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ORIGINAL PAPER

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Amino acid-decarboxylase activity of bacteria isolated from ice-preserved anchovies

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Abstract The amino acid-decarboxylase activity of bacteria isolated from *Engraulis encrasicholus* preserved in ice was investigated throughout 23 days of storage. A total of 140 bacterial isolates were studied, including 37 enterobacteria, 23 pseudomonads, 49 Gram+ catalase+ cocci, 27 lactic acid bacteria and 4 enterococci. The percentage of strains that decarboxylated amino acids was low, 12% on average. None of the Gram+ catalase+ cocci showed aminogenic activity, but all enterococci isolates did. The enterobacteria with aminogenic capacity were identified as *Enterobacter cloacae* and they simultaneously produced putrescine and cadaverine (up to 500 mg/l), but not histamine. Among pseudomonads, two species decarboxylated ornithine (producing 350–650 mg/l putrescine): *Pseudomonas cepacia* and *Pseudomonas fluorescens*. Lactic acid bacteria decarboxylated tyrosine, yielding up to 2,000 mg/l tyramine, and they were identified as *Lactobacillus brevis*, *Lactococcus lactis*, *Pediococcus pentosaceous* and *Enterococcus* spp. As a general rule, the positive Gram-negative isolates (enterobacteria and pseudomonads) were diamine producers and they were found only during the first 2 weeks of storage. In contrast, lactic acid bacteria and enterococci were mainly tyramine producers and they were isolated only at the end of storage.

Keywords Anchovy · Amino acid-decarboxylase · Putrescine · Cadaverine · Histamine · Biogenic amines

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Introduction

Biogenic amines (BA) are basic nitrogenous compounds that can cause food poisoning if ingested in large amounts or if human detoxification systems are inhibited or genetically deficient. They are formed by bacterial enzymes through the decarboxylation of precursor amino acids during food fermentation, which is due to technologically desirable microorganisms, but also to contamination through defective or improper handling, raw material or food spoilage [1]. BA formation not only requires precursors and the bacteria responsible for decarboxylation, but it is also influenced by several factors such as pH, water activity and temperature, which condition the growth and expression of the amino acid-decarboxylase activity of microorganisms [1, 2].

Fish spoilage is mainly caused by bacterial activity and some compounds, such as BA, can be formed by bacterial decarboxylation of precursor amino acids. In fact, BA are absent or found at very low levels in fresh fish and their formation is usually associated with spoilage [3]. Therefore, poor hygiene is probably the main factor involved in the formation of these compounds. Contamination with microorganisms bearing amino acid-decarboxylase activity can occur in any phase of the merchandise chain: aboard ship (in reused boxes or other potentially contaminated materials), during transport, at retail outlets and in the household. Fresh fish can be contaminated by a mixed bacterial population consisting of psychrotrophic Gram-negative bacteria like pseudomonads and enterobacteria and Gram-positive bacteria like micrococci, staphylococci and lactic acid bacteria (LAB) [4, 5].

The type of microorganisms present in food determines the type and amount of BA formed. Many enterobacteria and certain lactobacilli, pediococci and enterococci are particularly active during the formation of BA in food [6]. Indeed, enterobacteria have been mostly described as strong producers of histamine (HI) in fish [2, 7] and the diamines putrescine (PU) and cadaverine (CA) [8]. LAB have been mainly associated with the formation

of tyramine (TY) in fermented food such as dry sausages and cheese [9, 10].

Most reports have focused on HI because it is the main BA in fish, especially in pelagic species, associated with histaminic intoxication. Although HI is the only amine with a legally established maximum for fish [11, 12], other BA associated with fish decomposition, such as PU and CA, may enhance the toxic effects of HI [13].

Anchovy (*Engraulis encrasicholus)* is a pelagic fish particularly appreciated in Mediterranean countries. It can be consumed fresh, salted or marinated in oil or vinegar, and anchovies may accumulate HI and other BA during long storage times and throughout the ripening process [14]. However, the amino acid-decarboxylase activity of bacteria has been studied only in isolates from the ripened product. Thus, there is no information available about the aminogenic potential of microorganisms during the storage of fresh anchovies. We thus aimed to study the amino acid-decarboxylase activity of bacteria isolated from anchovy (fresh fish) preserved in ice during 23 days of storage.

Material and methods

Samples

Anchovies (*Engraulis encrasicholus*) were stored in self-draining boxes covered with flake ice (0 °C) replaced daily inside a refrigerator at 4 °C. Four anchovies were randomly sampled every 2–4 days throughout storage. Two trials were performed in the same conditions.

Microbiological analysis and isolation of colonies

Ten grams of a sample was mechanically homogenized with 90 ml 0.1% tryptone water (Merk, Darmstadt, Germany) for 2 min. Decimal dilutions were prepared and the appropriate dilution was inoculated to the corresponding medium [5]. Enterobacteria were enumerated in violet-red bile agar (VRBG, Oxoid, Unipath, Basingstoke, UK) and incubated with a double layer at 37 °C for 24 h; pseudomonads were enumerated on cetrimide agar (Sharlau, Barcelona, Spain) at 32 °C for 72 h; Gram+ catalase+ cocci (micrococci and staphylococci) on mannitol salt agar (Oxoid) at 32 °C for 72 h; LAB on Man Rogosa Sharpe agar (MRS, Oxoid) at 30 °C for 48 h anaerobically and enterococci on kanamycin aesculin azide agar base (Oxoid) at 37 °C for 48 h.

Several colonies of each microbial group were picked up and streaked on tryptone soy agar (Oxoid) (enterobacteria, pseudomonads, Gram+ catalase+ cocci) or MRS (LAB and enterococci) at 30 °C to obtain pure isolates.

Determination of the amino acid-decarboxylase activity

Pure cultures were incubated in decarboxylase broth as described by Bover-Cid and Holzapfel [15] and aminogenic activity was quantified throughout the analysis of BA following the high-performance liquid chromatography method reported by Veciana-Nogués et al. [16].

Identification of bacterial isolates

The colonies producing BA from the precursor amino acids were identified on the basis of the Gram and oxidase reaction, catalase production and biochemical tests using the appropriate API system (API 20E, API 20 NE or API 50 CH from Bio-mérieux, Marcy-l'Etoile, France).

Results and discussion

Table 1 shows the time course of microbial counts throughout 23 days of anchovy storage in ice. A total of 140 bacteria were isolated during storage, 17 of which formed noticeable amounts of BA. These microorganisms were identified and their amino acid-decarboxylase capacity was quantified (Table 2).

The number of enterobacteria increased throughout storage, achieving counts higher than 6log(cfu/g) at the end of the study period; however, only 8% of the isolates were decarboxylase positive. Ice-storage may have hindered the growth of BA-producing bacteria, since the microorganisms showing this ability are mainly mesophilic bacteria [17]. Therefore, the enterobacteria isolated here were mainly non-BA producers. Enteric microorganisms with amine- forming ability constitute a minor proportion of the fish flora and are difficult to isolate in high yields [18, 19]. All amine- forming enterobacteria found in this study were identified as *Enterobacter cloacae*. They simultaneously produce both diamines, especially CA. In contrast, none of the *Enterobacter cloacae* isolates showed the ability to form HI, in agreement with the observations of Lakshmanan et al. [8], who isolated bacteria belonging to the *Enterobacteriaceae* family that produced both CA and PU from fresh emperor fish. Likewise, *Enterobacter cloacae* strains isolated from salted anchovies lack histidine decarboxylase ability [20]. Although enterobacteria isolated from fish are frequently regarded as HI-forming bacteria, they are mostly isolates from fish kept under refrigeration or stored at excessive temperatures [7, 17, 21, 22]. In contrast, Kim et al. [19]

Table 1 Microbial counts, in log(cfu/g), throughout ice storage of anchovy. The values are the average ± the standard deviation of the two trials performed. Not detected (*ND*)

Days in ice	*Enterobacteriaceae*	*Pseudomonas*	Gram+ catalase+ cocci	Lactic acid bacteria	*Enterococcus*
0	2.55±0.16	2.54±0.51	3.14±0.76	2.74±0.01	ND
2	2.49±0.81	2.92±0.54	3.45±0.07	2.95±0.20	ND
5	2.96±0.50	3.74±0.39	3.55±0.78	2.57±0.12	ND
7	2.56±1.51	3.79±0.20	4.79±0.39	3.49±0.29	ND
9	3.58±0.64	5.05±0.35	5.64±0.06	3.74±0.36	ND
12	4.84±0.13	5.17±0.15	6.48±0.77	4.05±0.99	ND
15	5.73±0.21	4.88±0.14	6.94±0.84	5.30±0.07	ND
19	6.67±0.23	5.50±0.35	6.74±0.98	5.48±0.04	ND
23	6.36±0.71	6.28±0.45	7.09±0.61	5.59±0.23	1.48±0.43

Table 2 Aminogenic potential of bacteria isolated from anchovies stored in ice. The concentration in the decarboxylase broth is in units of milligrams per liter.

Species	No. of positives / Total isolates	Tyramine	Putrescine	Cadaverine	Histamine
Enterobacteriaceae	3/37				
Enterobacter cloacae		NEG[a]	592–814	852–1,160	NEG
Pseudomonas	3/23				
Pseudomonas cepacia		NEG	16–661	ND	NEG
Pseudomonas fluorescens		NEG	347–386	20–21	NEG
Gram+ catalase+ cocci	0/49	NEG	NEG	NEG	NEG
Lactic acid bacteria	7/27				
Lactobacillus brevis		NEG-826	NEG–20	NEG–42	NEG
Lactococcus lactis		789–797	NEG	NEG	NEG
Pediococcus pentosaceous		796–810	NEG	NEG	NEG
Enterococci	4/4				
Enterococcus spp		320–2,010	NEG	NEG	NEG

[a] Negative: not able to decarboxylate any amino acid in vitro

reported that no HI-forming bacteria were isolated from VRBG medium during the storage of pacific mackerel at 0 °C for up to 14 days. In agreement with these findings, there are fewer studies reporting HI accumulation in fish stored at 0 °C than during storage under refrigeration or at excessive temperatures. Indeed, the formation of HI at 0 °C has been generally reported when fish become unacceptable for human consumption [7, 23].

The levels of pseudomonads increased from 2log(cfu/g) to values close to 6log(cfu/g) and were slightly higher than those of enterobacteria during the first fortnight of storage. Thirteen percent of the isolates were amino acid-decarboxylase positive and were identified as *Pseudomonas cepacia* and *Pseudomonas fluorescens*. Both species decarboxylated ornithine, producing significant amounts of PU. *Pseudomonas cepacia* was a stronger PU producer than *Pseudomonas fluorescens,* which was also able to decarboxylate lysine, producing small amounts of CA. Lakshmanan et al. [24] also isolated pseudomonas from salted sardines able to decarboxylate both ornithine and lysine. In contrast, Du et al. [21] described HI production by *Pseudomonas fluorescens* isolated from tuna.

The Gram-negative bacteria isolates (both enterobacteria and pseudomonads) were potential producers of diamines (CA and PU) and they were isolated only during the first 2 weeks of ice storage.

The dominating group of Gram-positive bacteria was the Gram+ catalase+ cocci (staphylococci and micrococci), whose counts surpassed even those of Gram-negative populations; however, none of the isolates showed significant production of BA. Gram+ catalase+ cocci isolated from salted fish, however, have been reported to produce BA. Lakshmanan et al. [24] described that *Micrococcus luteous* isolated from salted sardines was the dominant halophilic amine-forming bacteria during ripening and produced CA. Hernández-Herrerero et al. [20] found that *Staphylococcus epidermidis* and *S. capitis* isolated during the ripening of salted anchovies are powerful HI-producing bacteria (up to 400 mg/l). In fresh fish, Baixas-Nogueras et al. [25] reported that most Gram+ catalase+ cocci isolates from hake stored in ice do not produce BA, although they grow to high counts throughout storage.

Twenty-eight percent of the 27 LAB isolates were able to decarboxylate amino acids, especially tyrosine. Two isolates showing a great tyrosine-decarboxylase activity were identified as *Lactococcus lactis* and *Pediococcus pentosaceous*. The other five positive isolates were *Lactobacillus brevis* and their aminogenic potential was variable, since three isolates were TY producers, while the remaining two did not decarboxylate tyrosine, but produced small amounts of CA and PU (lower than 50 mg/l). This variability confirms that the amino acid-decarboxylase capacity of bacteria depends on the strain. Dapkevicius et al. [26] found several LAB isolated from fermented mackerel that produced HI, TY, PU and/or CA.

Enterococci were found only at the last sampling point (day 23 of storage in ice). The four isolates were TY producers, the microbial group with the highest capacity to decarboxylate tyrosine (up to 2,000 mg/l TY). All enterococci were also able to produce β-phenylethylamine, but in much lower yields (below 50 mg/l), as described by Straub et al. [27] and Bover-Cid et al. [9]. Joosten and Northold [28] suggested the participation of the same enzyme in the production of both BA when TY is produced in large amounts, because the precursors phenylalanine and tyrosine have a similar chemical structure.

As a general rule, the Gram-positive bacteria (LAB and enterococci) isolates showed a higher potential to produce TY than diamines. Moreover, unlike Gram-negative bacteria, the decarboxylase positive strains among Gram-positive isolates were detected only after 2 weeks of storage. These findings are in agreement with the low and late occurrence of TY during the storage of anchovy [14].

Other BA (serotonin, agmatine and tryptamine) were analyzed, but they were not produced by any of the bacterial groups studied under the in vitro conditions applied. Spermidine and spermine were found at very low levels (below 5 mg/l) in the culture broth of all isolates. They are considered physiological amines needed for cellular growth [29]. These polyamines were also detected in the sterile decarboxylase medium at equivalent levels (data not shown), probably owing to the meat and yeast extract added.

Anchovy belongs to the *Engraulidae* family, which, like other pelagic fish, is characterized by a large quantity of free histidine in muscular tissue. This has been related to its high susceptibility to accumulate HI during spoilage, owing to the activity of histidine-decarboxylase positive microorganisms [7]. However, none of the isolated microorganisms studied decarboxylated histidine. Our results are in agreement with those of Lakshmanan et al. [24], who recorded PU- and CA-forming bacteria, but failed to detect HI-forming bacteria in sardine stored in ice. The lack of histidine-decarboxylating microorganisms may be due to the fact that prolific HI producers are mostly mesophilic bacteria, whose growth is not favored in the ice storage conditions used in this study.

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