish and their results have been very often ambiguous. This can be explained by the differences in the composition and the level of microorganisms in the fish. Histamine producing bacterial species and strains vary considerably in amounts of histamine formation, and the type of spoilage bacteria present depends on the aquatic environment. It has been reported that *M. morganii*, *K. pneumoniae*, and *P. vulgaris* are prolific histamine formers, producing >1000 mg/kg in the culture broth (López-Sabater et al., 1996; Rawles et al., 1996; Kim et al., 2001). These species have rarely been detected in fresh fish, but have mostly been isolated from fish spoiled under controlled storage conditions, above 20°C (Ababouch et al., 1991; Kim et al., 2001). Bacteria occurring naturally in marine environments such as *Photobacterium* spp., *Pseudomonas* spp., *Vibrio alginolyticus*, and *Aeromonas* spp. have indeed been frequently isolated from fish stored at refrigeration temperature for extended periods (Middlebrooks et al., 1988; Morli et al., 1988). However they are weak histamine formers, producing <500 mg/kg in the culture broth (Frank et al., 1985). Then, in raw fish histamine content is linked to the type of histamine-forming bacteria, the type of seafood, and temperature/time storage conditions (**Table 1**). Typically, boats fish overnight in a trip of up to 12 h. Storage is at ambient temperature until unloaded at the processing plant, with the first-caught fish being already stored for up to 10 h. Such a long period may cause histamine-producers to undergo nine doublings, an increase of 1000 times (three log scales) over the assumed initial level of 10/g or cm^2, reaching a level of 10000/cm^2 at fish surfaces or 10000/g in the gut (FAO, 2004).

In addition to their toxicological implications, BAs are related to fish spoilage, since they accumulate as a result of the proteolytic and amino acid decarboxylase activity of microorganisms

Table 1 | Histamine content in fresh fish stored at abused temperature/time conditions.

Fish	Temperature/time	Histamine (mg/kg)	Reference
Pacific mackerel	25°C for 48 h	2830.0	Kim et al. (2001)
Yellowfin tuna	22°C for 5 d	4533.0	Du et al. (2002)
Albacore tuna	25°C for 6 d	671.0	Kim et al. (2002a)
Mackerel	25°C for 24 h (inoculated with *Morganella morganii*)	4610.0	Kim et al. (2002b)
Albacore		3430.0	
Mahi-mahi		3340.0	
Salmon		255.0	
Skipjack tuna	21°C for 48 h	1533.0	Rossi et al. (2002)
Mackerel	32.2°C for 9 h	28.0	Shakila et al. (2003)
	32.2°C for 12 h	50.0	
	32.2°C for 16 h	100.0	
Mahi-mahi	26°C for 12 h	50.0	Staruszkiewicz et al. (2004)
Skipjack tuna	25°C for 10 h	10.0	
Yellowfin tuna	25°C for 12 h	10.0	
Yellowfin tuna	20°C for 24 h	111.4	Guizani et al. (2005)
Sailfish	25°C for 24 h	2240.0	Tsai et al. (2005a)
Milkfish		3990.0	
Anchovy	25°C for 24 h	1465.0	Visciano et al. (2007)
Pilchard		1106.0	
Mackerel	25°C for 24 h	2123.9	Kim et al. (2009)
Saury		1776.7	
Spanish mackerel		189.9	
Amberjack		36.6	
Tuna fish	25°C for 48 h (inoculated with *Morganella morganii*)	2000.0–4000.0	Tao et al. (2009)
	25°C for 48 h (inoculated with *Photobacterium phosphoreum*)	1500.0–1800.0	

(**Table 2**). The use of more than a single BA (i.e., a BA index that consists of a combination of BAs) can be used as a quality indicator for fish freshness. Some examples are the sum of cadaverine and putrescine (Stede and Stockemer, 1981), the index of Mietz and Karmas (1981), which considers the increases in putrescine, cadaverine and histamine levels along with the corresponding decreases in spermidine and spermine, as well as the index described by Veciana-Nogués et al. (1997a) for tuna, which includes putrescine, cadaverine, histamine, and tyramine. In their study, Baixas-Nogueras et al. (2005) used these indexes for the freshness evaluation of iced Mediterranean hake (*Merluccius merluccius*) in the chilling conditions as applied in the merchandising chain. Putrescine and cadaverine were the main amines accumulated, whereas histamine and tyramine were less abundant. Cadaverine was the amine best correlated with *S. putrefaciens*, the specific spoilage organism, while putrescine showed the most satisfactory correlation with the genus *Pseudomonas*. According to the

obtained results, the authors proposed a BAs index limit of acceptability in a range of 15–20 μg/g. The study of Veciana-Nogués et al. (1997a), indeed, considered BAs as hygienic quality indicators in tuna (*Thunnus thynnus*), a fish belonging to the *Scombridae* family and therefore, with high levels of free histidine in its muscle. So, the value of 50 μg/g for the sum of histamine, tyramine, putrescine, and cadaverine, which was not exceeded in samples stored at 0°C before organoleptic rejection, was proposed as a guiding limit value for tuna acceptance.

BIOGENIC AMINES IN PROCESSED SEAFOOD

BAs formation is possible during processes such as brining, salting, smoking, drying, fermenting, and pickling until the product is fully shelf-stable (**Table 3**). Refrigeration can be used to inhibit histamine formation during these processes (FDA, 2011). Samples of fermented fish products (fish sauce, fish paste, and shrimp paste) were analyzed for histamine content (Tsai et al., 2006), which was 394, 263, and 382 mg/kg, respectively. Three fish sauces, two fish pastes, and two shrimp paste products contained greater than 500 mg/kg of histamine. Moreover 7.4% of the tested samples contained >1000 mg/kg. The average content of various BAs in tested samples was less than 90 mg/kg. The fish paste Rihaakuru, which is an important condiment in the Maldives, could contain high concentrations of BAs, due to raw tuna, from which the product is made from, being subjected to temperature abuse. Twenty-eight samples of Rihaakuru (Naila et al., 2011), were analyzed for some BAs; in particular, histamine was detected at the highest concentration (5487 mg/kg). Tryptamine was not detected in most of the samples (only three samples contained <5 mg/kg) and phenylethylamine only occurred at low levels (<25 mg/kg). The authors supposed that the histamine found in Rihaakuru samples was most likely to have originated from Gram-negative bacteria growing in the fish before processing or within the fish during the early steps of manufacture. There are other processed seafood which have been investigated for BAs content. In southern China, three fish products are widely consumed: salted and fermented fish, canned fish, and packaged fish. Zhai et al. (2012) examined 49 fish products from the China market. The maximum total BAs content of lightly cured horse mackerel was 484.42 mg/kg compared to 167.86 mg/kg or less for the other salted and fermented fish products. In the Spanish mackerel sample, histamine was detected within the range of 15.74–28.70 mg/kg, whereas the maximum histamine level was 26.95 mg/kg in canned anchovies, 22.38 mg/kg in canned sardines and less than 10 mg/kg in all other canned samples tested (Zhai et al., 2012). Mah et al. (2002) found high levels of histamine (155–579 mg/kg) in fermented fish products made from anchovies, whereas Huang et al. (2010) reported large amounts of histamine in dried fish products (63.1–479.0 mg/kg).

Ripened, semi-preserved anchovies are prepared from fish of the *Engraulis encrasicholus* species, and are a common tradition in some Mediterranean countries. Pons-Sánchez-Cascado et al. (2005a) studied BAs in salt-ripened anchovies reporting that tyramine was the most abundant amine, reaching values up to 90 mg/kg, whereas histamine did not exceed 20 mg/kg. Then, the same authors analyzed samples of vinegar-marinated anchovies and reported higher values for tyramine than histamine (7.81 and 0.54 mg/kg, respectively) in 14 days of refrigerated storage (Pons-Sánchez-Cascado et al., 2005b).

The applicability of lactic acid bacteria (LAB) in fermenting whole fish has been demonstrated. The fermentation process for fish may fulfill the conditions required for abundant formation of BAs, i.e., availability of free amino acids, the presence of decarboxylase-positive microorganisms and conditions allowing bacterial growth, decarboxylase synthesis, and decarboxylase activity (Petäjä et al., 2000). Some fish sauce products, particularly those made from sardine and mackerel, often contain large quantities of histamine, about 1000 mg/l or greater (Tsai et al., 2006; Kuda and Miyawaki, 2010), as a result of the histidine decarboxylase of *Tetragenococcus* spp., a halophilic lactic acid bacterium. However, most studies (Thapa et al., 2006; Muñoz-Atienza et al., 2011) showed that in fermented fish products LAB produced no histamine or other BAs. Kuda et al. (2012) reported the possibility of regulation of histamine accumulation in salted and fermented fish products by the addition of halophilic LAB, like a starter culture, isolated from nukazuke (salted and fermented fish with rice bran). In a total of 200 isolates from nukazuke fish, 13 strains produced histamine in histidine containing broth (0.5%) at levels more than 200 μg/ml, whereas 130 isolates produced no histamine. Furthermore, 22 of the tested strains appeared to suppress histamine production (Kuda et al., 2012).

HISTAMINE FORMATION AND POISONING

Histamine poisoning occurs throughout the world and is perhaps the most common form of toxicity caused by the ingestion of fish (**Table 4**). However, reliable statistics about its incidence do not exist because poisoning incidents are often unreported due to mild symptoms, lack of adequate reporting systems, or misdiagnoses by medical personnel of histamine poisoning as a food allergy (FAO, 2004).

Many BAs have been found in fish, but only histamine, cadaverine, and putrescine have been identified as significant concerns with regard to fish safety and quality (Al Bulushi et al., 2009). Despite the widely accepted association between histamine and scombroid food poisoning, histamine alone appears to be insufficient to cause toxicity, and putrescine and cadaverine have been suggested to potentiate its toxic activity by inhibiting the intestinal histamine-metabolizing enzymes, diamine oxidase and histamine N-methyltranferase (Stratton et al., 1991). The onset of scombroid poisoning is typically from 10 min to 1 h following consumption of fish and can last from 12 h to a few days. The symptoms are variable and include peppery or metallic taste, oral numbness, headache, dizziness, palpitations, rapid and weak pulse, drop in blood pressure, difficulty in swallowing, and thirst. Also noteworthy are allergy-like symptoms such as hives, rash, flushing, and facial swelling (Hungerford, 2010). Symptoms involving the central nervous system such as anxiety are less frequently observed. Less specific symptoms such as nausea, vomiting, abdominal cramps, and diarrhea are also experienced (Gilbert et al., 1980). Serious cardiac and respiratory complications may be caused in individuals with preexisting conditions (Ascione et al., 1997). In a few rare cases hospitalization, including treatment for anaphylactic shock, has been required (Sanchez-Guerrero et al., 1997).

Table 2 | Levels (mean value, mg/kg) of BAs in raw seafood.

Fish	Temperature/ time	Cadaverine	Putrescine	Spermidine	Spermine	Tyramine	Histamine	Reference
Tuna	0°C	0.7	0.3	6.8	22.4	0.0	0.2	Veciana-Nogués et al. (1997b)
Herring	0°C/0 days	8.5	0.0	0.0	0.0	0.0	0.0	Özogul et al. (2002)
	0°C/16 days	237.2	39.7	4.5	3.4	4.2	271.4	
Rainbow trout	0°C/0 days	0.0	7.5	4.1	0.3	0.2	0.0	Chytiri et al. (2004)
	0°C/18 days	2.7	23.1	13.6	5.1	2.9	1.6	
Mediterranean Sea bass	0°C/0 days	0.0	0.0	8.8	14.4	0.0	0.0	Paleologos et al. (2004)
	0°C/16 days	6.5	3.1	0.0	0.0	4.3	0.0	
Sailfish	0°C	2.1	0.3	0.4	2.7	0.1	4.6	Tsai et al. (2004)
Indian anchovy	35°C/0 h	15.5	0.0	49.3	6.2	46.9	14.0	Yongsawatdigul et al. (2004)
	35°C/16 h	863.4	259.9	55.2	27.1	273.0	2007.0	
Mediterranean hake	0°C/0 d	0.8	1.7	3.5	4.6	0.6	0.1	Baixas-Nogueras et al. (2005)
	0°C/14 days	20.3	12.2	10.7	15.0	2.4	2.2	
Sardine	4°C/0 days	3.9	13.4	1.2	0.0	0.0	19.5	Özogul and Özogul (2006)
	4°C/15 days	100.4	114.0	7.6	2.9	16.3	203.0	
Alaska pollack	0°C	6.3	36.3	7.1	0.5	1.9	0.0	Kim et al. (2009)
Pacific cod		2.6	4.2	3.1	4.3	3.7	0.0	
Pacific herring		59.5	43.9	3.0	3.2	23.3	9.1	
Pacific mackerel		0.0	9.8	35.2	3.8	40.3	2.7	
Bandfish	0°C	9.9	15.0	1.8	4.4	0.7	0.6	Zhai et al. (2012)
Golden pompano		1.0	1.2	2.5	6.0	0.1	0.1	
Blue scad		54.3	42.5	2.8	1.9	29.6	20.0	
Mackerel		1.6	0.7	0.2	1.8	0.1	10.2	
Pacific saury		52.0	3.7	0.2	0.6	21.2	9.1	

Table 3 | Levels (mean value, mg/kg) of BAs in processed seafood.

Product	Cadaverine	Putrescine	Spermidine	Spermine	Tyramine	Histamine	Reference
Canned tuna	0.6	0.2	4.0	10.8	0.0	0.4	Veciana-Nogués et al. (1997b)
Anchovies in oil	38.3	7.6	2.3	7.9	21.6	12.6	Veciana-Nogués et al. (1997c)
Fish sauce	685.5	308.2	9.9	3.7	117.3	574.7	Yongsawatdigul et al. (2004)
Fish sauce	89.0	24.0	9.0	52.0	9.4	394.0	Tsai et al. (2006)
Fish paste	58.0	12.0	15.0	60.0	8.8	263.0	
Shrimp paste	80.0	40.0	36.0	43.0	3.7	382.0	
Dried milkfish	949.0	44.0	7.0	23.0	85.0	4097.0	Hsu et al. (2009)
Bullet mackerel	1.1	8.2	256.2	162.6	11.5	39.3	Huang et al. (2010)
Round scad	13.3	41.3	22.0	258.0	48.8	31.8	
Smooth-tailed trevally	145.0	63.3	0.0	11.5	59.2	210.7	
Pacific round herring	30.2	11.4	85.8	258.3	0.0	9.1	
Salted mackerel	2.0	0.0	5.5	2.0	6.0	0.9	Park et al. (2010)
Canned mackerel	7.8	2.3	4.3	1.8	4.7	1.4	
Canned tuna	1.7	1.8	3.0	4.4	3.2	1.4	
Canned salmon	1.2	0.2	1.0	4.7	1.1	0.0	
Fish paste	387.0	290.0	14.6	15.8	5.1	5080.0	Naila et al. (2011)
Salted escolar roe	17.2	21.8	51.3	40.7	24.8	6.2	Hwang et al. (2012)
Light cured horse mackerel	244.4	64.5	0.2	0.0	62.8	21.3	Zhai et al. (2012)
Canned bandfish	53.1	18.4	0.0	0.7	17.3	1.1	
Canned anchovy	23.9	2.8	2.0	1.1	3.0	19.8	
Salted ice fishes	5.8	51.1	47.7	51.8	0.4	0.1	

Table 4 | Outbreaks of scombroid poisoning: source, geographical location, period, and number of involved cases.

Source	Location	Period	No. cases	Reference
Canned tuna	USA	1973	254	Merson et al. (1974)
Mackerel, tuna, anchovies, sardines, marlin	Japan	1970-1980	4122	Taylor (1986)
Dried horse mackerel	Japan	1973	2656	Taylor (1986)
Tuna, mackerel	Italy	1979	250	Molinari et al. (1989)
Yellowtail	South Africa	1992	22	Müller et al. (1992)
Tuna (fresh/frozen, canned), mackerel	United Kingdom	1987-1996	243 (sporadic) 105 (general) 56 (family)	Scoging (1998)
Fish	USA	1993-1997	297	Olsen et al. (2000)
Yellowtail	South Africa	2004	19	Anonymous (2004)
Canned mackerel	Taiwan	2001	3	Tsai et al. (2005b)
Tuna	South Africa	2004	1	Auerswald et al. (2006)
Fish	USA	1998-2002	463	Lynch et al. (2006)
Swordfish	Taiwan	2004	43	Chang et al. (2008)
Dried milk fish	Taiwan	2006	3	Huang et al. (2010)
Fried fish cubes	Taiwan	2007	347	Chen et al. (2010)

According to the FDA guidelines (FDA, 2011), the toxicity and defect action levels of histamine, established for tuna, mahi-mahi, and related fish, are the 50 mg/100 g and 5 mg/100 g, respectively; the term "defect action level" refers to the level of histamine naturally or inevitably occurring in foods without, however, presenting a considerable hazard for humans. According to the EU Regulation No 2073/2005 nine samples should be taken from each batch of fish species of the following families: *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae.* These samples must fulfill the following requirements:

Mean value of all samples must not exceed 10 mg/100 g;
Two samples may be >10 mg/100 but <20 mg/100 g;
No sample may exceed 20 mg/100 g.

However, fish belonging to these families that have undergone enzyme ripening in brine may have higher histamine levels, but not more than twice the above values.

ANALYTICAL METHODS FOR BAs DETECTION

The number and variety of methods developed for laboratory histamine testing of fish and fishery products is impressive. In contrast to many of the other more potent seafood toxins, the relatively high action levels established for histamine in fish allow for its detection by a variety of different approaches ranging from simple and inexpensive thin layer chromatography (TLC) procedures to resource-intensive and more powerful liquid chromatography-mass spectrometry (LC-MS) methods (Hungerford, 2010). Most of the histamine separation methods applied in fish use reversed-phase high performance liquid chromatography (HPLC) with detection schemes based on pre-column derivatization (Mietz and Karmas, 1978; Hui and Taylor, 1983; Malle et al., 1996) or post-column derivatization (Glória et al., 1999; Brillantes and Samosorn, 2001) to produce fluorescent products or strong chromophores, but direct UV detection of histamine imidazole ring has also been applied (Shakila et al., 2001; Cinquina et al., 2004b). Other popular separation-based methods include ion chromatography (Cinquina et al., 2004a), capillary electrophoresis (Zhang and Sun, 2004), paper electrophoresis (Sato et al., 2002, 2006), TLC (Bajc and Gacnik, 2009), and gas chromatography-mass spectrometry (Marks and Anderson, 2006). In addition, there is a need for methods well suited to high-speed screening. The most rapid method for detecting histamine is based on flow injection analysis (FIA) and is capable of screening 60 sample extracts/hour (Hungerford et al., 1990). Enzymatic methods are attractive for their selectivity and flow injection has been used in combination with enzyme electrodes for easy automation (Watanabe et al., 2007). Many other commercial test kits are available, based on selective antibodies (Lehane and Olley, 2000; Köse et al., 2009). Commercial test kits based on immunoassay methods for histamine analyses became popular because of their user friendliness and reduced time requirements compared to those of traditional analytical techniques. Recently many authors (Köse et al., 2011; Tahmouzi et al., 2011; Tao et al., 2011; Hungerford and Wu, 2012) report different methods for rapid determination of histamine in fish.

CONTROL MEASURES

The FDA has issued industry guidelines aiming at establishing procedures for the safe processing and importing of fish and fishery products based on the hazard analysis and critical control points (HACCP) approach (FDA, 2011). According to the most recent HACCP guidelines for the control of histamine production, a core temperature of 4.4°C or less should be achieved and maintained throughout handling, processing, and distribution of potentially hazardous fish. The primary goal of these guidelines is the growth inhibition of spoilage bacteria capable of producing histamine through proper handling and chilling of fish (FDA, 2011). In order to achieve this objective, all boats should ice fish immediately after landing aboard the vessel so that the temperature at sites of microbiological concern is reduced at levels capable of controlling the growth of histamine-producing bacteria. Moreover, an ice-plant

could be built and ice made available at reasonable cost. Other spaces could also be modified so that the boats could be capable of carrying up to 100 kg of ice (FAO, 2004). It must be highlighted, however, that the time required to lower the internal temperature of fish after capture depends on a number of factors, including: (i) the harvest method; (ii) the size of the fish; (iii) the chilling method. Once chilled, the scombrotoxin-forming fish should be maintained as close as possible to the freezing point (or held frozen) until it is consumed (FDA, 2011).

At the processing plant, fish is gilled and gutted, then stored in ice until packed for air transport to the consumer country. Histamine decarboxylase activity could lead to a 10-fold increase in histamine during processing, air freight and marketing. The preventive measures for this step include controlling refrigeration temperature in the plant or performing proper icing during storage of raw material, in-process product as well as finished product (FAO, 2004). During processing of fish (butchering, cleaning, brining, salting, smoking, drying, fermenting, pickling, mixing, stuffing, packing, labeling, and staging), it is recommended that it is not exposed to ambient temperatures above 4.4°C for more than 12 h cumulatively, if it has been previously frozen or heat-processed sufficiently to destroy histamine-forming bacteria, or for more than 4 h in the other case (FDA, 2011). Given the heat-stable nature of histamine, the intended use of the product is not likely to affect the significance of this hazard. Recontamination of seafood with enzyme-forming bacteria in conjunction with temperature abuse may also allow for histamine formation following cooking. Thus, a conscientious sanitation program during seafood processing is of vital importance in order for recontamination events to be avoided.

Many recent studies proposed a new approach based on the employment of microorganisms or substances (additives, spices, disinfectants) able to inhibit histamine-forming bacteria. Mah and Hwang (2009a) studied the effects of food additives on BAs-producing strains of *Bacillus licheniformis* isolated from Myeolchi-jeot, with the greatest inhibitory effect being observed for glycine. The same product (Myeolchi-jeot) was ripened with the addition of a starter culture of *Staphylococcus xylosus*, which was shown to be capable of degrading histamine and tyramine (Mah and Hwang, 2009b), while the use of spices, in particular garlic, also showed an inhibitory effect (Mah et al., 2009). The development of post-harvest treatments for reducing histamine-forming bacteria in fish upon harvest is an important intervention strategy to prevent histamine formation in fish and control scombroid poisoning. Phuvasate and Su (2010) investigated the efficacy of treatments with electrolyzed oxidizing (EO) water and EO ice, containing 100 ppm chlorine. According to these researchers, soaking of fish (salmon) in EO water reduced *Enterobacter aerogenes* and *Morganella morganii* by 1.3 and 2.2 log CFU/cm^2 respectively, while soaking yellowfin tuna in EO ice reduced the same microorganisms by 2.4 and 3.5 log CFU/cm^2, respectively. Moreover, emerging methods potentially applied as control measures include the addition of starter cultures that degrade histamine, the application of hydrostatic pressure, irradiation, and packaging (Naila et al., 2010).

CONCLUSION

Seafood is susceptible to contamination by BAs-producing microorganisms at different points of the food chain. High levels of BAs can be prevented through the application of good hygiene practices and proper temperatures during handling, delivery and storage. Although BAs formation is the result of bacterial growth, the presence of these undesirable compounds, especially histamine, is not always noticed by consumers. In fact, while a fish with obvious spoilage will most likely not be consumed, a fish with a good appearance and no detectable spoilage odors may be consumed even if it contains a high histamine level. Thus, the application of appropriate control measures is fundamental for assuring seafood safety and such a responsibility is shared among the seafood catchers, processors, distributors, retailers, and merchants.

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Focused Review: Agmatine in fermented foods

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Polyamines (PAs) are ubiquitous substances considered to be bioregulators of numerous cell functions; they take part in cell growth, division, and differentiation. These biogenic amines are also involved in tissue repair and in intracellular signaling; in fact, because of their polycationic character, they interact to a large extent with membrane phospholipids and may play an important role in the regulation of membrane-linked enzymes. The intracellular polyamine content derives from the simultaneous regulation of the synthesis, catabolism, uptake, and elimination of the polyamines; furthermore, PAs are present in all cell types at different concentrations, but the highest levels are found in rapid-turnover tissues. In addition to spermidine, spermine, and putrescine, also agmatine (AGM), deriving from arginine and identified in mammals in the 1990s, is a polyamine and several studies have reported its potentially positive role in the production of secretagogues, and in neuronal, vascular, metabolic, and therapeutic functions. Because of the low arginine decarboxylase (ADC) activity in mammalians, the amounts of AGM found in their tissues can be only minimally ascribed to an endogenous *de novo* synthesis by ADC, while a substantial quantity of AGM may be of dietary origin. Several food products contain only small amounts of polyamines, while higher concentrations can be found in fermented foods. PAs could also be considered as indicators of freshness in fish and meat products; as these moieties are produced during food storage, it would seem to confirm the main role of microorganisms in their synthesis. In particular, high levels of AGM are present in alcoholic beverages, such as wine, beer, sake, which would seem to confirm the role of yeasts in AGM production. Although many biological functions have been attributed to polyamines, high levels of these compounds in foodstuffs can have toxicological effects; however, no safe level for the intake of polyamines in a diet has yet been established. In this paper the presence of AGM in different foodstuffs is discussed, also taking into account the various factors affecting its presence and concentration.

Keywords: agmatine, biogenic amines, fermented foods, foodstuffs, polyamines

INTRODUCTION

Biogenic amines (BAs) are ubiquitous substances occurring in every living cell. They play different roles in cellular metabolism: in particular, polyamines (PAs), such as agmatine (AGM), putrescine (PUT), spermine (SPM), and spermidine (SPD) are considered to be bioregulators of numerous cell functions, being involved in the process of cell growth, division, and differentiation (Galgano et al., 2003). These BAs are also involved in tissue repair and in intracellular signaling; in fact, because of their polycationic character, they interact to a large extent with membrane phospholipids and may play an important role in the regulation of membrane-linked enzymes. The intracellular polyamine content derives from the simultaneous regulation of the synthesis, catabolism, uptake, and elimination of the polyamines; furthermore, PAs are present in all cell types at different concentrations, but the highest levels are found in rapid-turnover tissues (Moinard et al., 2005). In mammals their diet also provides a daily supply of PAs and the distribution of the different PAs varies according to the food type. The highest amounts are present in fermented foods and beverages, especially in protein-rich foods, e.g., fish and fish products, meat and meat products, eggs, cheeses, fermented vegetables, fruits, nuts, chocolate, soybean products, and wine (Silla Santos, 1996). Moreover, also processing and additives can influence BA formation in foods: for example, BAs are heat stable compounds and their level is not significantly reduced by high temperature treatment (Naila et al., 2010).

Biogenic amines in foods are generated either as the result of endogenous amino acid decarboxylase activity in raw food materials or by the growth of decarboxylase-positive microorganisms under conditions favorable to enzyme activity (Halász et al., 1999a). As the microbial spoilage of food may be accompanied by the increased production of decarboxylases, the presence of BAs might serve as a useful indicator of food spoilage (Silla Santos, 1996).

Although many biological functions have been attributed to PAs, high levels of these compounds in foodstuffs can have toxicological effects. In fact, even if individually they are not considered toxic, PAs can enhance the effects of histamine (HIS) and tyramine (TYR) by interacting with the aminooxidases and interfering with detoxifying mechanisms. Similarly, other compounds, like alcohol and acetaldehyde, can enhance the toxic potential of BAs, since they promote the transportation of these moieties through the

intestinal wall. For these reasons, it is difficult to establish the safety threshold of BAs in a given product, because their toxicity does not depend merely on their presence (type and level), but also on individual detoxification mechanisms and on the occurrence of other compounds affecting their activity (EFSA Panel on Biological Hazards (BIOHAZ), 2011; Ruiz-Capillas and Jiménez-Colmenero, 2004).

ORIGIN OF AGMATINE

The presence of AGM in plants, bacteria, and invertebrates has been known for a long time, but its occurrence in mammals was reported for the first time only in the 1990s and since then growing attention has been focused on this polyamine (Moinard et al., 2005).

AGM is produced from L-arginine by arginine decarboxylase (ADC) and is a PUT precursor, one of the main BAs associated with microbial food spoilage. PUT can originate from arginine via ornithine decarboxylase, or another pathway is by AGM deamination involving three enzymes (AGM deiminase, PUT carbamoyltransferase, and carbamate). This pathway has been demonstrated only in a few microorganisms, such as *Pseudomonas aeruginosa* PAO1, *Enterococcus faecalis* ATCC11700, *Bacillus cereus* ATCC14579, and *Lactobacillus hilgardii* X_1B (Arena and Manca de Nadra, 2001; EFSA Panel on Biological Hazards (BIOHAZ), 2011; Landete et al., 2008). In particular, the amino-biogenic activity of the latter microorganism has been widely demonstrated, especially with regards to the production of AGM and PUT at high concentrations (EFSA Panel on Biological Hazards (BIOHAZ), 2011; Landete et al., 2008).

The presence of pyridoxal, Mg^{2+}, and Mn^{2+} can enhance ADC activity as well as that of other amino decarboxylases, but agmatine deiminase does not seem to be affected by the presence of these cofactors; conversely, arginine, glucose, and fructose have been shown to exert an inhibitory effect on AGM deamination, whereas high levels of AGM can enhance the production of PUT (Arena and Manca de Nadra, 2001; EFSA Panel on Biological Hazards (BIOHAZ), 2011; Landete et al., 2008). Landete et al. (2008) have reported a positive correlation between the presence of succinate, SPM and SPD, and PUT production in *P. aeruginosa* PAO1. Conversely, in the case of *E. faecalis* ATCC11700, *B. cereus* ATCC14579, and *L. hilgardii* X_1B succinate did not have any effect, while SPD and SPM always contributed to the reduction of AGM deamination.

The ability of bacteria to produce BAs is widely documented, whereas there are few reports regarding the formation of BAs by yeasts, even if considerable quantities of AGM and other BAs have been found in wines and other alcoholic beverages (Izquierdo-Pulido et al., 1996; Glória et al., 1998; Galgano et al., 2003, 2011). Caruso et al. (2002) have reported the ability of *Saccharomyces cerevisiae* to produce ethanolamine and AGM in inoculated grape must; also other yeast strains isolated from grapes and wines, belonging to the species *Candida stellata*, *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Brettanomyces bruxellensis*, are thought to be capable of producing AGM. In *Photobacterium phosphoreum* the presence of AGM and PUT has been reported to influence the occurrence of histamine (HIS), consequently inhibiting histidine decarboxylase activity (Karovicová and Kohajdová, 2005).

ROLE OF AGMATINE

Moinard et al. (2005) have reported that AGM concentrations in the brain have been found to be comparable to that of typical neurotransmitters, thus suggesting that AGM might be a neurotransmitter.

A variety of effects have been demonstrated in response to AGM *in vivo* and *in vitro*, but the biological significance of these effects is still under debate, because the concentrations at which the effects were induced were much higher than those present in blood and tissues (Molderings et al., 2002). However, AGM is known to have secretagogue properties, such as the modulation of insulin release and glucose metabolism, the stimulation of adrenaline, and noradrenaline secretion. AGM antagonizes some hyperalgesic states and enhances the dose-related analgesic effect of morphine (Moinard et al., 2005; Wade et al., 2008).

AGM appears to influence appetite regulation. The administration of a high dose of AGM to satiated rats triggered an increase in their caloric intake and their carbohydrate preferences, whereas AGM does not modulate caloric intake in hungry rats (Prasad and Prasad, 1996). AGM also plays an important role in polyamines homeostasis: by inhibiting ornithine decarboxylase, AGM inhibits smooth muscle proliferation (Moinard et al., 2005).

AGM is recognized as an inhibitor of nitric oxide synthase, an enzyme that, starting from arginine, catalyzes the production of nitric oxide (NO), an intercellular messenger implicated in the regulation of various physiological functions in central neurotransmission. It has been suggested that NO may be involved in the mechanism of anxiety, therefore NO inhibitors, such as AGM, may be a potential tool for the treatment of anxiety (Uzbay and Lal, 2002), even though Krass et al. (2008) failed to demonstrate the reported antidepressant and anxiolytic-like activity of AGM.

However, it is highly probable that AGM will play a role in the therapeutic treatment of a number of different diseases. In the treatment of cancer, it has been established that AGM has a antiproliferative effect due to its suppression of polyamine synthesis and cellular polyamine uptake through the induction of antizyme (Agostinelli et al., 2010).

It has also been suggested that AGM could play an important role in the control of ureagenesis, representing a significant contribution to the therapeutic removal of waste toxic nitrogen from the body. Moreover, AGM could be useful in the treatment of sepsis, because its administration to endotoxemic rats has been shown to prevent the decrease in blood pressure and renal function usually associated with sepsis (Moinard et al., 2005).

AGM is also able to protect brain mitochondria against the drop in energy capacity by the Ca^{2+}-dependant induction of permeability transition in rat brain mitochondria (Battaglia et al., 2010). Furthermore, AGM may also be considered as a regulator of mitochondria cell death. In addition, by increasing the expression of antizyme, a protein that inhibits polyamine biosynthesis and transport, AGM exhibits a regulatory effect on cell proliferation (Agostinelli et al., 2010).

The high content of AGM in the lumen of the gastrointestinal tract may also originate from the bacteria of the physiological gut microflora, as well as from pathogens such as *Helicobacter pylori*.

After oral administration of radiolabeled AGM to rats *in vivo*, radioactivity was retrieved in all organs investigated, as well as in blood and urine indicating that AGM is absorbed from the

stomach and the gut by means of an energy-dependent transport mechanism and distributed to the organs via the blood stream. Moreover, the accumulation of radioactivity in organs and distal gut luminal content decreases with the administration of increasing doses of PUT (Molderings et al., 2002).

Because of the low ADC activity in mammalians, only a fraction of AGM in the tissues of the organism can be due to endogenous *de novo* synthesis by ADC, while a substantial portion can be of dietary origin (Molderings et al., 2002).

AGMATINE IN FOODSTUFFS

Several food items contain only small amounts of PAs, such as AGM, while higher concentrations can be found in fermented foods. PAs can also be considered as indicators of freshness in fish and meat products, as they are produced during food storage, thus confirming the main role of microorganisms in their synthesis. In particular, AGM is present at high levels in alcoholic beverages, such as wine, beer, and especially in sake (114 mg/L; Okamoto et al., 1997), while low levels have been found in fermented non-alcoholic beverages, such as Turkish shalgam (Özdestan and Üren, 2010a). This fact could confirm the role of yeasts in AGM production.

In **Table 1** is summarized the AGM mean content in several foodstuffs.

WINE

The concentration of BAs in wine has been reported to range from a few mg/L to about 50 mg/L. The type and concentration of

Table 1 | Agmatine content in different foodstuffs (ppm).

Foodstuffs	Mean	Range	N ([A])	References
WINE				
Red wines	n.s.	n.d.–22	286	Galgano et al. (2003), Galgano et al. (2009), Galgano et al. (2011)
White wines	n.s.	n.d.–6.5	103	Galgano et al. (2003), Galgano et al. (2009)
BEER	12	0.5–42	211	De Borba and Rohrer (2007), Halász et al. (1999a), Izquierdo-Pulido et al. (1996), Kvasnicka and Voldrich (2006)
SAKE	114	n.s.	n.s.	Okamoto et al. (1997)
COFFEE				
Green coffee	n.d.	n.d.	n.s.	Cirilo et al. (2003)
Roasted	n.s.	n.d.–1.2	n.s.	Cirilo et al. (2003)
Instant coffee	0.4	0.4–5.3	68	Leite da Silveira et al. (2007)
CEREALS AND VEGETABLES				
Flour	n.d.	n.d.	5	Farkas and Hajós (1998)
Bread	3.3	n.d.–4.7	9	Farkas and Hajós (1998)
Soy sauce	n.d.	n.d.	4	Kirschbaum et al. (2000)
Soybean paste (Doenjang)	473	n.d.–5508	23	Shukla et al. (2010)
Miso	6.1	n.d.–30	5	Kirschbaum et al. (2000)
Sauerkraut brine	4.3	2.2–6.7	6	Halász et al. (1999a)
Fermented cabbage juice	n.d.	n.d.	5	Kirschbaum et al. (2000)
FISH AND DERIVED PRODUCTS				
Fresh fish	92	n.d.–401	13	Baker et al. (2010), Chotimarkorn (2011), Paarup et al. (2002), Ruiz-Capillas and Moral (2004)
Fish paste	n.d.	n.d.	3	Kirschbaum et al. (2000)
Cooked fish paste	29	n.d.–161	28	Naila et al. (2011)
MEAT AND MEAT PRODUCTS				
Fresh meat	0.8	n.d.–3.1	22	Bover-Cid et al. (2001), Hernández-Jover et al. (1996), Lorenzo et al. (2007), Ruiz-Capillas and Jiménez-Colmenero (2004)
Fermented meat	6.2	n.d.–43	23	Bover-Cid et al. (2001), Hernández-Jover et al. (1996), Lorenzo et al. (2007), Ruiz-Capillas and Jiménez-Colmenero (2004)
Cooked meat	5.4	n.d.–27	20	Hernández-Jover et al. (1996), Ruiz-Capillas and Jiménez-Colmenero (2004), Ruiz-Capillas et al. (2007)
DAIRY PRODUCTS				
Fermented milk (Kefir)	n.d.	n.d.	10	Özdestan and Üren (2010b)
Ripened cheese	1.1	n.d–18	69	Novella-Rodríguez et al. (2002), Vale and Glória (1998)
Fresh cheese	0.1	n.d.–1.3	13	
Grated cheese	1.2	n.d.–14	12	Vale and Glória (1998)

[A] Number of samples examined.

n.d., Not detected.

n.s., Not specified.

amines in wines depends on several factors, such as winemaking processes, time and storage conditions, raw material quality, and microbial contamination during winery operations. Moreover, some amines are normal constituents of grapes, and their level may vary with grape variety and degree of ripening, as well as with soil type and composition. Therefore, geographical characterization based on the BA content has also been proposed as a criterion to discriminate several types of wines from different countries or regions (Galgano et al., 2009, 2011). BAs usually investigated in wines are cadaverine (CAD), HIS, 2-phenylethylamine (2-PHE), PUT and tyramine (TYR); AGM, and ethanolamine can be abundant in wines, but they are rarely investigated. Low amounts of BA, as normal constituents of the raw materials, can be released in must from grapes during the winemaking process, and the BA concentration may increase as a consequence of alcoholic fermentation, yeast autolysis, malolactic fermentation, and wine aging, red wines usually having a richer amine content than white wines. Generally PUT and AGM can contribute significantly to the total amine content in alcoholic beverages, while CAD, SPM, and SPD are rare (Galgano et al., 2003, 2009, 2011). In particular, PUT is the most abundant BA in wine, while AGM is the most prevalent in beer (Alberto et al., 2007).

For AGM, the concentration has been reported to vary from not detected to 22 mg/L in red wines and from not detected to 6.5 mg/L in white wines, while PUT has been found in red wines at concentrations ranging from 0.6 to 21 mg/L and from not detected to 9 mg/L in white wines (Galgano et al., 2009). SPD and SPM originate from PUT, which derives from AGM; the latter is generally recognized as an intermediate in the PUT formation pathway, starting from arginine, the most abundant amino acid in grape. PUT can also derive from decarboxylation of ornithine. However, PUT levels increase during fermentation from must to alcoholic, to malolactic fermentation, thus supporting the hypothesis that the principal biosynthetic pathway for PUT formation is via arginine-AGM rather than via ornithine (Galgano et al., 2009). Moreover, arginine, from which AGM derives, is present in must at concentrations higher than those found in the finished wine (Galgano et al., 2009). In addition, wines with a low PUT content have been shown to possess a high level of AGM and total polyphenols and vice versa (Galgano et al., 2011). The phenolic compounds seem to be a natural way of reducing PUT formation in wine, because these moieties can protect the cells against oxidative stress (Alberto et al., 2007; Rodríguez et al., 2009).

BEER

PAs are natural beer constituents and are present in malt and yeast at higher concentrations then in hops (De Borba and Rohrer, 2007; Sohrabvandi et al., 2012), while TYR, HIS, and CAD are considered to be indicators of microbial contamination during brewing (Izquierdo-Pulido et al., 1996). In several beers AGM is the most abundant amine with concentrations ranging from 0.5 to 42 mg/L (Izquierdo-Pulido et al., 1996; Kvasnicka and Voldrich, 2006; De Borba and Rohrer, 2007). Barley variety, malting technology, and fermentation conditions can influence the BA content in beer both qualitatively and quantitatively. In particular, during wort processing, significant increases of AGM, and PUT have been observed, together with the decrease of SPD and SPM (Halász et al., 1999a).

COFFEE

The profile and levels of BAs in green and roasted coffee are different; in particular, the degree of roasting significantly influences the amine profile and levels. AGM has been detected in coffee roasted at a high temperature for a long time. In fact, extensive roasting (12 min at 300°C) contributes to AGM formation (1.2 mg/kg) by decarboxylation of arginine, which is present in green coffee at levels ranging from 2 to 5 g/100 g. Conversely, AGM was not found in American coffee, which was roasted for 6 min at 300°C. Furthermore, PUT is the most abundant amine in green coffee (10 mg/kg), while it is not present in roasted coffee (Cirilo et al., 2003). Also the processing of instant coffee can influence both the type and level of BAs; instant coffee contains low levels of AGM (0.4 mg/kg), while the most abundant BA is serotonin, followed by CAD, TYR, and SPD (Leite da Silveira et al., 2007).

CEREALS AND VEGETABLES

In cereal foods, in particular in flour and bread samples, PUT is reported to be the most abundant BA, at even higher levels than 40 mg/kg, while AGM has been found only in cereal derivates, in particular in different types of bread samples, at concentrations ranging from 3 to 5 mg/kg of dry matter (Farkas and Hajós, 1998). AGM is also a natural constituent of the seedlings of the winged bean, while is not found in the common bean nor in soybean (Morris, 2003). Since several varieties of molds, yeasts, and lactic acid bacteria are involved in the fermentation processes occurring in the production of several soybean products, and as the substrate is very rich in proteins, the formation of various amines might be expected during fermentation. Several studies have highlighted that BAs in fermented soybean products are most probably formed by the lactic microflora that remains active during fermentation (Kirschbaum et al., 2000). Among many fermented foods that have been consumed for thousands of years in Asian countries, soy sauce, and miso are the most important, and they are obtained by the fermentation of soybean with or without the addition of rice and wheat (in the case of soy sauce) or rice and barley (in the case of miso), using a mix of molds, yeasts, and lactic acid bacteria. In these products AGM has rarely been detected, whereas PUT is one of the most abundant amines that can be found (Kirschbaum et al., 2000). Conversely, in Doenjang, a traditional Korean soybean paste produced by the fermentation of naturally occurring bacteria and fungi, the presence of AGM was reported, in concentrations ranging from not detected to over 5500 mg/kg (Shukla et al., 2010).

Recently Özdestan et al. (2011) have investigated the BA content in Kumru, a traditional Turkish fermented cereal food produced from chickpeas. In this case not detectable amounts of AGM were found in the product, while other BAs, such as PUT, CAD, SPD, SPM, and HIS were present in all samples.

Fermentation temperature, salt concentration, and starter selection have a significant effect on BA production in sauerkraut (fermented cabbage). In particular, the AGM level seems to be dependent on the inoculum concentration of *Lactobacillus curvatus*. In sauerkraut brine, AGM was found to be the prevalent amine, reaching a maximum value of 12 mg/L (Halász et al., 1999b). Conversely, other authors have reported that lactic fermented cabbage juices do not contain AGM, while PUT, estimated at levels of up

to 360 mg/L, is the most abundant amine, with assessed levels up to 360 mg/L; Kirschbaum et al., 2000).

FISH AND FISH DERIVED PRODUCTS

SPM and SPD are usually the main PAs present in fresh tissues, at concentrations of less than 10 mg/kg. Depending on the fish species, the free amino acids present in the tissues, and the exposure conditions to spoilage bacteria, other amines can originate during storage or processing, reaching harmful levels, as reported for HIS in the case of animals belonging to the mackerel and herring families (Clifford and Walker, 1992; Önal, 2007). The existence of a synergic relationship between SPM, SPD, and HIS with potentially negative consequences on health, has been hypothesized (Silla Santos, 1996). Therefore, various indexes have been proposed for fish derived products, such as the "BA index," corresponding to the sum of the HIS, PUT, CAD, and TYR content (Veciana-Nogues et al., 1997), or the "quality index," directly related to the HIS, PUT, and CAD content and inversely related to the amounts of SPM and SPD (Mietz and Karmas, 1997). Moreover, several authors have indicated that AGM may be used as a freshness index, because immediately after catch AGM can not be detected or its level is very low, but then in various fish species the AGM content increases progressively during chilling storage, reaching concentrations of over 300 mg/kg after 7 days of storage (Yamanaka et al., 1987; Paarup et al., 2002; Baker et al., 2010; Chotimarkorn, 2011). The formation of BAs, commonly related to fish spoilage (HIS, TYR, CAD, and AGM), was found to be significantly higher in the first stage of ripening in ungutted anchovies than in gutted ones. Conversely, no differences have been observed regarding the content of SPM and SPD (Pons-Sanchez-Cascado et al., 2003). In Rihaakuru, a cooked fish paste, the AGM concentration is extremely variable, ranging from not detected to 161 mg/kg (Naila et al., 2011), whereas in other fermented and non-fermented fish paste, AGM is not detectable (Kirschbaum et al., 2000). Fish storage conditions can help to reduce the processes favoring the formation of BAs. In particular, refrigeration with flaked ice, traditionally applied to fish, significantly reduces AGM and the formation of other BAs compared with refrigeration at the same temperature, but without ice (Chotimarkorn, 2011). Protective atmosphere packaging (PAP) is frequently used to prolong the shelf-life of foodstuffs, and effectively reduces BA concentrations in different fish. In particular, Ruiz-Capillas and Moral (2004) have shown that for tuna a gas mixture containing 60% CO_2 is more effective in reducing these moieties than a gas mixture containing 40% CO_2. This was particularly true in the case of white muscle of tuna, where after 25 days of storage an atmosphere containing 60% CO_2 caused a 10-fold reduction of the AGM level compared with the control stored in air.

MEAT AND MEAT PRODUCTS

Biogenic amines can be found in processed meat products as a consequence of microbial activity related to fermentation occurring during processing, or to microbial contamination of poor quality raw meat. Therefore, in cooked meat products (non-fermented) BAs may represent a useful indicator for assessing the hygienic quality of the raw meat utilized for preparing these foodstuffs (Bover-Cid et al., 2001). Furthermore, the nitrosable secondary amines (AGM, SPD, and SPM) can form nitrosamines by reaction with nitrites, chemical agents considered to possess major carcinogenic properties. This is particularly important in some meat products with high polyamine levels and whose production process requires the use of nitrates and nitrites (Ruiz-Capillas and Jiménez-Colmenero, 2004). As with fish, also fresh meat normally contains PAs such as SPM and SPD, the levels of which may vary slightly in fermented and ripened meat products. In fresh meat AGM can be found at levels ranging from not detectable to about 3 mg/kg, while in fermented and ripened meat products the AGM content may range more widely from not detectable to about 43 mg/kg. However, it has been reported that in these products a low formation of PUT can be related to the presence of high levels of AGM, the latter being an intermediate metabolite in the PUT production pathway from arginine. Generally cooked meat products show lower polyamine concentrations than fresh meat (Hernández-Jover et al., 1996; Bover-Cid et al., 2001).

Dry-cured "lacón" is a traditional cured meat product made in Spain, following manufacturing processes very similar to those utilized in the production of dry-cured ham. During the manufacture of dry-cured lacón the content of BA may vary greatly, as is also the case with ham. In addition, higher levels of TRYPT have been found in fresh lacón pieces than in fresh meat, whereas AGM has not been detected. However, AGM was generated during the drying-ripening stage, both with and without additives and the values reached in the final product (around 8 mg/kg) were higher than those found in ham (Lorenzo et al., 2007).

The technology applied in meat processing may influence BA production. For example, sugar omission in the production of slightly fermented sausages is not recommended, because it may cause an increase in AGM and the accumulation of other BAs during manufacture and storage (Bover-Cid et al., 2001). Furthermore, different concentrations of BAs have been found in fresh, fermented, and cooked meat products, either treated with high pressure processing (HPP) or under PAP, which were closely related to the type of product and the processing conditions. In general, PAP has a positive effect in reducing BA formation in these products. In commercial meat products treated with HPP, higher BA concentrations, particularly AGM, have been reported compared with non-treated samples. This negative effect of HPP has also been observed in frankfurters, butifarra, cooked ham, and chorizo; for the latter product, it has been observed how commercially available non-treated samples may contain AGM concentrations of about 2 mg/kg, while chorizo treated by HPP may show a 20-fold increase in AGM content (Ruiz-Capillas and Jiménez-Colmenero, 2004; Ruiz-Capillas et al., 2007).

MILK AND DAIRY PRODUCTS

In milk, as in other fresh foods derived from animals, SPM and SPD are the prevalent PAs, whereas it must be reported that AGM was not detectable (Novella-Rodríguez et al., 2002). Also in industrial kefir, a milk product belonging to the category of mixed lactic acid and ethanol fermented beverages, AGM was not detectable. The estimated BA content in kefir, ranging from 2 to 35 mg/L, is generally lower than that found in other fermented foods. Short fermentation time, the use of starter culture, controlled production conditions such as heating at 90–95°C before fermentation

and a low microbial count in the raw material are the main reasons for the low BA content. Higher BA levels can be expected in artisanal or home-made kefir compared with industrial samples, because of worse hygienic conditions (Özdestan and Üren, 2010b).

Cheeses represent an ideal environment for the production of amines. The factors influencing the formation, accumulation, and type of amines are: the availability of amino acids, the presence of bacteria capable of decarboxylating amino acids, pH, salt concentration, water availability, temperature and duration of ripening and storage, bacterial density, the presence of cofactors, and amine catabolism. The pH of cheese is appropriate for BA production, generally between 5.0 and 6.5, depending on the age and type of the products. Cheeses are also rich in pyridoxal phosphate, which is required for amino acid decarboxylase activity. Studies have shown that in cheese the concentration of BAs decreases with increased fat content. This phenomenon has been attributed to changes in the water activity, which inhibits the growth of proteolytic bacteria, causing a reduction of free amino acid concentration in the medium (Ruiz-Capillas and Jiménez-Colmenero, 2004). Numerous bacteria, both intentional or adventitious and isolated from cheese, have been reported as BA producers (Vale and Glória, 1998; Novella-Rodríguez et al., 2002). Several outbreaks of HIS poisoning have occurred following the consumption of cheese; also cases of hypertensive crisis and migraine headache have been observed after the ingestion of cheeses with high levels of TRYPT and 2-PHE. The use of bacteriocin-producing starters can prevent the formation of HIS. Moreover, the addition of microbial isolates that degrade BAs in order to prevent the presence of hazardous levels of amines in the final products has been proposed. Although the type and levels of amines in each kind of cheese may vary considerably, AGM is a polyamine which is rarely present in cheeses (Vale and Glória, 1998; Novella-Rodríguez et al., 2002; Custódio et al., 2007).

CONCLUSION

PAs are produced in all living organisms and play an important role in cell growth and development, as well in protecting stressed cells. Our diet also provides a daily supply of PAs, and high PA concentrations in food can trigger toxic effects and intensify the toxicological effect of HIS. Moreover, the beneficial effects of the polyamine AGM for human health, in its various functions from neurotransmitter or neuromodulator, to stimulator of insulin release and tumor suppressor agent are well known. As only a limited amount of this amine is produced in mammal cells, food ought to contain a certain quantity of the AGM. However, the appropriate AGM level in different food types is a complex relationship which depends on numerous factors.

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Histamine poisoning and control measures in fish and fishery products

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Histamine poisoning is one of the most common form of intoxication caused by the ingestion of fish and fishery products. Cooking, canning, or freezing cannot reduce the levels of histamine because this compound is heat stable. All humans are susceptible to histamine and its effects can be described as intolerance or intoxication depending on the severity of the symptoms. The amount of histamine in food, the individual sensitivity, and the detoxification activity in human organism represent the main factors affecting the toxicological response in consumers. Histamine is the only biogenic amine with regulatory limits set by European Legislation, up to a maximum of 200 mg/kg in fresh fish and 400 mg/kg in fishery products treated by enzyme maturation in brine.

Keywords: histamine, fishery products, food poisoning, regulation

INTRODUCTION

Histamine is a biogenic amine produced in fish tissue through the decarboxylation of free histidine by exogenous decarboxylases released by microorganisms. This ability has been described in different genera, species, and strains of bacteria, both Gram positive and Gram negative (Ladero et al., 2010). Histamine is rarely found in fresh fish but its level increases with the progress of fish decomposition (Shakila et al., 2003). The microorganisms naturally present on the gills and in the gut of live fish start to grow upon death because the defense mechanisms are inactive. In particular histamine forming bacteria are able to grow more rapidly at high abuse than at moderate abuse temperatures. However once the enzyme histidine decarboxylase has been formed, it can continue to produce histamine also at or near refrigeration temperature, it remains stable in frozen fish and can be reactivated after thawing. Frozen temperature (−18°C or below) can stop the growth of bacteria and prevent any preformed histidine decarboxylase from producing histamine. Conversely histamine production is greater at high abusive temperatures (21.1°C or higher) particularly at temperatures near 32.2°C [Food and Drug Administration (FDA), 2011]. Cooking can inactivate both the enzyme and the microorganisms, but histamine which has been formed cannot be eliminated because it is heat stable [Food and Drug Administration (FDA), 2011].

Histamine poisoning is a food-borne disease characterized by a variety of symptoms similar to allergic reactions. The toxic effects of histamine are related to its normal physiological actions in the body. In particular the dilatation of the peripheral blood vessels results in hypotension, flushing, and headache, while the increased capillary permeability causes urticaria, hemoconcentration, and eyelids edema; the symptoms affecting the gastrointestinal system are due to the contraction of smooth muscles leading to abdominal cramps, diarrhea, nausea, and vomiting. Histamine exerts also a stimulatory action on the heart by increasing its contractility and exhibiting palpitations and tachycardia, while it is a potent stimulant of sensory and motor neurons producing pain and itching associated with the rash [Food and Agriculture Organization/World Health Organization (FAO/WHO), 2012].

The variability of symptoms can be linked both to the amount of histamine ingested and individual sensitivity. The ingestion of food containing small amounts of histamine has little effect in healthy individuals, but it can result in histamine intolerance in persons characterized by impairment of diamine oxidase (DAO) activity, either due to genetic predisposition, gastrointestinal diseases, or medication with monoamine oxidase (MAO) inhibitors (Maintz and Novak, 2007), whereas histamine intoxication can appear in everyone as a result of its high amounts in foods like fish and fishery products or ripened cheese [European Food Safety Authority (EFSA), 2011]. The symptoms of histamine poisoning can appear for few hours or a day but in rare case they can persist for some days. However, statistical data about its incidence are not available because the poisoning incidents are often underestimated due to mild or not recognized nature of illness and to inadequate systems to attribute food-borne diagnosis.

The histamine intoxication outbreaks between 2005 and 2010 using Rapid Alert System for Food and Feed (RASFF) were above 100 cases [European Food Safety Authority (EFSA), 2011]. The measures used to estimate the dose/exposure level causing histamine intoxication are generally based on the detection of the biogenic amine in the suspected food or on the patients reports. Moreover, the toxic effects of histamine are enhanced by the presence of other biogenic amines such as putrescine and cadaverine (Huang et al., 2010).

REGULATORY LIMITS

According to Commission Regulation EC No 2073/2005 (Regulation 2073/2005/EC) the limits for histamine have been established in fish species associated with a high amount of histidine, i.e., the families of *Scombridae*, *Scombresosidae*, *Clupeidae*, *Engraulidae*, *Coryfenidae*, *Pomatomidae*, both fresh and treated by enzyme maturation in brine. The sampling plan consists of a number of units comprising the sample (*n*) equal to nine and a number of sample units (*c*) equal to two, giving values between *m* and *M*. In particular the examined batch will be satisfactory when: (i) the mean value is less or equal to *m*; (ii) a maximum of *c*/*n* values are between *m* and *M*; (iii) no values exceed *M*. The sampling scheme of Food and Drug Administration offers more confidence that non-conforming lots will be detected, as reported in **Table 1**. Moreover EU assessed the limits of histamine for fishery products which have undergone enzyme maturation with brine manufactured from fish species associated with a high amount of histidine equal to 200 mg/kg and 400 mg/kg, for *m* and *M*, respectively.

For the detection of histamine also single samples may be taken at retail level. In such cases the whole batch should not be deemed unsafe based only on the result of one sample, unless the result is above *M*, as reported in Commission Regulation EU No 1019/2013 (Regulation 1019/2013/EU). The last Regulation amended Annex I to Regulation EC No 2073/2005 adding a maximum value for fish sauce produced by fermentation of fishery products, equal to 400 mg/kg. Since fish sauce is a liquid fishery product, histamine can be expected to be evenly distributed then a single sample can be examined.

The reports on histamine intoxication generally involve only a small number of individuals, so it is difficult to estimate the dose/exposure level in order to construct quantitative assessment of dose versus adverse response. A model used in the dose/response assessment can be based on volunteer challenge study, as reported by the EFSA biogenic amines report [European Food Safety Authority (EFSA), 2011]. In particular these studies aim to investigate the minimal dose of histamine that causes poisoning or intolerance symptoms, carefully monitored by medical professionals. Results from the limited number of studies suggested a potential no observed adverse effect level (NOAEL) of 50 mg histamine for the symptoms headache and flushing, but this was based on limited number of individuals: 66 healthy and 74 sensitive. Some healthy individuals did not show symptoms at concentrations up to six times higher than the NOAEL [European Food Safety Authority (EFSA), 2011]. Based on the above mentioned NOAEL and the consumption of a portion/size of 250 g of fish, the maximum concentration of histamine that would not cause an adverse effect would be equal to 200 mg/kg [Food and Agriculture Organization/World Health Organization (FAO/WHO), 2012].

Table 1 | Sampling schemes for histamine content in fishery products.

Sampling plan	*n*	*c*	Histamine (mg/kg)	
			m	*M*
European Union*	9	2	100	200
Food and Drug Administration	18	1	50	500

**Scombridae, Scombresosidae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae.*

PREVENTIVE AND HYGIENIC MEASURES

The risk of histamine poisoning could be controlled by applying basic Good Manufacturing and Hygiene Practices associated to an appropriate Hazard Analysis Critical Control Point (HACCP) system. According to European Legislation fish must be maintained at a temperature approaching that of melting ice as soon possible after harvest, in order to comply with freshness criteria and to avoid the growth of spoilage and histamine producing bacteria. All operations (heading, gutting, filleting, or cutting, etc.) should be carried out hygienically on board vessels. Moreover fresh fishery products must be kept at the above mentioned temperature during storage and transport in such a way as not adversely to affect food safety.

CONCLUSION

Fishery products can be involved in foodborne outbreaks by histamine when the application of Good Hygiene Practices and proper temperatures of storage failed to comply during the food chain. The regulatory systems have developed control strategies and monitoring procedures, such as sampling plan for fish species with a high amount of histidine, in order to assuring seafood safety.

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