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ORIGINAL PAPER

New insights into halophilic prokaryotes isolated from salting–ripening anchovies (*Engraulis anchoita*) process focused on histamine-degrading strains

Silvina Perez[1,2,3] · Silvia Elena Murialdo[2,4,5] · Irene Mabel Ameztoy[1,3] · Noemí Elisabet Zaritzky[6,7] · María Isabel Yeannes[1,2,3]

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Abstract

Salted and ripened fish foods are susceptible to cause histamine poisoning. The present study focuses on microbial histamine degradation from high salted fermented fishery products to deepen our understanding about this new and growing field of research. As a result of this first study related to salted–ripened anchovies (*Engraulis anchoita*), fifty seven moderate and extreme halophilic microbial isolates from salt and salted–ripened anchovy processes were characterized in terms of their phenotype and histamine-degrading capacity. Only 7%—4 isolates—were able to degrade histamine. None of the histamine-degrading isolates presented proteolytic and/or lipolytic activity. One of them designated A18 was chemotactic toward histamine, an interesting property not previously reported for that chemoattractant. However, the S18 and A18 isolates, genotypically identified as *Halobacterium* sp. and *Halomonas* sp. respectively, produced indole and/or H_2S, both undesirable characteristics associated to off-flavors occurrence. On the other hand, A28 and S20, identified as *Halovibrio* sp. and *Halobacterium* sp. respectively, presented desirable properties, such as cytochrome oxidase and catalase activity, and non-production of H_2S and indole. These strains also showed characteristics previously reported as dominant in the ripened stage. The results are promising, and A28 and S20 may have the desirable features to improve the anchovy salting–ripening process.

Keywords Salted and ripened anchovy · Halophilic prokaryotes · Phenotypic characterization · Chemotaxis · Histamine · 16S rRNA identification

Introduction

The salting and ripening process is a traditional practice used in Europe to preserve different fish species, which was later adopted in Latin-American countries using *Engraulis anchoita*. Like other naturally fermented foods, the process involves different microorganisms with dissimilar metabolic mechanisms in order to modify flavor and other sensorial characteristics that require the participation of several and diverse enzymatic activities (Campello 1985; Besteiro et al. 2000; Zgomba Maksimovic et al. 2018). Salted–ripened anchovy is preserved due to a high NaCl content (14–20%

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✉ Silvina Perez
silvinaperez@fi.mdp.edu.ar

[1] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Juan B. Justo 4302, 7600 Mar del Plata, Argentina

[2] Dpto. de Ingeniería Química y en Alimentos, Facultad de Ingeniería, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

[3] Grupo de Investigación Preservación y Calidad de Alimentos (GIPCAL), Facultad de Ingeniería, INCITAA, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

[4] Comisión de Investigaciones Científicas de La Prov. de Buenos Aires (CIC), Buenos Aires, Argentina

[5] Grupo de Ingeniería Bioquímica (GIB), Facultad de Ingeniería, INCITAA, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

[6] Dpto. de Ingeniería Química, Facultad de Ingeniería, Universidad Nacional de La Plata, La Plata, Argentina

[7] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, UNLP-CONICET-CIC, Mar del Plata, Argentina

w/w) and low water activity (a_w ~ 0.75). These conditions prevent growth of typical pathogenic and spoilage bacteria (Galdiero et al. 1997; Czerner and Yeannes 2014). The salting–ripening process of anchovies leads to the growth of a great diversity of halophilic or halotolerant indigenous microbiota capable of surviving and growing in hypersaline environments (Hernández-Herrero et al. 1999; Aponte et al. 2010; Czerner and Yeannes 2014; Felix et al. 2016; Perez et al. 2018).

However, salted and ripened anchovies are susceptible to the risk of containing histamine at different concentrations. Histamine is a biogenic amine which occurs endogenously in the human body and plays important physiological functions. Despite this, the ingestion of foods containing high levels of histamine can cause allergy-like food poisoning. This is among the most common seafood-related foodborne illnesses, due to the consumption of fish containing high levels of histamine, which in presence of other biogenic amines (e.g., putrescine and cadaverine) can potentiate histamine's toxic effect. The following guideline for histamine content of fish has been suggested: below 50 mg/kg is considered safe, between 50–200 mg/kg possibly toxic, 200–1000 mg/kg probably toxic, and greater than 1000 mg/kg toxic and unsafe for human consumption (Yeannes 1996; Lehane and Olley 2000). The histamine content in fish and fish products is regulated, and diverse sampling scheme and sanitary limits were established in different countries. The mean limit value of histamine for fishery products had been fixed at 50, 100, and 200 mg/kg in the USA, Argentina and the EU, respectively (FDA 2011; EC 2013; SENASA 2018). It should be noted that high levels of histamine in fish are not detectable by sensory spoilage indicators (Lehane and Olley 2000); besides, histamine is a thermally stable substance. Notably, the fermentation process may provide necessary conditions for formation of biogenic amines. Endogenous proteolytic enzymes and halophilic microorganism proteases increase levels of peptides and free amino acids in the muscle during ripening, enabling the rise of free histidine content which is the substrate for the histamine generation (Pons-Sánchez-Cascado et al. 2003; Rodrigues et al. 2003). In salted–ripened anchovy, our group (Ramirez et al. 1994) determined, for the first time, the presence of extreme halophilic bacteria with histidine decarboxylase activity, so histamine could be formed if conditions are given to allow microbial growth and the consequent enzymatic expression.

Biotechnological potential of halophilic microorganisms is under constant study (Margesin and Schinner 2001; Nercessian et al. 2015). Also, alternative methods to maintain low levels of histamine are of particular interest. In salted products, Alfonzo et al. (2017) and Kuda et al. (2012) explored starter cultures with suppressive effect on histamine accumulation. In the past, Paredi and Yeannes (1987) observed important decreases in histamine levels over time, which leads to inferring the presence of histamine-degrading microorganisms. More recently, few investigations were performed to study microbial histamine degradation in different high salted fermented fishery products (Tapingkae et al. 2010b; Zaman et al. 2014; Lee et al. 2016); however, there are no related studies about salted–ripened anchovies.

Microbial motility is associated with biofilm formation and acceleration of degradation compounds (Dang and Lovell 2016). Particularly, chemotaxis is the movement toward or away chemical concentration gradients (Paul et al. 2006; Murialdo et al. 2009). This characteristic has been reported as a competitive advantage, since chemotactic microorganisms have a sensory-response system that allow them to detect concentration gradients of certain chemical substances, and navigate toward more favorable areas finding optimal conditions for their growth and survival (Paul et al. 2006; Armitano et al. 2011). It is necessary, therefore, to analyze the presence of chemotaxis-guided bacterial movements toward histamine, which could influence the microbial attachment rate to (food-processing) surfaces, and enable bacteria to survive, grow, and improve the degradation kinetics of the toxic compound. However, bacterial chemotaxis toward histamine had been poorly studied and was only reported in relation to pathogenicity (Corral-Lugo et al. 2018).

Although the presence of histamine-degrading strains would be beneficial for the food safety of the salting–ripening anchovies, its possible effect on the sensory characteristics is also of great importance. In this sense, the process of typical preparations, based on indigenous microbiota from food products, could be standardized by the adequate selection of autochthonous starter cultures, because they are more competitive, well adapted, and with high metabolic capacities to beneficially impact quality and safety while preserving their sensory attributes (Margesin and Schinner 2001; Fontana et al. 2016; Zgomba Maksimovic et al. 2018).

On this basis, the present study was undertaken to assess autochthonous halophilic prokaryotic organisms (bacteria and/or archaea) isolated from the salting–ripening anchovy process. Relevant metabolic capacities, technological characteristics, possibility to degrade histamine, and chemotactic responses toward this compound were evaluated in order to find microorganisms with biotechnological potential and maintain sensorial quality and safety of the product.

Materials and methods

Selection of halophilic microbial cultures

Halophilic cultures were isolated from salt and manufactured anchovy (*Engraulis anchoita*) samples of salted–ripened processes performed in Mar del Plata industries for

selecting colonies with different enzymatic activities. Salt samples were taken from two food-grade commercial semi-coarse salts from a solar saltern located in the province of Rio Negro (Argentina) used for the salted–ripened anchovy manufacture. Whole specimens from the wet salting stage, beheaded and partially gutted salted–ripened anchovies were obtained from local factories (Mar del Plata, Argentina). Ripened fillets in oil were purchased from a local market. All samples were immediately analyzed in duplicate according to the procedure reported by Perez et al. (2018). Saline broth homogenates (NaCl, 150 g/L; meat peptone, 3 g/L; meat extract, 3 g/L) were prepared in duplicate, followed by enrichment steps by incubation at 35–37 °C for 30 min; then successive serial dilutions were performed (ICMSF 1983). Homogenates (0.1 mL) were spread onto the growth media ($MgSO_4.7H_2O$, 20 g/L; KCl, 2 g/L; trisodium citrate, 3 g/L; yeast extract, 10 g/L; casein hydrolysate, 7.5 g/L; agar, 20 g/L; Fe^{2+}, 10 mg/L; Mn^{2+}, 0.1 mg/L) supplemented with 150 and 200 g/L of NaCl (Sehgal and Gibbons 1960) in duplicate and incubated at 35–37 °C during 21 days. Colonies with different macroscopic characteristics (color, size, shape, and density) were re-streaked on fresh agar plates and incubated at 35–37 °C until growth. Pure isolates were transferred to growth broth ($MgSO_4.7H_2O$, 20 g/L; KCl, 2 g/L, trisodium citrate, 3 g/L; yeast extract, 10 g/L; casein hydrolysate, 7.5 g/L; Fe^{2+}, 10 mg/L; Mn^{2+}, 0.1 mg/L) with 150 or 200 g/L NaCl. Colony stocks were kept at 4 °C for further analyses.

Characterization of selected halophilic microorganisms

Cultures were submitted to several tests for their characterization, including those microbial properties which could exert an influence on the ripening process.

Cell morphology was distinguished on the basis of Gram staining (optic microscope), and the following biochemical tests were carried out: (a) cytochrome oxidase activity was confirmed by spotting a loopful of culture onto a disk impregnated with tetramethyl-*p*-phenylenediamine oxalate (Oxoid); (b) catalase reaction (H_2O_2, 3% v/v); (c) motility; (d) nitrate reduction; (e) citrate utilization was tested on Simmons´ citrate agar (Britania); (f) carbohydrate fermentation test for glucose; (g) hydrogen sulfide (H_2S) production was verified by inoculation on TSI medium (Britania); and (h) indole production was tested employing peptone broth and Kovacs's assay (MacFaddin 1980).

Proteolytic and lipolytic activities were evaluated by streaking pure cultures on milk agar (yeast extract, 3 g/L; meat peptone, 5 g/L; agar, 15 g/L; milk, 10 ml/L) and on a solid medium containing tributirin, respectively. Plates were incubated at 35–37 °C for 10 days. Positive activities were indicated by clear zones around the streaks (FIL IDF 73 1974).

The ability to produce histamine by decarboxylation of its precursor (histidine) was qualitatively examined through the histidine decarboxylase test. Cultures were inoculated on a slanting surface of a solidified selective medium (tryptone, 5 g/L; yeast extract, 5 g/L; L-histidine, 27 g/L; $CaCO_3$, 1 g/L; agar, 20 g/L; bromocresol purple, 0.06 g/L; pH 5.3), and incubated at 35–37 °C during 10 days. Colonies that produced a turn of the medium toward violet were considered positive (Niven et al. 1981; Ramirez et al. 1994).

The culture media used for previous tests were supplemented with NaCl to a final concentration of 150 or 200 g/L (similar to the place of isolation), and with K^+ (10 mg/L) and Mg^{2+} (0.1 mg/L) in order to provide the specific nutrients needed by halophilic microorganisms (Sehgal and Gibbons 1960).

Capacity to perform chemotactic movement was tested by dotting pure cultures on swarming plates. Soft agar was composed of agar (4 g/L), mineral salts (NaCl, 175 g/L; $MgSO_4.7H_2O$, 20 g/L; KCl, 5 g/L; CaCl ($6H_2O$), 0.2 g/L), and tryptone (0.025% w/v) and yeast extract (0.025% w/v) as substrates. Plates were incubated at 35 °C and monitored for 10 days. Positive chemotactic response was confirmed when isolates formed a concentric ring around the inoculation point which spread along the surface to the edges of the plates following the concentration gradient generated by consumption (Adler 1966; Wolfe and Berg 1989; Englert et al. 2009).

The salt concentration range for microbial growth was determined by inoculating growth media with increasing levels of NaCl (0, 1, 5, 7.5, 10, 15, 17.5, 20, and 25% w/v), and subsequent incubation at 35–37 °C. Growth of a microorganism indicated a positive result (Sehgal and Gibbons 1960; ICMSF 1983). All analyses were carried out in duplicate.

Histamine-degrading isolates

In order to identify histamine-degrading isolates, stock cultures were resuspended in 5 mL of halophilic broth (NaCl, 175 g/L; tryptone, 5 g/L; $MgSO_4.7H_2O$, 20 g/L; yeast extract, 4 g/L; KCl, 5 g/L; $CaCl.6H_2O$, 0,2 g/L; pH 7.0–7.2) (IRAM 1988) and incubated at 25 °C under orbital agitation (160 rpm) until growth was observed. Then, as a step of microorganism acclimation to the histamine presence, histamine dihydrochloride (Sigma–Aldrich) was added up to a histamine concentration of ~ 800 mg/L, and maintained at 25 °C for 7 days (160 rpm). Acclimated cultures were inoculated (Britannia calibrated loop) in 5 ml of halophilic broth with histamine concentration of ~ 800 mg/L and incubated at 25 °C for 14 days (160 rpm). Subsequently, 3 mL samples were taken and an equal amount of HCl (1 M) was

added (Tapingkae et al. 2010b; Zaman et al. 2014) to stop the reaction, the suspension was centrifuged at 9000 g for 10 min, and supernatants were kept at – 20 °C until histamine was analyzed. The same procedure was performed without bacterial inoculum ("control"). Analyses were done in duplicate.

Histamine quantification

Supernatants were analyzed in order to quantify the histamine content by high performance liquid chromatography (HPLC). For this purpose, 5 g of sample was transferred to a 50 ml graduated tube and brought to volume with methanol:water solution (70:30, v/v). It was homogenized, centrifuged, filtered through Whatman paper N°91 and syringe filters (pore size 0–0.22 µm), and placed in vials for injection. The analyte was quantified on an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) with multiple wavelength Diode Array Detector (G1315D) and C18 column (150 mm, 5 µm, Zorbax SB-C18). The mobile phase was made by combining 87% v/v solvents A (KH_2PO_4, 3.26 g/L; K_2HPO_4; 2.86 g/L; sodium decanesulfonate, 0.48 g/L; pH 6.6) and 13% v/v B (acetonitrile, HPLC grade). The flow rate, injection volume, column temperature, and wavelength detection were 1 mL/min, 20 µL, 35 °C, and 215 nm, respectively. Analyses were done in duplicate.

Statistical analysis

Histamine quantification results were analyzed by one-factor ANOVA (microbial strain) with a significance level of 5%. The Tukey test was applied for comparison of means with a significance level of 5%. The R project software (R Developer Core Team 2008) was used.

Histamine degradation

The histamine degradation percentage was estimated in cultures that presented histamine degradation capacity ($p < 0.05$). It was expressed as percent degradation in the supernatant, where the quantification of the biogenic amine was performed. It was calculated as follows:

$$D(\%) = \frac{X_c^H - X_m^H}{X_c^H} \cdot 100.$$

Where X_c^H is the content of histamine (mg/L) in the supernatant of the negative control (without microbial inoculum); X_m^H is the content of histamine (mg/L) in the supernatant of samples treated with microbial inoculants.

Chemotaxis toward histamine of histamine-degrading strains

Histamine-degrading strains were screened for their ability to sense and respond behaviorally toward histamine as a possible chemoattractant as previously described. Soft agar contained agar (4 g/L), mineral salts, and histamine as substrate (0.5 g/L). A negative control was included without this additional nutrient. Swarming plates were incubated at 35 °C and formation of a concentric ring around the inoculation point was monitored during 10 days (Adler 1966; Wolfe and Berg 1989; Englert et al. 2009).

Molecular characterization of histamine-degrading strains

Bacterial and archaeal molecular identification of histamine-degrading strains were carried out based on 16S rRNA gene sequence analysis as detailed in (Table 1). Isolated colonies were sent to Macrogen (South Korea) for identification

Table 1 Bacterial and archaeal identification. The columns from left to right indicate: method of strain identification, Taq polymerase used, PCR primers, and amplification programs

Procedure	Taq polymerase	Primers	Program		
			T (°C)	Time	Cycles
Bacterial identification	EF-Taq (SolGent, Korea)	27F 5′ (AGA GTT TGA TCM TGG CTC AG) 3'	95	2 min	1
			95	1 min	35
			55	1 min	
		1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3'	72	1 min	
			72	10 min	1
Archaeal identification	Dr. MAX DNA Polymerase (Doctor protein INC, Korea)	21F 5′ (TTCCGG TTGATCCTG CCGGA) 3'	94	5 min	1
			94	30 s	35
			Variable	20 s	
		1492R 5′ (TAC GGY TAC CTT GTT ACG ACT T) 3'	72	40 s	
			72	7 min	1

analysis. Genomic DNA samples were extracted using a QIAamp DNA mini kit (QIAgen, Germany). The PCR reaction was performed with 20 ng of genomic DNA (the template) in a 30 µL reaction mixture (primers and Taq polymerase) in a DNA Engine Tetrad two Peltier Thermal Cycler (BIO-RAD). PCR products were purified, Sanger-sequenced with the BigDye terminator v3.1 sequencing kit, added to Hi-Di formamide (Applied Biosystems, Foster City, CA) (incubation at 95 °C for 5 min and 5 min on ice), and analyzed by 3730XL automated sequencer (Applied Biosystems, Foster City, CA). The 16S rRNA gene sequences were examined and checked for quality using EzBioCloud (NCBI databasewww.ezbiocloud.net/identify) to determine their taxonomic affiliation (Yoon et al. 2017). Sequences with the lowest cut-off values (e-value) were selected as the isolates phylogenetically most closely related to the analyzed isolate. Although the 16S rRNA genes were partial sequences, a high level of confidence was established at the genus level (Clote and Backofen 2000).

Results and discussion

Technological and safety characteristics of selected halophilic cultures

Fifty-seven halophilic colonies were selected based on macroscopic characteristics (color, size, shape, and density): thirty were obtained from salt samples, seventeen from whole fish specimens sampled during the wet salting stage, and ten from salted–ripened anchovies (beheaded anchovies and fillets in oil). Most microorganisms were rod and coccoid shaped (47.4 and 43.9%, respectively) and a low proportion was disc shaped (8.8%). About 95% of the isolates were Gram -stain negative, and 47% were motile cells as shown by motility assay in tubes with semisolid agar medium. Seventy-three percent reduced nitrate, 36% fermented glucose and only 8% utilized citrate. These tests contribute to our knowledge of the metabolic diversity of the microbial ecosystem, which may contribute to the management of the product for improving its characteristics without altering other desired properties.

The majority of the cultures tested positive for cytochrome oxidase activity (86%) and catalase reaction (88%). These capacities are considered as important functional properties of microbial starter cultures for meat fermentation. The catalase enzyme has antioxidant properties because it catalyzes decomposition of hydrogen peroxide, a reactive form of oxygen involved in unsaturated fatty acids oxidations. Together with cytochrome oxidase activity, this would promote oxygen reduction present inside the fermentation barrel, preventing rancidity (Geisen et al. 1992; Perez et al. 2018). Also, 38% of the isolates possessed lipolytic activity and 30% proteolytic activity. Proteolysis in salted fish is related to texture, and a collaborative role of endogenous fish enzymes and microbial enzymes in the process has been proposed, where the former release peptides that are substrates for the latter (Czerner and Yeannes 2014). Moreover, microbial lipolysis would promote the production of free fatty acids during ripening, affecting flavor development (Triqui and Reineccius 1995). It must be considered that these activities in excess could lead to quality loss or spoilage (Czerner and Yeannes 2014).

In relation to chemotactic navigation capacity, different responses were observed as shown in (Fig. 1): non-chemotactic response (I), where only visible growth was observed with the naked eye at the inoculation point, and chemotactic response (II–V), when a concentric ring around the inoculation point given by net displacement over time was exhibited. In 56% of the isolates, this response was consistent with the characteristic chemotactic movement (Murialdo et al. 2009). Chemotaxis has been poorly studied in this context. However, evaluating chemotactic response of microorganisms is of great interest since this property helps microorganisms, under the right conditions, to find optimal conditions for their growth and survival (Paul et al. 2006).

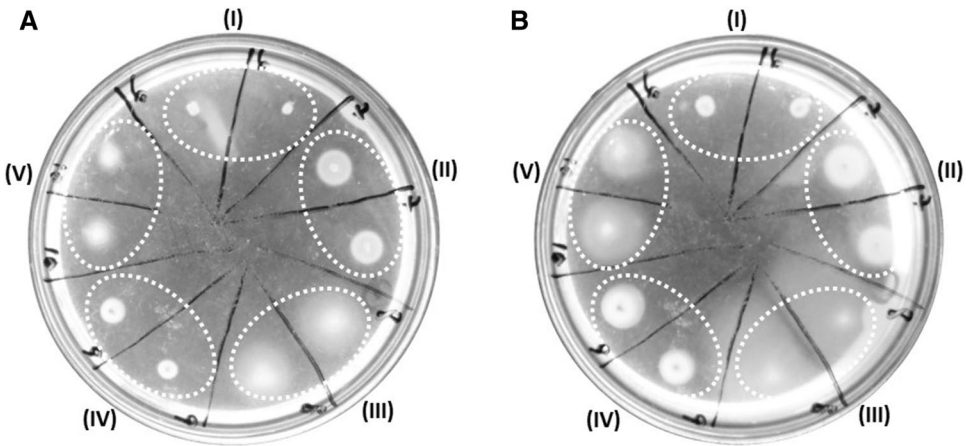

Fig. 1 Different responses of isolates on swarming plate at 24 and 48 h, A and B, respectively. I: no-chemotactic. II, III, IV, and V: characteristic rings of chemotactic cells

Unexpectedly, more strains showed characteristic rings of chemotactic movement in the Petri dishes than those that were positive in the traditional tube motility test. This could be attributed to differences in culture media composition, to the texture of the support, and to oxygen availability (Morales-Soto et al. 2015; Hölscher et al. 2015). Perhaps, the gelation in a small vessel (tube) had a sudden cooling rate as compared to agar plates. A slow and constant cooling rate provides uniform gel particles, while abrupt cooling leads to microcrystals that are heterogeneous in shape and size, affecting the homogeneity of nutrient distribution and oxygen availability. A reduced contact area between air and support in the tubes compared to plates could affect oxygen availability (Blanch and Clark 1996). This opens new paths to further studies by associating chemotactic response with medium composition and aerophilic behavior.

Regarding the salt requirements (Fig. 2), it was observed that the highest microbial growth was achieved at a NaCl range of 17.5–20% w/v, while in the presence of smaller amounts of NaCl, the number of microorganisms decreased progressively. The population decreased more sharply at 25% w/v of NaCl. Only 6% of the isolates grew without the presence of salt (NaCl), showing that they were halotolerant, and remaining cultures were obligate halophiles (DasSarma and DasSarma 2012). Moderate and extreme halophiles were predominantly found, as expected in accordance to the characteristics of the samples from which the isolates were derived: salt samples and anchovies with a high salt content and low a_w (Czerner and Yeannes 2014; Felix et al. 2016). The salt requirement is an important aspect to take into account for the selection of the most proper microorganisms for the process and product under study. It must be considered that the NaCl content of the product is 14–20% w/w and its a_w is ~ 0.75 (Czerner and Yeannes 2014), so that only microorganisms adapted to these conditions can develop.

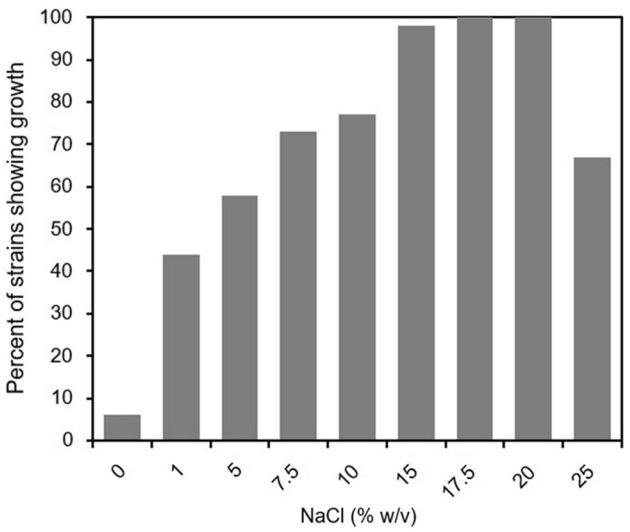

Fig. 2 Microbial salt requirement: percentage of isolates that grew at different NaCl content (% w/v) in the culture medium

Many isolates were capable of production of hydrogen sulfide (H_2S) and indole (35 and 60%, respectively). Both characteristics are undesirable because of their implication on off-odors development and consequent spoilage of salted fish products (Huss and Valdimarsson 1990). Regarding the food safety, 46% of the isolates resulted positive in the histidine decarboxylase test, which means that they could form histamine under suitable conditions, being a strictly undesirable property (Ramirez et al. 1994; Fontana et al. 2016).

Histamine-degrading halophilic isolates and their characterization

The fifty-seven halophilic cultures were analyzed for their histamine degrading capacity, using a halophilic broth rich in specific nutrients for halophilic microbial growth (Sehgal and Gibbons 1960; IRAM 1988), a NaCl content of 17.5% w/v based on the salt content of salted–ripened anchovy (Czerner and Yeannes 2014), and a histamine concentration of 800 mg/L to evaluate the ability of the isolates to grow in the presence of high histamine contents.

Significant differences ($p<0.05$) in final histamine contents for different isolates were found. As shown in (Table 2), isolates S18, S20, A18, and A28 caused a significantly higher ($p<0.05$) decrease in histamine compared to the control without microbial inoculation. These cultures showed degradation between 6.2 and 9.7%. Few halophilic microorganisms with this ability had been found in the past (Tapingkae et al. 2010b; Lee et al. 2016).

The cultures that degraded histamine (Table 2) included both red halophilic cultures (S18, S20 and A18) and cream-beige shades culture (A28) (Table 3). It should be noted that Huss and Valdimarsson (1990) related the development of red halophilic bacteria to the appearance of off-flavors, so it is important to identify the strains and

Table 2 Histamine-degrading capacity of halophilic prokaryotes

Treatment	Isolation source	Residual Histamine (mg/L)		Histamine degradation percentage (%)
Control	–	396.5 ± 4.95	A	–
S18	Salt	372.0 ± 1.41	BC	6.2
S20		358.0 ± 4.24	C	9.7
A18	Anchovies from wet salting stage	364.5 ± 4.95	BC	8.1
A28		366.5 ± 6.36	BC	7.6

*Mean ± standard deviation. Different letters indicate significant differences ($p<0.05$) in the histamine concentration due to microbial degradation according to Tukey test. Quantified histamine contents correspond to the supernatant diluted with 1 M HCl (ratio 1:1)

Table 3 Phenotypic characterization of histamine-degrading prokaryotes

Isolate	Colony colour	Cell morphology	Nitrate reduction	Citrate	Motility	Indole production	H_2S production	Salt requirement (% w/v)
S18	Red	Coccoid shaped	–	–	–	+	–	> 17.5
S20	Red	Coccoid shaped	–	–	–	–	–	> 10
A18	Red	Rod shaped	+	+	+	+	+	1–20
A28	Cream	Disc-shaped	+	–	–	–	–	> 15

investigate the phenotypic characteristics of each isolate in relation to spoilage and safety.

Histamine-degrading isolates were rod, coccoid, and disc shaped. They all were Gram stain negative and did not ferment glucose; A18 and A28 were the only cultures capable to reduce nitrate, and A18 showed motility and utilized citrate.

The histamine-degrading isolates demonstrated desirable biochemical features, such as cytochrome oxidase and catalase activity, that help prevent the oxidation of fatty acids and rancidity in the product (Geisen et al. 1992; Gøtterup et al. 2007). None of them had proteolytic and/or lipolytic capacities, properties that in excess have been related with quality loss or spoilage (Czerner and Yeannes 2014). Proteolytic and/or lipolytic activities in starter cultures for the production of fermented products have been studied in order to decrease the ripening time, but results have not been consistent (Casaburi et al. 2008). None of our isolates showed histidine decarboxylase activity, so these would not affect the safety of the product (Fontana et al. 2016; Torracca et al. 2018).

Regarding the chemotactic analyses, S18 and S20 did not show motility, while A18 and A28 showed characteristic rings in swarming plates with nutrients as tryptone and yeast extract. A18 formed a ring in swarming plates with histamine as chemoattractant compared to the negative control. It is the first study where this property is reported in extremely halophilic bacteria. It would be interesting to analyze whether or not chemotaxis-guided bacterial movements enable bacteria to improve histamine degradation. S18 required at least 17.5% NaCl w/v, so it could proliferate in the product only when that level of salt is reached, while the remainder of the cultures could develop in a wider range of salinity.

The S18 and A18 isolates were capable of producing indole and/or H_2S, both of which are undesirable. The first one in excess possesses a characteristic foul odor and the second one is a volatile compound with a rotten egg odor at low concentrations, so they are related to the occurrence of off-flavors (Huss and Valdimarsson 1990). Despite these characteristics, chemotaxis toward histamine could be an advantage for A18 to degrade histamine in situ. A28 and S20 isolates did not present these characteristics, and they showed the dominant characteristics found in the ripened stage during the salted–ripened anchovy production, that is lack ofH_2S production and lack of motility, glucose fermentation, indole production, and cytochrome oxidase activity (Perez et al. 2018). A28 exhibited chemotactic movement capacity toward yeast extract and tryptone, and although this characteristic had not been detected toward histamine, it may facilitate several interactions between microorganisms and the environment and contribute to the microbial community structure and biofilm formation (Chet and Mitchell 1976). A28 and S20 thus possess characteristics that could improve the ripening process and contribute to maintaining lower levels of histamine in salted–ripened products.

16S rRNA gene sequence-based identification of histamine-degrading isolates

The histamine-degrading isolates were identified based on their 16S rRNA gene sequences (Table 4). Isolates S18 and S20 belonged to archaeal genus *Halobacterium*, while isolates A18 and A28 corresponded to bacteria with a high percentage of similarity with *Halomonas* sp. and *Halovibrio*

Table 4 16S rRNA gene-based identification of histamine-degrading halophilic prokaryotes

Culture	PCR primers	Identity percentage (%)	Top-hit taxon	Identified organism	GenBank accession number
S18	21F 1492R	95.52	*Halobacterium salinarum*	*Halobacterium* sp.	MK634472
S20	21F 1492R	94.93	*Halobacterium salinarum*	*Halobacterium* sp.	MK634473
A18	27F 1492R	99.18	*Halomonas utahensis*	*Halomonas* sp.	MK634470
A28	27F1492R	98.26	*Halovibrio denitrificans*	*Halovibrio* sp.	MK634471

sp., respectively. The genera of the identified strains have been related to salted and ripened seafood products (Jiang et al. 2014; Jung et al. 2016; Alfonzo et al. 2017). Chemotaxis to different compounds has been reported in other *Halomonas* strains (D´Ippólito et al. 2011).

Histamine-degrading capacity in hypersaline conditions has been reported in few microorganisms only. Tapingkae et al. (2010b) and (2010a) observed that *Natrinema gari* (halophilic archaeon) exhibited ability to reduce histamine with improved performance by immobilizing whole cells. In fermented fish products, Lee et al. (2016) and Zaman et al. (2014) observed that the bacteria *Bacillus polymyxa* and *Staphylococcus carnosus* exhibited this capacity. However, in this work, isolates were exposed to much higher histamine contents (~ 800 mg/L). Strains of the genus *Halobacterium* have been suggested as potential starter cultures to maintain low levels of histamine during the maturation of *E. encrasicolus* (Alfonzo et al. 2017), but their potential as histamine degraders remains unknown. Nevertheless, strains of *Halobacterium* and *Halovibrio* genera have not yet been associated with histamine degradation. In the *Halomonas* genus, only the strain "*Halomonas shantousis* SWA25" has been related to the degradation of biogenic amines (Jiang et al. 2014).

Based on these findings, the isolation and investigation of autochthonous culturable microorganisms resulted in a suitable methodology to examine the microbiota of the salted–ripened anchovies and explore their biotechnological potential to develop moderately and extremely halophilic prokaryotic organisms as potential starter strains for the process. Several authors have been employed cell concentrates and low histamine concentrations, or tried to define optimal conditions for the degradation of histamine in fermented fish products, but salted–ripened anchovy has not yet been previously used in such studies. Our isolates were exposed to a high initial histamine content (~ 800 mg/L). The strains were not only capable of surviving, and reproducing at this high histamine concentration and extreme salt content, but also showed histamine degrading ability. This is an important finding because low initial microbial concentrations are used as starters in the food industry, and they must face significant histamine concentration that could be present in fish-based foods. By reducing the toxic load and increasing the microbial population, the quality and safety of the product could be benefited. In addition, two unexplored fields are reported in the present work: microbial histamine degradation performed by autochthonous halophilic microorganisms from salted–ripened anchovies, and their chemotactic behavior toward histamine.

The finding that strain A18, a *Halomonas* sp., was chemotactic toward histamine may contribute to the development of new fields of study, linking degradation with chemotactic movement toward histamine, biofilm formation, colonization facilitators, or interactions with other native microorganisms. Strains S20 and A28, identified as an archaeon *Halobacterium* sp. and a bacterium *Halovibrio* sp., respectively, possessed desirable in vitro properties in order to be used as potential starter cultures. They could contribute to maintain lower levels of histamine in salted–ripened products without the production of off-flavors. These strains are good candidates for further investigation in situ during the traditional salted–ripened anchovy production.

The in vitro characterization of microbial cultures allowed determining which strains isolated from salted–ripened anchovy may be more appropriate for improving the product of interest. This also enables the generation of a database to select the proper microorganisms to be employed in the production of salted fermented foodstuffs.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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