Changes in Bacterial Counts and Biogenic Amines during the Ripening of Salted Anchovy (Engraulis encrasicholus)

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Abstract Samples of salted anchovies (Engraulis encrasicholus) were taken at different stages during industrial ripening. The changes of bacterial counts and biogenic amines (histamine, tyramine, agmatine, putrescine, and cadaverine) contents were studied throughout the process. Bacterial growth was generally inhibited during ripening, with the occurrence of Enterobacteriaceae spp., probably related to hygienic failures. The changes in biogenic amines showed a decrease concentration trend, except with putrescine. The salt-ripened anchovy fillets exhibited low bacterial load (0.01-1.85 log CFU/g) and moderate biogenic amine contents (1.05-33.5 mg/kg). Relevant amine quality indicators such as histamine and tyramine showed levels, respectively, lower than those of the regulation and recommendation. This study indicated also that samples of salt-ripened anchovy fillets can be considered as safe.

Keywords: anchovy, ripening, bacterial flora, biogenic amines, histamine

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1. Introduction

Biogenic amines are low-molecular-weight organic bases showing biological activity [1]. Several biogenic amines (serotonin, histamine, and tyramine) have important physiological effects in humans, but consumption of foods containing high amounts of these compounds can also have toxic effects [2]. The main monoamines are histamine, a vasoactive [3], and tyramine, a potent vasoconstrictor [4]. Furthermore, diamines and polyamines as agmatine, spermine and spermidine are reported to be potentially carcinogenic by converting to nitrosamine [5]. Despite the widely accepted association between histamine and scombroid food poisoning, histamine alone appears to be insufficient to cause toxicity, and diamines (putrescine and cadaverine) have been suggested to potentiate its toxic activity by inhibiting the intestinal histamine-metabolizing enzymes (diamine oxidase and histamine N-methyltranferase) [3]. Biogenic amines are primarily formed in foods by microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases [2,6]. The free amino acids either occur as such in foods or may be liberated through proteolysis. In addition to the availability of these precursors, biogenic amines accumulation in foods require the presence of microorganisms with amino acid decarboxylases and favourable conditions for their growth and decarboxylation activity [7]. Regulated limits have been established only for histamine in fish and fish products. Tunisian and European regulations have fixed a maximum average value of 200 mg/Kg in a group of nine samples of fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species belonging to the Scombridae, Clupeidae, Engraulidae, Coryphaenidae, Pomatomidae and Scombresosidae families [8,9].

Anchovy is a pelagic species belonging to the Engraulidae family. It is one of the most significant captured species in the world fisheries consisting of about 10.4% of total captured marine fish in 2010. Engraulis encrasicholus (Linnaeus, 1758) species ranks the third amongst the common anchovy species captured with a production of about 0.5 million tons in 2012, after Peruvian anchovy (Engraulis ringens) and Japanese anchovy (Engraulis japonicus), with, respectively 4.7 and 1.3 million tons [10]. Stocks of European anchovy (Engraulis encrasicholus) are overexploited or at their optimal level of exploitation in almost all Mediterranean fisheries [11]. However, this species is considered under-exploited in Tunisian waters, which is the only representative of the Engraulidae family in Tunisia. Influenced by its economic importance in European markets, a revision of the Tunisian fisheries policy was carried out to improve the exploitation of this resource [12].

Ripened semi-preserved anchovies are prepared in Mediterranean area from fish of the Engraulis encrasicholus species [13]. In Argentina, Chile and Peru, the species
used are Engraulis anchoita and Engraulis rigens [14]. The process goes back to ancient times and is a common tradition in some Mediterranean countries (Spain, Italy, Greece, France, and Morocco) [13], and in Tunisia. They are prepared by a process of two stages: salting and ripening, taking at least 2 months [15]. The first includes diffusion of the salt into the fish and elimination of water. The second is slower and involves a series of complex biochemical processes that can be grouped broadly into proteolysis, lipolysis, and lipid oxidation [16]. Through ripening progression, the general characteristics of the anchovy are greatly changed. At the end of the process, the fish acquires a soft consistency along with the development of a pink color and a strong characteristic flavor [13]. Finally, after desalting, anchovies are filleted and immersed in oil. These anchovy fillets are much appreciated by Tunisian consumers and are highly requested in the international market.

One of the health risks associated with the consumption of this product is histamine and other biogenic amines development that can be formed during handling and storage of raw anchovy or during subsequent ripening, and storage steps. Although, ripened anchovies are not a common cause of histamine intoxication, various previous reports and experimental studies showed that these products still present health risks around the world. Two outbreaks of scombroid poisoning caused by consumption of semi-preserved anchovies were reported, one in 1980 on anchovies containing levels up to 3060 mg/kg of histamine [17] and another by Hernández-Herrero et al. [18] who detected 680 mg/kg of histamine both in Spanish semi-preserved anchovies. Lee et al. [19] even found higher levels of histamine, ranging from 1976 mg/kg to 2720 mg/kg. Apart from the high quantity of free histidine naturally present in the muscular tissues of anchovies [20], the ripening process (proteolysis process), results in the release of more peptides and free amino acids, including histidine [16], that will be available for bacterial decarboxylation [21]. Furthermore, the low pH (4.0–5.5) achieved in salted anchovies, is favourable for enhancing the amino acid decarboxylase activity [22].

Several previous studies on microbial and biogenic amines during the anchovy ripening process were carried out in a laboratory scale [16,23]. However, no data on biogenic amines, particularly, histamine, tyramine, cadaverine, putrescine and agmatine in salted anchovies captured in Tunisian waters and sampled from private companies, is available. In fact, it is known that the properties of fish products vary widely according to country and industrial processes. Therefore, the present study was carried out to understand the changes in the microbial flora load and the contents of histamine and other biogenic amines along the salting and ripening process, and to determine the quality of the end product.

2. Materials and Methods

2.1. Sampling

Anchovies (Engraulis encrasicolus) were caught in the southern coasts of Tunisia and processed in some Tunisian industrial plants for salting. The samples were collected from the three steps of the ripening process (for the duration of 2 months): S1: after salting (pre-salted whole anchovies); S2: intermediate stage after 30 days (eviscerated anchovies) and S3: after ripening (salt-ripened fillets immersed in olive oil). All anchovies were transported under refrigerated conditions (4 °C ± 1 °C) by plane to the laboratories of Portuguese Institute for the Sea and Atmosphere (IPMA, I.P.), in Lisbon, Portugal, and analyzed within 24 hours for microbiological analysis and for biogenic amines determination.

2.2. Microbiological Analysis

Ten grams of each sample were taken aseptically and homogenized for 2 min using a stomacher (Seward Lab. Systems Inc., USA) with 90 mL of tryptone-salt broth (TSB). The homogenates were serially diluted with 9 mL of TSB and the appropriate dilution was inoculated to the corresponding medium. Total viable count (mesophilic bacteria) and sulphite producers were enumerated on Lingby iron agar base (Scharlau, Spain) incubated at 30°C for 72 h; Enterobacteriaceae spp. enumeration in double layered violet red bile glucose (Oxoid, UK) incubated at 37°C for 24-48 h, followed by confirmation tests; coliforms enumeration on chromocult agar (Merck, Germany) incubated at 37°C for 24 h, enterococci enumeration on KF streptococcus agar (Merck, Germany) incubated at 37°C for 24-48 h; halophilic bacteria enumeration on halophilic agar (in-house preparation) incubated at 30 °C for 30 days. Histamine-forming bacteria were detected on modified Niven medium according to Bover-Cid and Hozapfel [24] incubated at 37°C for 4 days, followed by confirmation tests. A minimum of five representative colonies from each plate were picked and further streaked on tryptone soy agar (Oxoid, UK) supplemented with 2% NaCl to obtain pure cultures. The purified strains were then tested for Gram with 3% of KOH, oxidase reaction, catalase production and oxidative-fermentative OF test (Oxoid, UK) supplemented with glucose. For all performed experiments, results were expressed as CFU/g.

2.3. Biogenic Amines Analysis

2.3.1. Standards and Reagents

Biogenic amines standards (histamine, cadaverine, tyramine, putrescine and agmatine), internal standard hexanediamine, o-ptalaldehyde and Brij 35 were obtained from Sigma-Aldrich (USA). Acetonitrile, 1-octane acid sulfonic sodium salt, sodium acetate from Merck (Darmstadt, Germany) and mercaptoethanol from Fluka (Berlin, Germany). Ultrapure water was obtained with a Millipore-Milli-Q equipment (Germany).

2.3.2. Reversed-phase High Performance Liquid Chromatography (RP-HPLC) Procedure

Five biogenic amines included histamine, cadaverine, putrescine, tyramine and agmatine contents on the samples were determined, in duplicate, by high performance liquid chromatography (Agilent 1100, Series G1322A) (Agilent, Germany) procedure on a C18 reversed-phase column (220 x 4.6 mm, 5µ) involving post-column derivatization with OPA and fluorescence detection according to Veciana-Nogués et al. [25]. Twenty five grams of samples were taken from each lot separately and homogenized in 50 mL of 10% trichloracetic
2.3. Changes in Bacterial Counts

Apart from the nature of the flora, the bacterial load throughout ripening of salted anchovy was low, ranging between undetectable and 3.65 log CFU/g (Table 1). Rodríguez-Jerez et al. [26] also detected a low bacterial load during ripening. Along the ripening stage, coliforms and enterococci were not detected in all analyzed samples (0.01 log CFU/g) and a consistent decrease of mesophilic microorganisms (3.65 log CFU/g to 1.85 log CFU/g) was observed. Halotolerant bacteria increased from 0.75 log CFU/g in S1 to 2.26 log CFU/g in S2, followed by a decrease after immersion of the salted anchovies in oil (S3). Histamine-producing bacteria showed a similar pattern to halotolerant bacteria. The enumeration of H$_2$S-producing bacteria was fairly constant along the process (0.01-0.54 log CFU/g), and Enterobacteriaceae spp. increased on stage 2 and 3 (0.01-1.83-1.75 log CFU/g). In a similar study, Rodriguez-Jerez et al. [26] noted that the counts of different bacterial groups decreased during the first two weeks of ripening, but later stabilized. Also no counts of faecal coliforms were reported during the fish ripening, and the Enterobacteriaceae spp., that initially did not appear after two weeks, were detected at final phases.

Table 1. Mean Values of Bacterial Flora (log CFU/g) of Anchovy Samples at Different Stages of Ripening

| Bacterial flora                  | Ripening stage |       |       |
|---------------------------------|----------------|-------|-------|
|                                 | After salting  | Intermediate stage | After ripening |
| Mesophilic bacteria             | 3.65           | 2.76   | 1.85  |
| H$_2$S-producing bacteria       | 0.01           | 0.54   | 0.50  |
| Enterobacteriaceae spp.         | 0.01           | 1.83   | 1.75  |
| Coliforms                       | 0.01           | 0.01   | 0.01  |
| Enterococci                     | 0.01           | 0.01   | 0.01  |
| Halotolerant bacteria           | 0.75           | 2.26   | 0.01  |
| Histamine-producing bacteria    | 0.01           | 3.45   | 1.10  |

In samples from pre-salted whole anchovies (S1), average bacterial counts varied between undetectable and 3.65 log CFU/g. Indeed, anchovy microflora is affected by conditions and length of storage before processing, and it also depends on the pre-salting stage. According to Martínez and Gildberg [27], fresh anchovy is susceptible to spoilage caused by both microbiological and chemical reactions. Its small size and the spread of gut enzymes and microbes when stomach bursts are the main cause. Even under ice preservation (day$_0$ to day$_2$), Pons-Sánchez-Cascado et al. [28] noted that the levels of Enterobacteriaceae spp. and Pseudomonas spp. increased throughout storage, achieving counts higher than 6 log CFU/g at the end of the study period.

If bacterial growth of this stage depends on handling of raw anchovy, the most significant parameter at pre-salting stage is the rapid salt absorption and $a_w$ reduction, followed necessarily by changes in bacterial flora, as reported by Pons-Sánchez-Cascado et al. [29] who observed that microbial counts during ripening differed significantly from those of fresh anchovy. Indeed, as shown by Hernández-Herrero et al. [18], after salting, salt content reached 19.3%. Additionally, Filsinger [30] reported that during this stage, $a_w$ is generally reduced from 0.99 in raw fish, to 0.80-0.84. At this stage (S1), samples exhibited 3.65 log CFU/g as average value of mesophilic bacteria. Previously, Rodríguez-Jerez et al. [26] have observed, after two weeks of ripening, 1.86 log CFU/g for total aerobic mesophilic. According to these authors, NaCl presented a significant influence on the mesophilic count. On the other hand, Enterobacteriaceae spp., coliforms and enterococci were not detected. On a similar note, Hernández-Herrero et al. [18] observed that Enterobacteriaceae spp. and enterococci counts decreased sharply (p<0.05) during the first week of ripening, from 2.8 and 2 log CFU/g to 0.5 and 0.7 log CFU/g, respectively. These authors explained also the early decreases of Enterobacteriaceae spp. counts during ripening by their low tolerance to the high-salt environment. Additionally, Rodríguez-Jerez et al. [26] reported that the faecal coliforms did not appear after first two weeks of ripening. Oxygen concentration and specially sodium chloride concentration were the main causes for the decrease in faecal coliforms. Likewise, H$_2$S-producing bacteria and histamine-producing bacteria were not detected. According to Wheaton and Lawson [31], as the fish salt content increased above 1%, bacteria associated with spoilage of fresh fish were increasingly stressed. At salt concentrations of 6-8% most of these bacteria would die or at least stop growing. Vieties et al. [32] reported that the strong decrease in water activity due to the immersion of fish in solutions with high concentrations of NaCl inhibits the growth of undesirable microorganisms.

Conversely, halotolerant bacteria reached 0.75 log CFU/g. Comparably, after the 1st week of experimental ripening (9 week process at 20°C), moderate halophilic bacteria were reported to reach 2.87 log CFU/g [16]. Obviously, although salt prevents growth of common bacteria, other microorganisms may not be affected by its presence. Occurring naturally in the outer layer of skin, gills and intestines of marine fish [33], halophilic bacteria diffuse into anchovy flesh during pre-salting and following stages. Pre-salting stage is believed to cause the selection of halotolerant bacteria, possibly on account of a more suitable water activity for growth of the most halophilic bacteria.
At the final stage (S3) of ripening, salt-ripened anchovies are rinsed and washed, drained and afterwards filleted and immersed in olive oil barrels. As reported by Hernández-Herrero et al. [34], the salt content increases from 0.3% in raw fish to 18% in ripened products and then decreases to 10% to 12% during desalting and packing of the products with oil in cans. The salt rate decreasing is generally attributed to the absorption of olive oil by the fillets. In present study, anchovy fillets in this stage were characterized by low bacterial density as the bacterial load varied, depending on the nature of flora, between 0.01 log CFU/g and 1.85 log CFU/g. As reported by several authors, salt is the main factor preventing bacterial growth and providing food safety. Microbiological analysis revealed the lack of coliforms, enterococci and halotolerant bacteria, and the presence of mesophilic bacteria, Enterobacteriaceae spp., histamine-producing bacteria and H2S-producing bacteria at average rates, respectively, of (1.85, 1.75, 1.10 and 0.50) log CFU/g. According to Rodríguez-Jerez et al. [26], the majority of the anchovy microorganisms studied were inhibited following the eleven week of ripening. Previously, Vieites et al. [35] established also that anchovies that have been prepared following a traditional salt-ripening process without any thermal treatment generally have lower microbial loads than do non-processed fish.

With an average value of 1.85 log CFU/g, mesophilic flora observed a decrease compared to previous ripening stages. Presenting a similar behaviour, bacterial counts in canned anchovy recalled by the FDA were consistently below the detection limit of 2 log CFU/g [36]. Halotolerant bacteria were not detected at the end of our experiment. In other ripening processes reported by Hernández-Herrero et al. [16], where it has not been made experiment. In other ripening processes reported by Hernández-Herrero et al. [18], the extremely halophilic bacteria, Enterobacteriaceae spp., histamine-producing bacteria and H2S-producing bacteria at average rates, respectively, of (1.85, 1.75, 1.10 and 0.50) log CFU/g. As reported by several authors, salt is the main factor preventing bacterial growth and providing food safety. Microbiological analysis revealed the lack of coliforms, enterococci and halotolerant bacteria, and the presence of mesophilic bacteria, Enterobacteriaceae spp., histamine-producing bacteria and H2S-producing bacteria at average rates, respectively, of (1.85, 1.75, 1.10 and 0.50) log CFU/g. According to Rodríguez-Jerez et al. [26], the majority of the anchovy microorganisms studied were inhibited following the eleven week of ripening. Previously, Vieites et al. [35] established also that anchovies that have been prepared following a traditional salt-ripening process without any thermal treatment generally have lower microbial loads than do non-processed fish.

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Histamine-producing bacteria appear to be sensitive to NaCl concentration, simulating the behavior of halotolerant bacteria as there was a level decrease from S2 to S3 stage (3.45 log CFU/g to 1.10 log CFU/g). The presence of histamine-forming bacteria in canned anchovies was not also detected by Lee et al. [19]. As reported by Chauquy and El Marrakchi [39], H2S-producing bacteria are virtually absent after anchovy capture, which is also in compliance with present study (not detected in stage 1), but occurred during ripening and at final S3 stage (0.54-0.50 log CFU/g). Its low value indicated that salted anchovy can be subject to proteolytic flora growth. Usually indicative of degradation, proteolytic flora seems to be associated with bacterial proteolysis, a factor contributing to the sensory changes during ripening. In this study, the development of proteolytic flora may be related to proteolysis phenomena of ripening mechanisms.

Moreover, average value of Enterobacteriaceae spp. at stage 3 was 1.75 log CFU/g, approximatively the same of the previous stage (1.83 log CFU/g). This is in conflict with the finding of Vieites et al. [35] and Veciana-Nogués et al. [40], where Enterobacteriaceae spp. were not detected in Spanish anchovies immersed in oil. Nevertheless, our result is consistent with that of Rodríguez-Jerez et al. [26], who found that Enterobacteriaceae spp., which disappeared after two weeks, were detected in the final stages of maturation (0.5 log CFU/g). Handling performed after ripening, including desalting and filleting, prior to oil immersion, could result in secondary contamination. Filleting material and waiting time in temperature above 10°C are the most origin.

In summary, four different microbiological profiles accompanied the ripening process: (1) microorganisms that were not detected in all maturation stages (coliforms and enterococci), (2) microorganisms that show a continuous decrease (mesophiles), (3) microorganisms that presented development during the process (Enterobacteriaceae spp., H2S-producing bacteria and histamine-producing bacteria), and (4) microorganisms that observed no significant evolution (halotolerant bacteria).

### 3.2. Changes in Biogenic Amines

During the course of ripening, agmatine (95.5-32.25 mg/kg) was the dominant amine, followed by tyramine (42.45-28.75 mg/kg), cadaverine (43.4-19.6 mg/kg), putrescine (38.26 - 12.35 mg kg⁻¹), and histamine (5.25-1.05 mg/kg) (Table 2). According to Pons-Sánchez-Cascado et al. [22], tyramine was the most abundant biogenic amines in all batches during 26 weeks of experimental ripening. This amine has been also reported by Veciana-Nogués et al. [40] as the major amine found during the salt-ripening process and the major amine of ready-to-eat salt-ripened anchovies.

#### Table 2. Biogenic Amines Mean Content (mg/kg) of Anchovy Samples at Different Stages of Ripening

| Biogenic amines | After salting | Intermediate stage | After ripening |
|-----------------|--------------|--------------------|---------------|
| Histamines SD†  | 5.25±2.47    | 2.23±2.98          | 1.05±0.63     |
| Cadaverines SD  | 43.40±1.13   | 27.46±25.98        | 19.60±4.30    |
| Putrescine SD   | 12.35±2.19   | 38.26±9.32         | 33.5±2.40     |
| Tyramine SD     | 42.45±10.05  | 30.66±14.91        | 28.75±1.48    |
| Agmatine SD     | 95.5±52.89   | 43.7±35.24         | 32.25±7.65    |

† SD, standard deviation.

Evolution of biogenic amine content during ripening depended on the amine considered. The highest content of agmatine (132.9 mg/kg), cadaverine (57.4 mg/kg) and putrescine (56.1 mg/kg) were greater than those obtained by Pons-Sánchez-Cascado et al. [22] for ready-to-eat product, respectively, 57.45 mg/kg, 47.84 mg/kg and 17.20 mg/kg. However, highest putrescine content was lower than that obtained by Draisci et al. [41], who qualified putrescine as dominant amine. Moreover, the highest content of tyramine (52.5 mg/kg) was lower than that reported by Pons-Sánchez-Cascado et al. [22] (84.75 mg/kg). Although fish of the family Engraulidae are...
characterized by a large concentration of free histidine in muscle [20], the highest content of histamine was 7.0 mg/kg, which is lower than those reported by several authors. The maximum concentration observed by Pons-Sánchez-Cascado et al. [22] was 19.35 mg/kg, whereas those of Draisci et al. [42] and Hernández-Herrero et al. [34] were, respectively, lower than 30 mg/kg and 50 mg/kg. Furthermore, Fuselli et al. [43] also found that histamine concentration was below 40 mg/kg at every stage of the Engraulis encrasicholus marinating process.

During the first period of ripening (S1), average values of agmatine, tyramine, cadaverine, putrescine and histamine of pre-salted whole anchovies were, respectively, (95.5, 42.45, 43.4, 12.35, and 5.25) mg/kg. As in this study, Pons-Sánchez-Cascado et al. [29] distinguished, at this stage, two groups: agmatine, cadaverine and tyramine which reached 20-30 mg/kg, and histamine and putrescine whose contents did not exceed 10 mg/kg. The relatively important amounts, particularly, of agmatine, tyramine and cadaverine, could be, first of all, related to their accumulation during storage, prior transformation. If we consider particularly the case of agmatine, this amine can be naturally elevated due, at first, to its physiological role in anchovy muscle [44]. Additionally, as shown by Pons-Sánchez-Cascado et al. [45], agmatine is the prevailing amine in raw material, exhibiting the highest content (10 mg/kg). In contrast, histamine, cadaverine, putrescine and tyramine and putrescine contents are very low (< 1 mg/kg) or not detected in raw material [22]. Nevertheless, during spoilage of anchovy at 8 °C and 22 °C, Veciana-Nogués et al. [40] demonstrated that histamine, cadaverine, tyramine, and putrescine were important amines formed. For instance, these authors observed that histamine contents in anchovies reached 194 mg/kg in 22 h at 20 °C and 181 mg/kg in 56 h at 8 °C.

If amines accumulation at early period of ripening depends on handling of raw anchovy, the most significant parameter following the pre-salting stage is the changes in bacterial counts as reported by several authors. Given microbiological status of this stage (S1), it seems that amines production is essentially dependent on mesophilic (3.65 CFU/g) and halotolerant (0.75 log CFU/g) bacteria.

In light of our results, raw anchovies used for processing were of adequate quality, and pre-salting stage was undergone with correct handling. In fact, histamine is extremely stable once it is formed in the food. It is not easily removed or destroyed by food-preparation processes, such as freezing or salting [46]. Additionally, according to Hernández-Herrero et al. [18], salt content may reach 19.3%, after salting, a concentration which inhibits necessarily almost all histamine-forming bacteria at this stage. On the other hand, processing prior sample analysis (capture, on board preservation, reception, ice storage and, particularly, pre-salting) may have been the main cause of noticeable increase in amine rates, especially agmatine, cadaverine and tyramine, which reflects some signs of fish deterioration. Accumulation of relatively large amounts of amines during the early stages of ripening, particularly agmatine, tyramine and cadaverine, which are usually related to microbial spoilage, might thus be explained, by their formation during the first days of anchovy deterioration, or by their synthesis in muscle before the fish is saturated with salt.

After ripening (S3), average values of agmatine, tyramine, cadaverine, putrescine and histamine were, respectively, (32.25, 28.75, 19.6, 33.5 and 1.05) mg/kg. Agmatine, tyramine, and putrescine were major biogenic amines found in salt-ripened anchovy fillets. Previous works by Veciana-Nogués et al. [40] showed also low or moderate contents of biogenic amines in anchovy immersed in oil immediately after packaging, 45.3, 21.6, 38.3, 7.6 and 12.6 mg/kg, respectively, for agmatine, tyramine, cadaverine, putrescine and histamine. In contrast, our results were higher than those of just packed marinated anchovies, where average values were (1.43, 5.61, 0.37, and 0.51) mg/kg, respectively, for agmatine, tyramine, cadaverine, and putrescine [45]. These latter attributed the low values not only to the low formation of amines during the marinating process, but also to the vinegar extractive effect. In anchovy fish sauce, Kirschbaum et al. [47] reported a higher level of histamine (720.1-750.7 mg/L), cadaverine (100.8-280.5 mg/L), tyramine (330.7-730.9 mg/L), and putrescine (100.8-190.7 mg/L).

The average value of histamine of finished product is close to the result of Hernández-Herrero et al. [16] who observed 5.36 mg/kg of histamine at 9 weeks of experimental ripening. In our study, accumulation of important amounts of histamine cannot take place during the ripening. In fact, fresh raw anchovy and proper handling during ripening were obviously the origin of low content of histamine in the end product. Histamine content in the samples showed a decrease during the ripening process (5.25-2.23-1.05 mg/kg). Hernández-Herrero et al. [16] observed also a decrease in histamine content, which was significant (p < 0.05) only during the first week of the ripening process. According to these authors, histamine diffuses, probably, from the fish into the brine. Additionally, Sebastio et al. [48] found that processing of ripened anchovies into fillet semi-preserves in oil did not increase the concentration of histamine. They evidenced a slight reduction in histamine content due to the operations involved in processing of the salt-cured fish, especially scaling, centrifugation and filleting, preceding preservation in oil.

Moreover, our results were in agreement with those of Pons-Sánchez-Cascado et al. [22] where tyramine level was higher than that of histamine. In later case, tyramine reached values up to 90 mg kg⁻¹, whereas histamine did not exceed 20 mg kg⁻¹. Lee et al. [19] reported also that canned anchovy products may contain high levels of biogenic amines, such as tyramine and spermine, despite low levels of histamine accumulation in the products. These results can be explained by the presence of sodium chloride, which activates tyrosine decarboxylase activity and inhibits histidine decarboxylase activity, as reported by Santos [1].

The cadaverine content of final product is slightly lower than those of Veciana-Nogués et al. [40] and Pons-Sánchez-Cascado et al. [22] for ready-to-eat products, which were, respectively, 38.3 and 47.84 mg/kg. On contrary, the putrescine average is higher than those reported by Veciana-Nogués et al. [40] and Pons-Sánchez-Cascado et al. [22], where putrescine content reached, respectively, 7.6 mg/kg and 17.20 mg/kg for ready-to-eat product. As reported by Brink et al. [2], the presence or an increased amount of putrescine is usually associated with high occurrence of Gram negative bacteria usually resulting from a mishandling process, or insufficient hygiene. In this study, the occurrence of Enterobacteriaceae spp. in
same samples (1.75 log UFC/g) could, particularly, explain the increase in putrescine levels. While the agmatine content was slightly higher than that of Veciana-Nogués et al. [40] (21.6 mg/kg), it was lower than the mean value obtained by Pons-Sánchez-Cascado et al. [22] for ready-to-eat product (84.75 mg/kg). Comparably, this amine reached 161 mg/kg in Rihaakuru, a cooked fish paste, whereas in other fermented and non-fermented fish paste, agmatine is not detectable [47].

Overall, our results showed two profiles: (1) amines (histamine, cadaverine, tyramine and agmatine), which showed a progressive decrease, and (2) putrescine that increased during ripening. Our results are, globally, consistent with the finding of Veciana-Nogués et al. [40] who showed also that amines contents have not increased during anchovy maturation up to 22 weeks. Likewise Pons-Sánchez-Cascado et al. [29] reported also that the formation of biogenic amines during ripening was moderate. According to these authors, after an important amines formation phase prior salt saturation of fish, production of amines is stopped or reduced except putrescine which increased slowly until the 10th week of maturation.

As previously referred build-up of biogenic amines may occur during salting and brining of anchovy, though identification of amines-degrading bacteria in fermented foods [49] characterises amines production during the course of anchovy ripening as a complex phenomenon governed by multitude of antagonist mechanisms of formation and decomposition, that need further investigation.

3.3. Correlation Study

It was reported that the amount and type of biogenic amines formed in food depends on the nature of the substrate and type of microorganisms [6], [50]. The Pearson correlation was used to establish whether there was a relationship between bacterial counts and the presence of biogenic amines.

Our results revealed that histamine (r = 0.968), tyramine (r = 0.848) and agmatine (0.936) depend exclusively on mesophilic bacteria (Figure 1). According to the equations established by Pons-Sánchez-Cascado et al. [28], the best correlation for content of biogenic amines with bacterial load was that for tyramine and mesophilic bacteria (r = 0.89); and the next best was between histamine and mesophilic counts (r = 0.88). Conversely, no relationship between growth of Enterobacteriaceae spp. and histamine formation was established. Along the process of salt-maturation, El Filali et al. [51] did not establish a correlation between Enterobacteriaceae spp. and histamine, although they have found that the company, which correspond to the high values of histamine, also recorded high levels of enteric bacteria. Hernández-Herrero et al. [34] observed that levels of histamine were negligible before Enterobacteriaceae spp. counts reached 5 log CFU/g, which are the minimum counts reported to be able to produce significant amounts of histamine. Globally, Veciana-Nogués et al. [50] did not observe a relationship between growth of Enterobacteriaceae spp. and amine formation because those counts were very low and remained constant during storage of anchovies immersed in oil.

As previously referred build-up of biogenic amines may occur during salting and brining of anchovy, though identification of amines-degrading bacteria in fermented foods [49] characterises amines production during the course of anchovy ripening as a complex phenomenon governed by multitude of antagonist mechanisms of formation and decomposition, that need further investigation.

**Figure 1. Regression line of histamine with mesophilic bacteria correlation**

In contrast, putrescine depend mainly on Enterobacteriaceae spp. (r = 0.991) and H2S-producing bacteria (r = 0.994) (Figure 2a and b). Cadaverine was correlated positively with mesophilic bacteria (r = 0.980) and H2S-producing bacteria (r = 0.921) (Figure 3). These observations may be consistent with the decomposing capacity of these bacterial groups and with the potential quality indicator of cadaverine in fishery product. Cadaverine and putrescine are commonly used as indicators of spoilage and toxicity in fish.

**Figure 2. Regression line of putrescine with Enterobacteriaceae spp. (a) and with H2S-producing bacteria (b) correlations**
histamine level was far below than regulatory limit of 200 mg kg\(^{-1}\) allowed by Tunisian and European regulations, established for fishery products, which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine [8], [9], and was lower than the stricter acceptable histamine value of 50 mg/kg, established by The US Food and Drug Administration [46]. Similarly, tyramine level was below to the range value of 100-800 mg/kg, recommended by Nout [55]. Quality wise, mainly cadaverine level, which has been proposed as chemical indicator of the hygienic conditions of raw material and/or manufacturing practices, was below recommended value of 30 mg/kg [1,2]. Currently, no recommendations about the levels of putrescine have been suggested.

Even though anchovies in oil, are a semi-preserved product obtained without any heating process to stabilize the end product and storage of ripened anchovies immersed in oil at refrigeration temperatures delays but does not prevent amine formation [50] the manufacture process provides the product a relative stability. In summary, marked by a low bacterial density, a weak content of histamine, and presence of other biogenic amines at moderate values, salt-ripened anchovy fillets were declared fit for human consumption. All these results indicate that salt-ripened anchovies can be controlled by strict use of good hygiene in both the handling of raw materials and manufacturing process. The Tunisian companies, from which samples were obtained, are known to apply strict self-controls for their product using the HACCP system. In fact, fishery products are regulated in Tunisia in accordance with the hazard analysis and critical control point (HACCP) principles [56].

In parallel to this approach dealing with amines individually, the use of several amines expressed as biogenic amine index (BAI) has been suggested as an indicator of the quality of fish sample. According to several authors, BAI (expressed as the sum of histamine + putrescine + cadaverine + tyramine) correlates well with the organoleptic quality of tuna and sardines [57]. Calculated according this formula, the sum of biogenic amines of salt-ripened anchovy fillets (103.45 mg/kg) was lower than those of other fermented food, which were (582-588, 177-334 and 281-283) mg/kg, respectively, for fish sauce, cheese and fermented sausages [58].

3.4. Quality of Finished Product

The salt-ripened anchovy fillets were marked by low bacterial load (0.01-1.85 log CFU/g). Level of mesophilic microorganisms (1.85 CFU/g) was lower than the limit (m) of 5 log CFU/g established by Tunisian regulation for semi-preserved of unpasteurized fish product [8], and by Spanish regulation for salted fish [54]. Enterobacteriaceae spp. count (1.75 CFU/g) was closely below the allowable limit of 2 log CFU/g established for salted fish [54]. Although Enterobacteriaceae spp. family include environmental species that appear in the food manufacturing structure, these species do not present health risks. However, their presence may sometimes mean the presence of specific pathogens. Being a hygiene criterion of the manufacturing process, the levels of mesophilic bacteria and Enterobacteriaceae spp. indicate that the microbiological quality of the process is satisfactory.

Otherwise, the end product recorded amines rates ranged between 1.05 mg/kg and 33.5 mg/kg, for all considered amines. In fact, the interest in biogenic amine content of ripened anchovy lies in safety and quality issues. From a toxicological point of view, mean histamine level was far below than regulatory limit of 200

![Figure 3](image)

**Figure 3.** Regression line of cadaverine with mesophilic bacteria correlation

No relationship was observed between halotolerant bacteria and histamine. However, Karnop [52] reported that histamine formation can be related with remaining enzyme activity from halophilic microorganisms, even when low bacterial counts are found. Moreover, the best correlation between halotolerant bacteria and amines production was observed with putrescine (0.360).

All the correlations found between amines and bacterial groups, are consistent with the microbial origin of biogenic amines. In this study, the decreasing trend of histamine, cadaverine, tyramine and agmatine could be particularly related to the decrease in mesophilic bacteria. As reported by Nei [53], the producing process, mainly in salt-ripening treatment, limits the bacterial development at the origin of histamine production. Karnop [52] reported that increases in histamine could be expected only when the total bacterial count of anchovy samples increased more than 5 log CFU/g during storage. Although some correlations were detected between microbial groups and amines, presumably, amines accumulation depends on a complex interaction of factors.

4. Conclusion

The current study provided valuable information on bacterial and biogenic amines occurrence during ripening of salted anchovy sampled from Tunisian industry. Taking into account all microbiological results, it could be concluded that bacterial growth is generally inhibited during ripening. It also revealed that salted anchovy can be highly prone to contamination, particularly, with Enterobacteriaceae spp., probably related to hygienic failures during ripening manipulations. Nevertheless, it can be said that salt-ripened anchovy fillets analysed presented a notorious low bacterial density.

Within the scope of our study, it can be also concluded that, with the exception of putrescine, levels of other amines, agmatine, tyramine, cadaverine and histamine observed a decreasing trend. The increase of putrescine level during ripening is probably linked to the observed
growth of Enterobacteriaceae spp.. Histamine was a minor amine throughout the ripening.

Agmatine, tyramine, and putrescine were major biogenic amines found in salt-ripened anchovy fillets. Characterised by a final content of histamine lower than regulatory limit and final content of other amines lower than recommended levels, end products were considered as safe.

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