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Investigation of *Halococcus morrhuae* in salted-ripened anchovy products

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Abstract

In this work, the incidence of *H. morrhuae* in salted-ripened anchovy products was assessed. Moreover, the microbial biochemical activity possible related with sensorial and physicochemical changes that takes place during the process was explored. A type strain of *H. morrhuae* (ATCC 17082) was used as reference and a detailed phenotypic characterization of the species was carried out. Samples of salted-ripened anchovy taken from barrels at the end of ripening (78) and fillets of salted-ripened anchovy (22) were analysed for microbial counts and isolation of presumptively *H. morrhuae* colonies. Cluster analysis of the biochemical tests revealed that none of the 38 isolated strains with colony morphology and cell arrangement similar to the type strain would be classified as *H. morrhuae*. However, a number of the isolated strains were very closely related with the *H. morrhuae* type strain, for which it could correspond other species of the genus *Halocuccus*. A remarkable result is that a high percentage of the isolates were proteolytic and/or lypolitic, capable to decarboxylase amino acids and produce indol and H_2S. These activities, depending on the intensity, can be directly related with the expected physicochemical changes that occur during ripening or even with quality loss and spoilage.

Introduction

Salting is one of the oldest treatments in food preservation and is still extensively used. In the presence of salt, some pelagic fish species, such as anchovy and herring, may suffer physicochemical modifications that lead to the obtention of a product called "ripened" or "matured". Salted-ripened fish products persist in the world trade and have even increased their sales over time. Belonging to this category, salted-ripened anchovy is a traditional product with typical sensorial characteristics: firm consistency, reddish colour, juicy texture and characteristic odour and flavour (Filsinger *et al*., 1982; Triqui and Zounic, 1999; Pastous Madureira *et al*., 2009; Czerner *et al*., 2011). It is produced from the species *Engraulis encrasicholus* in the southern countries of Europe and from *Engraulis anchoita* and *Engraulis ringens* in Latin American countries. Argentina is a pioneer country in the exploitation and processing of *E. anchoita* for human consumption, being salted-ripened anchovy the main product manufactured. This product is well positioned in the external market and is exported as a commodity in barrels mainly to Spain, Peru, the United States, Italy and Morocco, whereas a small amount is locally processed to supply the domestic market (Pastous Madureira *et al*., 2009; SAGPyA, 2009).

Salting-ripening involves a preliminary operation of brining, where whole fish is immersed in saturated brine reaching a first equilibrium water and salt contents in the muscle, with a water activity (a_w) value between 0.80-0.84 (Filsinger *et al.*, 1987; Yeannes, 2006). Following this, anchovies are handling beheaded and gutted, placed in barrels, alternating layers of fish and salt, and pressed reaching in the muscle a final a_w value of about 0.75 (Filsinger *et al.*, 1982). The water content in salted-ripened anchovy is between 45 and 53 g $100g^{-1}$ and the salt content varies in the range 14-18 g $100g^{-1}$ (Filsinger *et al*., 1987; Yeannes, 1996; Yeannes and Ameztoy, 1998-2005; Yeannes, 2006).

Although the high salt and the low moisture contents prevent the growing of pathogenic and common spoilage bacteria, other type of microorganisms, such as halophiles, may not be affected by its presence. In this sense, studies have shown that the microflora developing in this type of products is dominated by halotolerant and halophilic microorganisms (Campello, 1985; Villar *et al*., 1985; Yeannes, 1991; Yeannes, 1996; Vilhelmsson *et al.*, 1997; Hernández-Herrero *et al.*, 1999b; Ramirez *et al.*, 2000; Rodrigues *et al.,* 2003; Yeannes *et al,* 2006; Felix *et al*., 2006a; Czerner and Yeannes,

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2014). Furthermore, extremely halophilic archaea belonging to the Halobacteriaceae familiy are involved in salted fish products spoilage, causing the condition known as "pink" or "redding" (Ramirez *et al.* 1997; Vilhelmsson *et al.,* 1997; Ramirez *et al*., 2001; Felix *et al.*, 2006a, Ramirez, 2009). The genus Halococcus and Halobacterium are responsible of the appearance of a pink-coloured slime in salt, brines and salted fish products as well as off-odours and flavours normally associated with spoilage (hydrogen sulphide and indole) (Huss and Valdimarson, 1992; Gram and Huss, 1996). Due to their involvement in microbial spoilage at high salt concentrations, the development of these genera in salted fish may be associated to economic losses to these industries. In addition, species belonging to these genera have been associated with histamine formation (Yeannes, 2006; Ramirez, 2009).

In contrast, Aponte *et al.* (2010) reported a positive influence of the halophilic archaea *Halobacterium salinarium* and *Haloarcula marismortui* in traditional salted-ripened anchovy production, which could enhance the safety with regard to the histamine formation during ripening and improve the sensory attributes of this product. Moreover, Czerner and Yeannes (2014) found that some of the moderately and extremely halophilic strains isolated during the salting-ripening of anchovy process presented proteolytic and lipolytic activities. Concurrently, sensory descriptors stayed within the normal expected parameters, with no off-odour or flavour development. Thus, the authors concluded that bacterial proteolysis and lipolysis could be playing an important role on the development of the typical sensory characteristics of this product. However, an excessive development of bacteria with those characteristics could leads to quality losses or even the spoilage of this product.

Consequently, an adequate evaluation of salted fish contamination and the characterization of microorganism involved in their spoilage would allow to control the growth of red halophiles and to enhance the shelf life of this kind of products. In this sense, local investigations had been conducted in order to isolate and identify the characteristic extremely halophilic bacteria growing on salted-ripened *E. anchoita* produced in Argentina (Felix *et al.* 2004; Yeannes *et al.* 2005; Felix 2009). Preliminary results indicate that *Halococcus morrhuae* would be one of the species with major incidence in this product (Yeannes *et al.* 2003; Felix *et al.* 2006a; Felix *et al.* 2006b; Felix *et al*., 2011). This red haloarchea have been previously reported in salterns (Ramirez *et al.* 1997, Rodrigues *et al.* 2003; Ramirez, 2009) and also in other salted fish products (Villar *et al.*, 1985; Prasad and Seenayya, 2000; Wang *et al.* 2007). In this work a systematic investigation was effectuated to assess *H. morrhuae* in salted-ripened anchovy produced in Argentina. The two main objectives were: 1) to verify the incidence of *H. morrhuae* in salted-ripened anchovy and 2) to identify its possible participation on the sensorial and physicochemical changes that takes place during the process, by means of the assessing of microbial activities towards different substrates.

Materials and Methods

Halococcus morrhuae *type strain characterization*

A type strain of *H. morrhuae* (ATCC 17082) was used as reference in order to obtain a full phenotypic characterization of the species and thus to get an internal standard for comparative purposes. The lyophilized type strain was purchased from the Spanish Type Culture Collection (Valencia, Spain) and recovered according to the supplier instructions. The type strain was cultured in Gibbons broth plus NaCl 15% and incubated at 37ºC until exponential growth (Felix et al., 2006b). Fresh culture was plated in surface (0.1 mL) in Gibbons agar with different NaCl concentrations (0M to 6M) (ICMSF, 1983). The colony morphology, the resistance to NaCl concentration and the time needed for growing under the different conditions were monitored. Biochemical tests detailed below were carried out in order to fully characterize the type strain.

Salted-ripened anchovy sampling and culture conditions

A total of 100 samples of salted-ripened anchovy products were collected from different factories of Mar del Plata city, Argentina. Two different type of samples were assessed: salted-ripened anchovy taken directly from the barrel at the end of the ripening period (beheaded and partially gutted) (78 samples) and fillets of salted-ripened anchovy in oil (22 samples).

For extremely halophilic bacteria counts and isolation, a complex medium based on the formulation of Gibbons and his collaborators (Holt, 1989) was used. The media composition was as follows: $MgSO_4.7H_2O$, 20 g/L; KCl, 2 g/L; trisodium citrate, 3 g/L; yeast extract, 10 g/L; acid casein peptone, 7.5 g/L; agar, 20 g/L; Fe^{2+}; 10 ppm; Mn^{2+}; 0.1 ppm; NaCl, 200 g/L. This medium provides the specific nutrients needed for the growing of this type of bacteria. It has been previously employed in the study of the incidence of halophiles in salted-ripened

anchovy, showing a good performance compared to other complex medium (Yeannes *et al.*, 2003 and 2005, Felix *et al*., 2006a; Yeannes *et al.* 2011).

Aseptically, 10 g of fish flesh were excised from 6-8 different anchovies, homogenized in 90 mL of sterile salt broth (ICMSF, 1983) and serially diluted up to 10^{-4}. Then, 0.1 mL of each dilution was used to spread Gibbons agar (Holt, 1989). The plates were incubated at 37ºC during 14 days in sealed polyethylene bags. Colonies were then counted and their appearance was observed (IRAM 1988, Nº 15.139). The characteristic *H. morrhuae* red to orange pigmented colonies were picked up and purified by the conventional streaking technique on Gibbons agar to obtain pure isolates (Holt, 1989).

Phenotypic identification of pigmented halophiles bacteria

To obtain fresh cultures for identification tests, strains isolated from salted- ripened anchovy and fillets in oil were inoculated in modified Tryptic Soy Broth (TSB, Merck). According to Gibbons *et al.*, K^+ and Mg^{2+} ions are required for the growth of these bacteria; thus the TSB medium was modified by adding NaCl (200 g/L), $MgSO_4.7H_2O$ (20 g/L) and KCl (2 g/L) (Holt, 1989). Tubes were incubated at 37ºC during 14 days.

All isolates were initially tested for Gram staining according to Holt (1989) and cell morphology and arrangement were examined. Isolates corresponding to cocci Gram negative, grouped in pairs, tetrads or irregular clusters were considered as presumptive *Halococcus morrhuae* (Holt, 1989).

For biochemical characterization the following tests were carried out: motility, catalase and cytochrome oxidase reactions, hydrolysis of starch, Tween 80 (ICMSF, 1983), hydrolysis of gelatine (Mossel and Moreno García, 1985), nitrate reduction, indole production, urease, sulfhydric acid production (Triple sugar iron agar, Britania), citrate (ICMSF, 1983), fermentation of lactose, sucrose and glucose and production of hydrogen sulphide in Triple Sugar Iron medium (TSI, Britania), lysine decarboxylase and arginine dihydrolase activities (Mac Faddin, 1980). Each isolate was also tested for the ability to ferment the following carbohydrates: glucose, lactose, sucrose, galactose, fructose, maltose, mannitol, sorbitol and trehalose (Gibbs and Skinner, 1966). Their proteolytic and lipolytic characteristics were determined by plating in milk and tributirin agar, respectively (F.I.L-I.D.F 73, 1974). All the above mentioned tests were carried out by incubating the cultures at 37ºC. NaCl (200 g/L), $MgSO_4.7H_2O$ (20 g/L) and KCl (2 g/L) were added to the corresponding mediums.

Analysis of phenotypic data

The software Statistica version 5.1 (Statsoft, Inc., Tulsa, United Estates) was used to analyse and cluster data based on 23 biochemical tests for all isolates. The Euclidean distance was used as similarity measure with the un-weighted pair group method arithmetic averages clustering algorithm. The type strain of *Halococcus morrhuae* was included as reference (ATCC 17082).

Results and Discussion

Description of the growing characteristics of the H. morrhuae *type strain*

The growing characteristics of the type strain (ATCC 17082) were assessed in our laboratory. This characterization established an easy comparative parameter under well-defined experimental conditions. The optimal growing conditions were determined specifically for this species and therefore used for the investigation of its presence in salted-ripened anchovy samples. Moreover, it allowed identifying the *H. morrhuae* colony morphology and the growing behaviour and, posteriorly, uses it for comparison with strains isolated from anchovy samples.

Plates with Gibbons agar were inoculated with the reference strain (ATCC 17082), as detailed in the Materials and Methods Section, and observed daily during the whole incubation period (14 days). Results showed in Figure 1 correspond to the time needed to observe the characteristic *H. morrhuae* red to orange pigmented colonies. Initially colonies appear translucent and the characteristic pigment was progressively acquired during incubation. The shortest growing time was observed at NaCl 2.5 and 3.4 M (150 and 200 g/L NaCl), therefore these salt concentrations was considered the optima for its growth. Moreover, the colonies morphology on plates was circular, convex, with entire margin, rough and opaque, presenting an intense red to orange color which facilitates the visualization and the microbial count. The growth of *H. morrhuae* observed in Gibbons agar with NaCl 1.5 and 4.3 M required more days than in the aforementioned salt concentrations, being observed after seven days at 37ºC. For 4.3 M and higher salt concentrations a reduction of the number of colonies growing in plates was observed concomitant with longer time for development of characteristic colonies. Below 1.5 M, no colonies development was observed.

Although Bergey´s Manual of Systematic

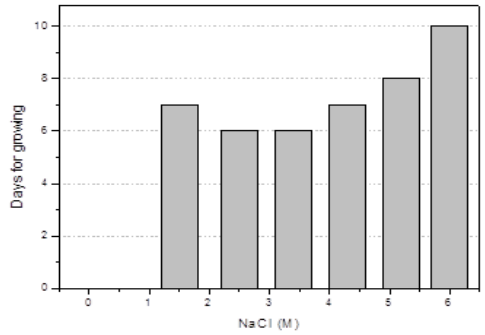

Figure1. Growing time for the *Halococcus morrhuae* type strain (ATCC 17082) in Gibbons medium with different NaCl concentration

Bacteriology describes the composition of the Gibbons medium and incubation temperature, no specific information is given about the incubation period (Holt, 1989). Thus, in this paper it was taken as reference the Norm IRAM 15139 (IRAM, 1988) used by national control laboratories, which establishes an incubation of 14 days at 37°C in a culture media for halophilic bacteria. The findings arisen from this study had permitted the establishment of the specific incubation period for the type strain under the laboratory conditions, which resulted highly dependent on the NaCl media concentration.

The optimum NaCl content determined for the type strain in vitro agrees with the salt levels reached in salted-ripened anchovy, which could allow the development of extreme halophiles in this product. These results are consistent with those emerging from the salt tolerance tests carried out on salted-ripened anchovy and fillets in oil (Czerner and Yeannes 2011).

Exploration of the presence of Halococcus morrhuae *in salted-ripened anchovy*

A total of 97 pigmented strains with colony morphology similar to *H. morrhuae* were isolated from salted-ripened anchovy and fillets of anchovy in oil. Among them, 65 strains were Gram negative cocci, but only 38 strains isolated from anchovy samples taken from the barrels (H&G) showed the *H. morrhuae* characteristic cellular morphology and cell arrangement. Of the remaining, 22 strains were Gram negative rod-shaped and 10 strains, Gram negative cocobacillus.

The phenotypic characteristics were used to cluster the 38 isolated strains and are shown as a dendogram (Figure 2). The isolates were clustered at a linkage distance 2.1 into two major groups (A and B), two pairs (Group C: 211-10, 3A; Group D: 1025-3, S25/311) and one single strain (511) (Group E). Group A consisted of 8 strains (5I, MP3II, MP7II, DS3, LID2II, 4, 15II, DS1) associated with the

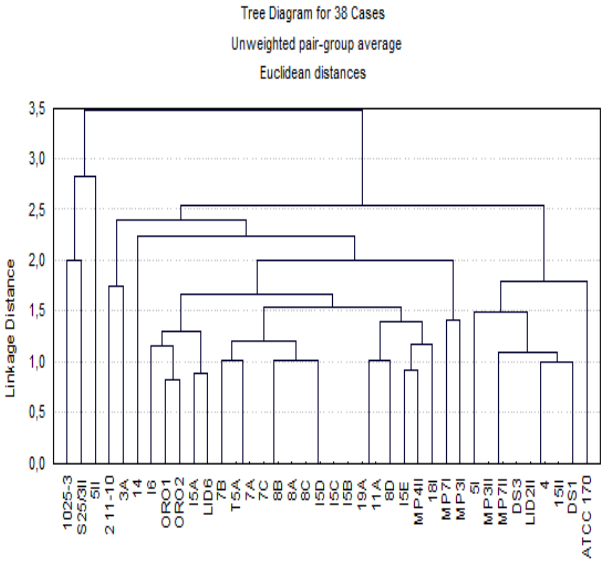

Figure 2. Dendogram showing the hierarchical clustering of the isolated red halophilic gram negative cocci bacterial strains, based on their phenotypic characteristics

reference strain ATCC 17082 at a very short linkage distance. Thus, these strains were very similar to the ATCC 17082 whereby it could be inferred that belong to genus *Halococcus* but not to the species *Halococcus morrhuae*. Table 1 shows the biochemical properties that distinct the strains from *H. morrhuae.* Unlike *H. morrhuae*, all the strains were not capable to utilize citrate as a carbon source and to produce the enzyme lysine decarboxylase. Other biochemical characteristics that differed from the type strain were the indole production and the proteolytic and lipolytic activities. As *H. morrhuae*, all the strains were non-mobile; it had catalase, oxidase, urease and arginine dihydrolase activity. It could not use glucose, lactose, sucrose, galactose, fructose, maltose, mannitol, sorbitol and trehalose as sole carbon sources. They were capable to hydrolyze starch and Tween 80, to reduce nitrate and to produce H_2S from thiosulfate.

Recently, novel species belonging to the *Halococcus* genus have been described. Between them, *H. qingdaonensis* (Wang *et al*., 2007), *H. hamelinensis* (Goh *et al.*, 2006) and *H. thailandensis* (Namwong *et al*., 2007) were isolated from hyper saline environments as sea-salt and fish sauce and classified as a novel species. Therefore, molecular studies would be necessary to identify the isolates obtained in this paper or determine if it belongs to novel species.

Cluster B, which contained 23 isolates, could be subdivided into five subgroups (B1 to B5): subgroup B1: I6, ORO1, ORO2, I5A, LID6; subgroup B2: 7B, T5A, 7A, 7C, 8B, 8A, 8C, I5D; subgroup B3: I5C, I5B, 19A; subgroup B4: 11A, 8E, I5E, MP4II, 18I; subgroup B5: MP7I, MP3I. Subgroup B1

Table 1. Biochemical characteristics that differentiate strains grouped in Cluster A from type strain of *Halococcus morrhuae* (ATCC 17082)

Characteristic	DS1	DS3	MP7II	15II	4	5I	MP3II	LID2II	ATCC 17082
Indole production	0	1	1	0	0	0	1	0	0
Proteolityc	1	0	0	0	0	1	0	0	1
Lipolitic	0	0	0	0	0	1	0	0	0
Lysine	1	1	1	1	1	1	1	1	0
Citrate	0	0	0	0	0	0	0	0	1

0: negative reaction; 1: positive reaction

yielded negative results for nitrate reduction and Tween 80 hydrolysis and was uniquely positive for acid production from glucose. Subgroups B2 and B4 showed negative reaction to H_2S production and nitrate reduction. The last subgroup mentioned did not present Tween 80 hydrolysis. Subgroup B3 did not hydrolyze Tween 80 or reduce nitrate and additionally did no presented lysine decarboxylase and arginine dihydrolase activities. Finally, strains corresponding to subgroup B5 were the only positive for motility.

A high percentage of the isolated strains showed metabolic characteristics that might contribute to spoilage of salted anchovy products. Twenty nine percent of the isolated strains showed proteolytic activity and 10.5% presented lipolytic activity. Even though these activities are related to the positive physicochemical changes that take place during the ripening process (Czerner and Yeannes, 2014), an excess would lead to development of off-flavors. Lipolysis generates free fatty acids that can be readily oxidized and produce rancidity whereas non-protein nitrogen produced by proteolysis would contribute to increase TVB-N content (Huss, 1995) or provide substrate for biogenic amines formation. It is noticeable the high percent of strains showing indole (18.4%) and hydrogen sulphide production (52.6%) which are compounds produced from the degradation of proteins and sulphur containing amino-acids and are directly responsibly of off-odours development (Huss, 1995). For its part, decarboxylase activity of bacteria is not only a spoilage topic, but also an important safety one. For example, putrescine and cadaverine, which are indicators of fish spoilage (Mietz and Karmas, 1977, Rodriguez *et al.*, 2003) and are produced by the activity of the enzymes ornithine decarboxylase and lysine decarboxylase, have been identified as potentiators that enhance the toxicity of histamine to humans by depressing histamine oxidation (Halasz *et al*., 1994). Among the isolated strains, 62% were positive for arginine and lysine decarboxylase activity.

Conclusions

Based on the analysis of the phenotypic characteristics, the species *H. morrhuae* has not been found in the salted-ripened anchovy samples analyzed. However, a number of the isolated strains were closely related with the *H. morrhuae* type strain, for which it could correspond other species of the genus *Halocuccus*. Further studies are in progress in order to accurately identify those isolates.

An important percentage of the isolates showed *in vitro* metabolic characteristics directly related to the physicochemical changes that takes place during the ripening process. The results obtained indicate that the extremely halophilic bacteria isolated in this study could play an active role on the ripening process or even contribute with spoilage. This fact reaffirms the idea that a right balance of the microflora is necessary for the obtention of a product with the desired sensorial characteristics and the expected shelf-life.

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