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Bacterial Contribution to Salted Anchovy (Engraulis Anchoita Hubbs & Marinni, 1935) Ripening Process

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Keywords: salted ripened fish; halophilic bacteria; lipolysis; proteolysis; TMAO reduction.

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Abstract

The bacterial populations of the salted anchovy ripening process and its potential role on the development of the typical sensory characteristics of this product were studied. Salted anchovy samples were taken during the ripening process and submitted to microbiological analyses in order to monitor the evolution of bacterial groups. According to the results obtained, the ripening process was dominated by moderate halophilic bacteria. Moreover, many of the isolated strains showed proteolytic, lipolytic, and trimethylamine oxide (TMAO) reductase activities. These activities contribute to the development of the typical flavor of this product and to the increase of total volatile bases observed during ripening.

Keywords: salted ripened fish; halophilic bacteria; lipolysis; proteolysis; TMAO reduction.

1. INTRODUCTION

Salting is a method that has been applied for fish preservation since ancient times. In the presence of salt (NaCl), the flesh of some fatty fish species may undergo chemical and physicochemical changes, leading to a process called ripening or "*anchoado*." Salting and ripening of different pelagic species is a common and traditional practice in European countries (Borgstrom, 1965; Steffánson y Guðmundsdóttir, 1995). Among this type of product, salted-ripened anchovy (*Engraulis encrasicholus*) produced in Mediterranean countries has the highest international relevance. In the last century, after European immigration, this procedure was adopted in Latin American countries, especially Argentina, where the raw material used is the species *Engraulis anchoita.* Sensory characteristics of this product are similar to European

salted-ripened anchovy: reddish color and characteristically pleasant flavor and odor. Currently, 90% of the Argentinean production is exported as a commodity in barrels to countries, such as Spain, Portugal, France, and Morocco, which reprocess the fish to obtain anchovy fillets in oil. This product is also appreciated in the United States, Mexico, and Brazil, where it is imported ready to eat (Madureira et al., 2009). The manufacturing process involves a preliminary operation of wet salting (brining), where traditionally, whole fish is immersed in saturated brine until osmotic equilibrium is achieved in the muscle. During this stage, water activity (a_w) is reduced from 0.99, corresponding to raw fish, to 0.80-0.84 (Filsinger, 1987; Czerner and Yeannes, 2010). Following this, anchovies are beheaded and partially gutted (leaving gonads and pyloric caeca), placed in barrels of alternating layers of fish and salt, and pressed, reducing the a_w to 0.75 (Filsinger et al., 1987; Yeannes, 1996). Ripening implies several chemical and physicochemical changes, both in the lipid and the protein fraction, and requires 3 to 12 months depending on the fish species and the manufacturing technology used (Durand, 1981; Campello, 1985; Filsinger et al., 1982, 1984, and 1987; Roldán et al., 1985; Hernández-Herrero, 1999a and 1999b). As a result of these changes, the product acquires firm consistency, reddish color, juicy texture, and a characteristic odor and flavor (Filsinger et al., 1982). Different studies indicate that compounds derived from proteolysis and lipolysis would contribute to the development of the characteristic flavor. In this sense, the relationship between total and non-protein nitrogen (NPN) has been proposed as an objective index to evaluate the degree of ripening (Durand, 1981; Hernández-Herrero, 1999a; Besteiro et al., 2000; Pons-Sánchez-Cascado et al., 2005). Moreover, an increase of the ester index (related to free fatty acid content) has been observed during ripening, showing a good correlation with the development of the sensory characteristics

(Filsinger et al., 1982; Roldán et al., 1985). This fact is very important since it constitutes the prior step to free fatty acid oxidation which gives rise to numerous volatile compounds that would be responsible to a large extent for the salted-ripened anchovy's characteristic flavor (Triqui and Reineccius, 1995; Czerner, et al., 2011). Another objective index that is usually employed to evaluate the degree of ripening is the total volatile basic nitrogen content (TVB-N), which has shown a very good correlation with both the ripening time and the sensory evaluation (Filsinger et al., 1984; Hernández-Herrero et al., 1999a). The TVB-N fraction includes ammonia, monoethylamine, and dimethylamine, along with trimethylamine (TMA). The last is generated in fresh marine fish by the reduction of trimethylamine oxide (TMAO) by specific spoilage bacteria (Gram and Huss, 1996). Nevertheless, the origin of the increased TVB-N during the ripening of salted anchovy has not been determined yet.

The mechanisms of transformations taking place during ripening are still under analysis. In this sense, different and complementary theories have been proposed about the contribution of tissular and microbial enzymes to this process (Voskresensky, 1965; Campello, 1985; Hernández-Herrero et al., 1999b; Besteiro et al., 2000; Triqui and Reineccius, 1995). Taking into account the microbiological aspect, different studies indicate that the ecosystem of this type of product is dominated by halophilic microorganisms (Campello, 1985; Villar et al., 1985; Hernández-Herrero et al., 1999a; Moschetti et al., 2006; Félix et al., 2004 and 2007). Salted-ripened anchovy is characterized by a high NaCl content (14 – 20 g/100 g) and a water activity value near 0.75 (Filsinger et al., 1987; Yeannes, 1996) which prevents the growth of pathogenic bacteria, such as *C. botulinum*, and typical spoilage bacteria of fresh fish (Gram and Huss, 1996). Halophilic microflora includes a great diversity of the microorganisms prokaryote, eukaryote,

and *archaea*, which are able to survive and grow on hyper saline environments such as solar saltern, salt packs, brines, and heavily salted food. These organisms have adaptive strategies allowing them to withstand osmotic stress, maintaining high intracellular concentrations of salt, and synthesizing compatible solute in order to balance their osmotic pressure. They can be classified according to their behavior toward salt into moderate halophiles, with optimal growth between 0.5 and 2.5 M salt (3 – 15 %), and extreme halophiles, growing between 3 and 5 M salt (18 – 30 %). On the other hand, bacteria able to grow in the absence of salt, as well as, in the presence of relatively high salt concentrations are named halotolerant (Ventosa et al., 1998).

Even though participation of microorganisms during the ripening process has been suggested (Villar et al, 1984; Hernández-Herrero et al., 1999a), systematic investigations have rarely been performed. Actually, studies of extreme halophilic *archaea* have been generally focused on their implications in spoilage, associated with the "pink" condition which can produce off-odors and flavors in this product (Gram and Huss, 1996; Félix et al., 2007). Another aspect that has been often investigated is the bacterial production of histamine and other biogenic amines, which are considered a hazard in this product (Yeannes, 1996; Pons-Sánchez-Cascado et al., 2005). In this sense, several extreme halophilic bacteria with histamine-forming potential have been isolated from salted-ripened *E. anchoita* (Yeannes, 2003). Furthermore, Pons-Sánchez-Cascado et al. (2005) also isolated different microorganisms that were able to decarboxylase amino acids and produce biogenic amines, especially histamine during the salted *E. encrasicholus* ripening process. On the other hand, Aponte et al. (2010) recently reported that two selected extreme halophilic *archaea* strains could have a positive influence in the production of salted-ripened

anchovy from a sensory point of view and because of their inhibitory effect on histamine formation.

In this context, the aim of this research was to characterize the bacterial populations that grow during the ripening process and to analyze the potential bacterial role on the development of the sensory characteristics of salted-ripened anchovy (*E. anchoita*), based on their proteolytic, lipolytic, and trimethylamine oxide TMAO-reductase activities.

2. Materials and methods

2.1 Preparation of salted-ripened anchovy and sampling

Anchovy (*Engraulis anchoita*) used for the experiments were caught near Mar del Plata, Argentina (38º S, 57º 33' W) during the harvest season and kept in bins with ice until they arrived at the laboratory. Fish was classified as 28-34 pieces per kg.

Three batches of salted anchovy were studied during ripening, in which modifications on the operative conditions were introduced. Two of them (batches A and B) were processed according to the traditional method, for which whole fish (about 40 kg for each batch) was immersed in saturated brine for 24 hours (one part of brine, one part of fish). Then, anchovies were manually beheaded and partially gutted (H&G) (leaving gonads and pyloric caeca) and disposed in plastic tins (approximate capacity: 5 kg), according to the traditional process named “head-tail”. The tins were packed with alternate layers of fish and salt, finishing with a layer of salt (final salt to fish ratio, 1:5). A plastic disk was placed on the top layer of salt, and weights were put in place

in order to maintain the fish under constant pressure. For the first 8 to 10 days, a pressure of 160 g/cm^2 was applied to achieve a rapid loss of water. Then, some blocks were removed in order to achieve 140 g/cm^2 and 80 g/cm^2 for batches A and B, respectively. This level of pressure was maintained up to the end of the maturation process. In order to evaluate the effect of viscera on the microbial ecosystem of the ripening process, a third batch (batch C) was filleted previous to the brining stage. After that, fillets were immersed in saturated brine for 24 hours, then placed in barrels, alternating layers of fish and salt (same conditions as described for batches A and B), and pressed at 80 g/cm^2. Each batch consisted of 6 plastic tins.

Tins were kept for 395 days in an adiabatic chamber for ripening. Samples of approximately 350 g (~15-20 anchovies) were aseptically taken along the ripening process from three different tins selected at random, i.e. 45-60 anchovies (~ 1 kg) were sampled for each batch at each sampling time. For sample extraction, the two superior layers of fish were discarded.

2.2 Microbiological analyses

2.2.1 Microbiological counts

For microbiological analyses, 10 g of salted-ripened anchovy muscle taken from 6-8 different anchovies were homogenized with 90 mL of sterile salt broth (meat extract, 3 g/L; meat peptone, 5 g/L; NaCl, 150 g/L) (ICMSF, 1983). The homogenate was incubated at 35-37ºC for 30 minutes as an enrichment step to recover stressed cells. Two independent homogenates were obtained for each batch at each sampling time. Following this, decimal dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were prepared with the same solution and spread into the growth media in duplicate. Gibbons medium

($MgSO_4.7H_2O$, 20 g/L; KCl, 2 g/L; trisodium citrate, 3 g/L; yeast extract, 10 g/L; casein hydrolizate, 7.5 g/L; agar, 20 g/L; Fe^{2+}, 10 ppm; Mn^{2+}, 0.1 ppm) was used for halophilic populations monitoring (Holt, 1989). Counts in medium with increasing salt concentration (NaCl 30, 50, 70, 100, 150, and 200 g/L) were performed in order to evaluate the growth ability of the microbial groups at different salt contents. Hereafter, G3, G5, G7, G10, G15, and G20 will be used to identify the Gibbons medium with 3, 5, 7, 10, 15, and 20% NaCl, respectively. Plates were incubated at 35-37ºC until growing.

Samples were also analyzed for the presence of Gram-positive/catalase-positive cocci (micrococci and staphylococci), due to the high salt tolerance of these genera. Thus, 10 g of sample were homogenized with 90 mL of sterile peptone water. Decimal dilutions were prepared and inoculated on the surface of the selective and differential medium mannitol salt agar (MSA) (Britania) at 35-37 ºC for 72 h. Two independent homogenates were obtained for each sample and plated on MSA in duplicate.

2.2.2 Determination of proteolytic, lipolytic, and TMAO-reductase activities

Representative colonies growing in the plates were selected on the base of their color, size, density, and shape, picked up and streaked on Gibbons media with 3, 5, 7, 10, 15, and 20% NaCl, depending on the NaCl concentration of the media from which they were isolated. Plates were incubated at 35-37ºC to obtain pure isolates. Then, they were transferred to Gibbons broth ($MgSO_4.7H_2O$, 20 g/L; KCl, 2 g/L; trisodium citrate, 3 g/L; yeast extract, 10 g/L; casein hydrolizate, 7.5 g/L; Fe^{2+}, 10 ppm; Mn^{2+}, 0.1 ppm) and stored at 4ºC for further analyses.

Isolated strains were analyzed for their proteolytic, lipolytic, and TMAO-reductase activities. Proteolytic activity was determined by streaking pure culture in milk agar, with the following composition: yeast extract, 3 g/L; meat peptone, 5 g/L; agar, 15 g/L. After sterilization of the mixture, 1 mL of solution of skim milk (10 % v/v) was added for each plate (FIL IDF, 1974). Clear zones around the streaks were regarded as positive reactions.

Lipolytic activity was studied in a solid medium containing tributirin (FIL IDF, 1974). The plates were inoculated and incubated at 35-37ºC. The positive reaction was indicated by clear zones around the colonies growing in the plates.

TMAO-reductase ability was determined in the following semisolid medium: meat peptone, 20 g/L; meat extract, 3 g/L; yeast extract, 3 g/L; NaCl, 150 and 200 g/L; KH_2PO_4, 4 g/L; K_2HPO_4, 5.75 g/L; $MgSO_4$, 0.5 g/L; and agar, 4 g/L (pH 6.8). After sterilization, the medium was cooled down to 45ºC, and 5 g/L of trimethylamine oxide was added (Gram et al., 1987). Rezasurin (0.001 g/L) was added to the medium as a pH indicator. The medium was fractionated into tubes, puncture inoculated, and covered with 1 mL of a mixture of vaseline and part of paraffin at a ratio 2:1. Tubes were incubated at 35-37ºC. The shift of the medium to yellow was regarded as positive to TMAO-reduction.

All media were supplemented with 150 g/L NaCl, KCl, and Mg^{2+} in order to provide the specific nutrients needed by halophilic bacteria. Analyses were done in duplicate.

2.2.3 Characterization of bacterial isolates

The cell morphology and arrangement of the isolates were determined on the basis of the Gram staining, morphology (phase contrast microscope), and motility (Holt, 1989; Mac Faddin, 1980).

Furthermore, cultures were submitted to the following biochemical tests for genera identification: cytochrome oxidase activity by spotting a loopful of culture on a disk impregnated with tetramethyil-*p*-phenylenediamine oxalate (Oxoid), catalase reaction (H_2O_2 3 % v/v), nitrate reduction (Mac Faddin, 1980), citrate utilization on Simmons citrate agar (Britania), indole production (Mac Faddin, 1980), inoculation in OF medium (Merck, Darmstadt, Germany) for determining oxidation and/or fermentation of glucose ability, and inoculation in TSI medium (Britania) which allows the investigation of the production of acid and gas from glucose, lactose, and sucrose and simultaneously the production of H_2S. The media used for biochemical tests were also supplemented with NaCl to a final concentration of 150 g/L KCl and Mg^{2+}.

All biochemical tests were carried out in duplicate.

2.3 Physicochemical analyses

Anchovy samples taken during the ripening process were analyzed for their water content by oven drying at 105 ± 1ºC until constant weight (AOAC, 1993) and salt (NaCl) content, according to the Mohr´s method (Kirk et al., 1996). The water activity was measured in the anchovy muscle by a digital hygrometer (Aqualab, model CX-2T, Decagon, Pullman, WA, USA). For pH determination, 10 g of sample were homogenized with 10 ml of distilled water, according to

AOAC (1993) and measured with a digital pH-meter (Instru® RS-232) equipped with a combined glass electrode. TVB-N was determined by direct distillation, according to the method described by Gianinni et al. (1979). Total nitrogen was determined in 0.5 g of sample by Kjeldhal (AOAC, 1993). For non-protein nitrogen (NPN) determination, 25 g of sample were treated with 25 mL of trichloroacetic 7.5%, then centrifuged at 3000 rpm and filtered. The extraction procedure was repeated once on the precipitate. An aliquot of 10 mL of the extract was analyzed for its nitrogen content by Kjeldhal (AOAC, 1993). All analyses were conducted in triplicate.

2.4 Sensory evaluation

The progress of ripening was sensory evaluated by a six-member trained panel, according to the Table proposed by Filsinger et al. (1982) in which the parameters flavor, odor, flesh color, flesh consistency, and adherence of fillets to back-bone are assessed using a 8-point quality scale (0, fresh anchovy; 8, over-ripened anchovy). The score 0 corresponds to characteristic fresh fish flavor, odor, and color, with a very elastic and damp texture and flesh adhering firmly to the backbone. The score 6 corresponds to a fully-ripened product, which presents a pink tone uniformly distributed, a ham-like flavor, a characteristic anchovy odor, and compact fillets that are easily separated from the spine. The score 8 corresponds to a product with off-flavor and odor, a dark red or black color with red or black blots, and a flimsy flesh consistency that gets torn in the filleting process.

Anchovies for sensory analysis were rinsed to remove the excess salt and dried with absorbent paper. For each sampling time, anchovies were selected at random and three-digit coded. Two panel replications were carried out on each sample.

2.5. Statistical analysis

Analysis of variance was carried out to find effects of the processing method and the time of ripening on the microbiological counts, physicochemical analyses, and sensory scores. Difference between means was analyzed using the Tukey test for post hoc comparison. Analyses were performed using STATISTICA 6.0 (Statsoft, Inc., Tulsa, OK, USA).

3. RESULTS AND DISCUSSION

3.1 Growth of bacterial groups as a function of the NaCl concentration in the culture medium

The evolution of the bacterial groups growing at different salt concentrations was examined throughout the salting-ripening process. The total counts registered in batch A, considering medium with all salt concentrations, showed an increase during the ripening process from 1.0 x 10^4 CFU g^{-1} to 6.4 10^4 CFU g^{-1}. Furthermore, the prevalence of different bacterial groups was modified during ripening. During the first month, counts were recorded only in the culture mediums with the lower NaCl concentrations. Counts at 15 and 20% NaCl were registered after 34 and 134 days of ripening, respectively, showing an increasing trend during the remaining ripening period. Additionally, the highest counts were obtained at these salt

concentrations; after 395 days of ripening, counts were 2.8 and 2.4 x 10^4 CFU g^{-1} in G15 and G20, respectively. Counts at 5, 7, and 10% NaCl were < 8.5 x 10^3 CFU/ g showing minor fluctuations during the ripening process ($p > 0.01$).

It is well known that the intermediate a_w value, high salt content, and relatively acid pH value determines the microbial activity in salted anchovy (Campello, 1985; Villar et al., 1985; Hernández-Herrero et al., 1999a). In this experiment, the water content sharply decreased during the first week of ripening from ~ 75 g 100 g^{-1} to 48.59-53.83 g 100 g^{-1}, accompanied by an increase of the NaCl content to values between 15.65 and 19.49 g 100 g^{-1} ($p < 0.01$). Thereafter, water and NaCl contents remained constant until the end of the period. In addition, a_w values dropped immediately after salting to approximately 0.75 and remained constant during the ripening stage ($p > 0.01$), creating adequate conditions for the changing of the bacterial populations. The pH also showed modifications during the first week of ripening, decreasing up to 5.60-5.75 ($p < 0.01$) (See Table 1). Taking into account these conditions, it could be inferred that counts obtained on G3, G5, G7, and G10 at the beginning of the ripening process could correspond to marine or moderate halophilic bacteria, which resist and adapt to the extreme environmental conditions but show better growth at lower salt concentrations. Throughout ripening, moderate and extreme halophilic bacteria gradually adapt to the environment while increasing the availability of nutrients due to physicochemical changes, such as proteolysis, that take place during this process. These considerations could explain the delay in registering counts at 15 and 20% NaCl, in addition to the slow growing characteristic of extreme halophilic bacteria (*archaea*) if compared to bacteria (Holt, 1989).

Similar results were obtained for batches B and C. At 15% NaCl, counts were registered after 73 days of ripening, whereas 127 days were required to obtain counts at 20% NaCl. Thereafter, counts at 15% NaCl were similar to those obtained at 20% NaCl ($p > 0.01$). Furthermore, total counts (G15 + G20) were similar in both batches, reaching a final value of 5.2 x 10^4 CFU/g in the batch processed according to the traditional method (batch B) and 6.8 x 10^4 CFU/g for batch processed as fillets (batch C). These results were analogous to those obtained in batch A, which would reaffirm the existence of a delay in the development of the halophilic microflora in this product, related to its ability to adapt to the environment. In this sense, Fuselli et al. (1998) determined that typical bacterial flora of marinated anchovy required four months to adapt and develop in the product (*E. anchoita*) (pH = 4.2).

Furthermore, the initial load of Gram-positive/catalase-positive cocci (counts in MSA) was 4.9 x 10^3 UFC/g for batch B, increasing to 4.8 x 10^4 UFC/g during the ripening process. On the other hand, no counts were initially detected in batch C. Thereafter, the number of Gram-positive/catalase-positive cocci increased from 1.5 x 10^4 UFC/g to 4.3 x 10^4 UFC/g. This difference could be related to the presence of viscera in the traditional process (batch B) that would constitute a contamination source of this type of bacterial group (Gram and Huss, 1996). Thus, the removal of viscera in fillets could eliminate much of the Gram-positive/catalase-positive cocci. After salting, the environment changed and became selective for halophilic microflora, so the development of Gram-positive/catalase-positive cocci was favored. The results obtained for counts in MSA are in line with those reported by Pons Sánchez-Cascado et al. (2005) for salted-ripened *E. encrasicholus.* The authors indicated that Gram-positive catalase-

positive cocci were the dominant group during the salting-ripening process, with a population of about 10^3 UFC/g.

According to the results obtained, the adaptation of microorganisms to the media (salted anchovy) leads to an increase in the total counts during ripening. Among them, the predominant bacterial groups corresponded to moderate and extreme halophilic bacteria which presented the best growing ability at high salt concentrations.

3.2. Cultural and biochemical characteristics of the representative colonies

In general, colonies presented different external characteristics depending on the salt concentration of the culture medium and the time of ripening. Colonies growing in G3, G5, G7, and G10 showed similar characteristics at the beginning of the ripening process. Among them, beige creamy colonies with diameter > 4 mm and wavy edges predominated. Throughout ripening, a prevalence of small (d = 1-2 mm), bright orange colonies with translucent appearance were observed on G5, G7, G10, and G15. Other colonies, colored in shades of brown and larger than the previous ones were also noticed. According to the counts obtained, this type of colony was more abundant on G15. White, flat surface, and smooth colonies were also observed growing in these media. Furthermore, small, pink and coral colored colonies developed on G20. It must be said that the descriptions given above correspond to the majority of colonies.

Colonies were classified according to the NaCl concentration of the medium from which they were isolated. Thus, colonies isolated from medium with 3 and 5% of NaCl (G3 and G5) would be classified as halotolerant or weakly moderate halophilic bacteria (Group 1), colonies growing

at 7-15% NaCl (G7, G10, and G20) would correspond to moderate halophilic bacteria (Group 2), and finally, colonies isolated from Gibbons medium with 20% NaCl (G20) would belong to the extreme halophilic bacteria group (Group 3).

As shown in Figure 1, Group 2 presented the greatest diversity of representative colonies. Comparatively, the number of strains growing on G20 (Group 3) was low, and it isolated 2 to 5 representative colonies.

According to the counts obtained and the number of representative colonies isolated from the media with different salt content, it could be inferred that the dominant microflora of the ripening of salted anchovy is represented by moderate halophilic bacteria. On the other hand, counts at 20% NaCl were obtained towards the end of the ripening process, showing a limited diversity of colonies that on the basis of their pigmentation and morphology could be classified as extreme halophilic bacteria.

Some of the isolated strains were identified on the basis of their cultural and biochemical characteristics, according to Bergey´s Manual of Systematic Bacteriology (Holt, 1989). Table 2 shows the genera identified in different samples taken throughout the salted anchovy ripening process.

The genera identified were found in different samples taken throughout the desalting-ripening process. *Salinicoccus*, *Micrococcus*, *Mesophilobacter*, and *Paracoccus* may be classified as moderate halophilic bacteria and *Marinobacter* as extreme halotolerant bacteria. These genera

have been isolated from marine sources and natural and artificial brines (Holt, 1989), therefore they could be contaminants proceeding from fresh anchovy or salt used in the process.

Previous studies in salted-ripened *E. anchoita* identified the species *Pediococcus halophilus* as the predominant bacterium in the curing process (Villar et al., 1985). However, other studies have reported greater diversity in the bacterial flora during and at the end of this process. In this sense, the presence of Gram-positive catalase-positive cocci and lactic acid bacteria was observed during salted *E. encrasicholus* ripening (Sánchez-Cascado et al., 2005). In addition, different species of extreme halophilic archaea have been isolated from this product (Félix et al., 2004 and 2007).

3.3. Proteolytic, lipolytic, and TMAO-reductase activity

A total of 82 strains classified as moderate halophilic bacteria were isolated and studied for their proteolytic and lipolytic activities. Among them, 5% showed proteolytic activity, whereas 15% presented lipolytic activity. Twenty-five per cent were proteolytic and lipolytic simultaneously. Both activities can be related to the physicochemical changes taking place during the ripening process. In this sense, an increase in the non-protein nitrogen content was determined during ripening of the three assessed batches (Figure 2), indicating proteolysis. These results are in accordance with the information given by other authors for *E. anchoita* (Filsinger et al., 1978) and *E. encrasicholus* (Durand, 1981; Hernández-Herrero et al., 1999a and 1999b; Besteiro et al., 2000). In the light of the results obtained, it could be inferred that the bacterial proteolytic activity would add to the endogenous proteolysis of this type of product, cooperating with the ripening process. In the same way, bacterial lipolysis would contribute to increased free fatty

acids and rancidity, which has been related to the development of the typical sensorial characteristics of this kind of product (Hernández-Herrero et al., 1999a; Triqui and Reineccius, 1995) and specifically with *Engraulis anchoita* products (Filsinger et al., 1982; Roldán et al., 1985; Czerner et al., 2011). However, an excess of both bacterial proteolytic and lipolytic activities would lead to development of off-flavors. In this sense, Gram and Huss (1996) determined by sensory assessment that counts of halophilic bacteria over 10^5 UFC/g imparts in this product a strong putrefaction odor. Furthermore, Félix et al. (2007) have reported the presence of deteriorating extreme halophilic bacteria in salted-ripened anchovy fillets (*E. anchoita*).

On the other hand, among 30 analyzed strains corresponding to moderate halophilic bacteria, 67% were positive to TMAO-reductase activity, whereas among 16 isolates of extreme halophilic bacteria, 25% gave positive results and 31% weak positive. TMAO is found in all marine fish species as a part of the NPN fraction. A number of specific spoilage bacteria (*Shewanella putrefaciens, Photobacterium phosphoreum,* and *Vibrionaceae)* are able to utilize TMAO as the terminal electron acceptor in an anaerobic respiration resulting in off-odors and flavors due to formation of TMA. This compound is part of the TVB-N, widely used as an indicator of fresh fish quality (Gram et al., 1987; Gram and Huss, 1996). In this work, an increasing trend of TVB-N was determined throughout ripening for the three batches studied, reaching a final value between 135.6 and 174.0 mg-100 g^{-1} (See Figure 3). These TVB-N values are elevated compared to fresh fish, but average sensory scores obtained after 395 days of ripening were 5.6, 5.1, and 4.5 for batches A, B, and C, respectively, corresponding to a semi-ripened product (Figure 4). In addition, judges did not detect off-odors in these products. The

TVB-N values obtained are in line with those previously reported for salted-ripened *E. anchoita* and *E. encrasicholus* (Filsinger et al., 1984; Hernández-Herrero et al., 1999a; Pons-Sánchez-Cascado et al., 2005). Thus, volatile bases are naturally generated during the process, contributing to the increased TVB-N value, but did not lead to off-flavor development in these products. Even though Hernández-Herrero et al. (1999a) suggested that the increase in TVB-N might be related to the growth of halophilic bacteria, no attempts have been made to verify this hypothesis. The results obtained in this work indicated that bacterial reduction of TMAO would contribute to the increased TVB-N. Moreover, taking into account that moderate halophilic bacteria are predominant during most of the ripening and show the highest percentage of strains positive for TMAO-reduction, it could be inferred that this bacterial group would be primarily responsible for TMA formation, and therefore, would have a great influence on the development of the characteristic flavor.

CONCLUSIONS

The salted anchovy (*E. anchoita*) ripening process is dominated by moderate halophilic bacteria, showing great diversity in the colony morphology. On the other hand, extreme halophilic bacteria were observed toward the end of this process.

Considering the high percentage of moderate halophilic strains that showed proteolytic, lipolytic, or both activities simultaneously (45%), a microbial contribution to the ripening process could be inferred. In addition, an elevated number of strains with the ability of reducing TMAO were observed, which could explain the increase of the TBV-N content during the ripening process. The findings obtained in this work suggest that moderate halophilic bacterial populations found

during the salted anchovy ripening process would have a positive influence on the development of the desired sensory characteristics of this product. According to the growing behavior of different halophilic bacterium groups (moderate and extreme halophiles), it seems to be a cooperation between them that gives rise, at least in part, to the changes in sensory attributes observed during the ripening process.

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Table 1. Physicochemical parameters determined on fresh and salted-ripened anchovy

		Water content	NaCl content	a_w	pH
		g/100g	g/100g		
Batch A[1]	Fresh	75.64 ± 0.23	0.23 ± 0.04	0.996 ± 0.001	6.45 ± 0.03
	Salted-ripened	48.59 ± 0.17	19.49 ± 0.38	0.746 ± 0.003	5.67 ± 0.02
Batch B[2]	Fresh	75.49 ± 0.34	0.26 ± 0.01	0.995 ± 0.001	6.03 ± 0.01
	Salted-ripened	52.65 ± 0.28	17.80 ± 0.17	0.759 ± 0.002	5.63 ± 0.02
Batch C[3]	Fresh	75.49 ± 0.34	0.26 ± 0.01	0.995 ± 0.001	6.03 ± 0.01
	Salted-ripened	53.83 ± 0.10	15.65 ± 0.07	0.749 ± 0.006	5.60 ± 0.01

Mean ± standard deviation

[1] Traditional process: brining whole anchovy, ripening H&G. Ripening pressure: 140 g/cm^2

[2] Traditional process: brining whole anchovy, ripening H&G. Ripening pressure: 80 g/cm^2

[3] Modified process: brining and ripening fillets. Ripening pressure: 80 g/cm^2

Table 2. Bacterial genera isolated during the ripening of salted anchovy (*E. anchoita*)

Characteristics					
Number of colonies isolated	6	5	12	4	5
Pigment and colony appearence	Opaque creamy white	Creamy light pink	Opaque beige-light brown to yellow. Irregular Edge	Pale pinkish white to beige	Not pigmented Translucent
Cell morphology	cocci	cocci	bacilli	Short bacilli or coccobacilli	cocci
Gram staining	+	+	-	-	-
Motility	-	-	-	+	-
Catalase reaction	+	+	+	+	+

Oxidase reaction	+	+	+	+	+
Simmons citrate	-	-	-	+	-
Growing at 7.5 % NaCl	+	+	+	+	+
Fermentation of:					
Glucose	-	-	-	-	-
Lactose	-	-	-	-	-
Sucrose	-	-	-	-	-
TSI	alcaline	alcaline	alcaline	alcaline	alcaline
OF	oxidative	fermentative	fermentative	oxidative	oxidative
Nitrate reduction	-	+	+	+	+

H_2S production	-	-	-	-	-
Indole	-	-	+	-	-
Family	*Micrococcaceae*	*Staphylococcaceae*	*Pseudomonaceae*	*Alteromonadaceae*	*Rhodobacteraceae*
Genera	***Micrococcus* spp.**	***Salinicoccus* spp.**	***Mesophilobacter* spp.**	***Marinobacter* spp.**	***Paracoccus* spp.**

Figure captions:

Fig. 1 Number of representative colonies isolated throughout ripening of Batch A, classified according the NaCl content of the culture medium. G3, G5, G7, G10, G15, and G20: Gibbons medium with 3, 5, 7, 10, 15, and 20 % NaCl, respectively.

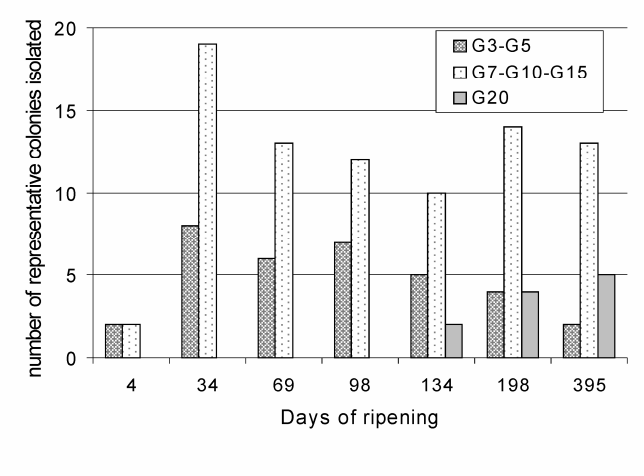

Fig. 2 Non protein nitrogen content (NPN) during salting-ripening anchovy.

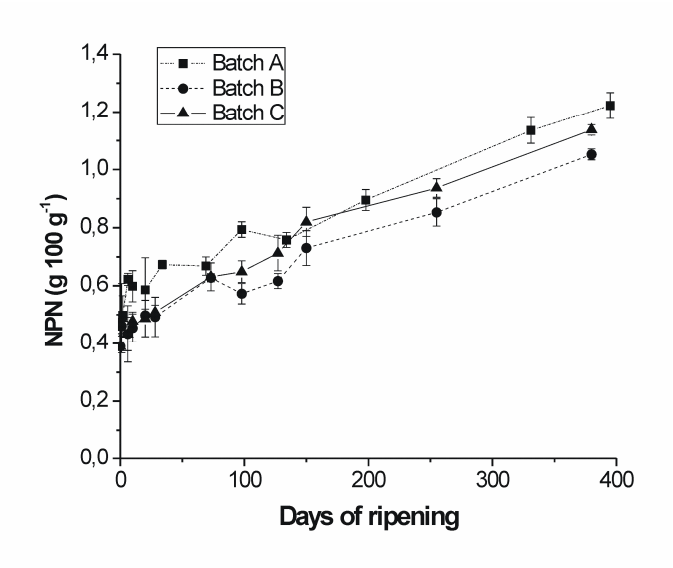

Fig. 3 Total volatile basic nitrogen content (TVB-N) during salting-ripening anchovy.

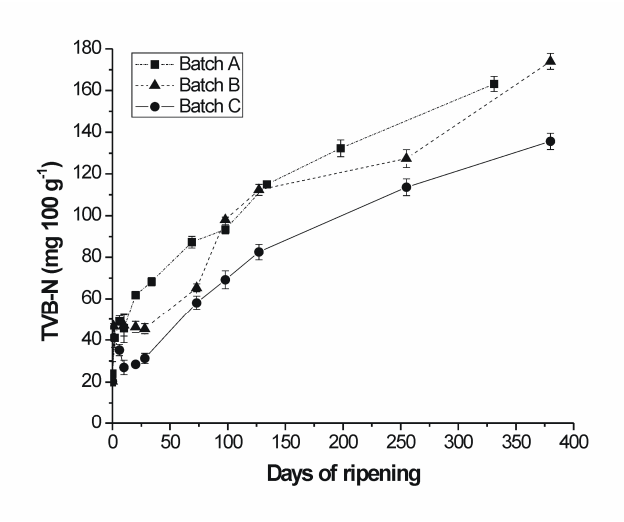

Fig. 4 Evolution of average sensory scores during salting-ripening anchovy.