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Effect of Salt Concentration and Extremely Halophilic Archaea on the Safety and Quality Characteristics of Traditional Salted Anchovies

Antonio Alfonzo[a], Walter Randazzo[a], Marcella Barbera[a], Ciro Sannino[b], Onofrio Corona[a], Luca Settanni[a], Giancarlo Moschetti[a], Andrea Santulli[c], and Nicola Francesca[a]

[a]Dipartimento Scienze Agrarie e Forestali, Università degli Studi di Palermo, Palermo, Italy; [b]Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Perugia, Italy; [c]Dipartimento Scienze della Terra e del Mare, Università degli Studi di Palermo, Palermo, Italy

ABSTRACT

Extremely halophilic archaea (EHA) might play an important role in salted fish production. So far, limited information has been available on the effect of EHA and salt concentration on the safety and quality characteristics of salted anchovies. Eight *Halobacterium salinarum* strains were isolated from different sea salt samples and subjected to phenotypic and genotypic characterization. The strains were then inoculated into fresh salt before addition to anchovies. A total of 18 experimental productions were performed. The inoculated trials showed the lowest counts of undesired microorganisms. In particular, salted anchovies produced with *Hbt. salinarum* H11 showed the lowest histamine concentration as well as the highest sensory scores. Differences in terms of volatile organic compounds (VOCs) were estimated among trials. Furthermore, multivariate analysis showed that experimental production performed with a reduced amount of salt (175 g of sea salt per kg of anchovies) did not affect the final quality of salted anchovies. The strain *Hbt. salinarum* H11 produced salted anchovies with well-appreciated organoleptic features. Thus, the addition of EHA and the use of a lower amount of sea salt might represent a valuable alternative to the traditional method for production of salted anchovies.

KEYWORDS
Extremely halophilic archaea; *Halobacterium salinarum*; histamine; salted anchovies; salt concentration

Introduction

In the Mediterranean basin, the production of salted anchovies (*Engraulis encrasicolus* L.) is a traditional process that is performed to preserve the fish (Aponte et al., 2010; Hernandez-Herrero et al., 1999). A total of 490,000 tons of anchovies were captured in Europe during the year 2012, and 91,803 tons were transformed by salting and/or brining (FAO, 2014). Traditionally, production of salted anchovies begins with beheading and gutting of fresh fishes. After that, the anchovies are disposed “head-tail” in glass jars (technically known as “arbanelle” with 3 L volume capacity), and salt is added (about 500 g of salt per kg of anchovies) layer by layer. Karaçam et al. (2002) demonstrated that the amount of salt used (22–26% w/v brine solution) for the production of brined anchovies is of considerable importance; in particular, the product obtained with higher concentration of salt is more appreciated by consumers. So far, the amount of salt used during anchovy productions is determined only by a traditional and popular recipe based on a salt/fish ratio of 1:2 (Aponte et al., 2010). Karaçam et al. (2002) investigated the quality of anchovies brined at different concentrations; they showed that different amounts of salt added to brine might significantly affect

CONTACT Nicola Francesca nicola.francesca@unipa.it Dipartimento Scienze Agrarie e Forestali, Università degli Studi di Palermo, Viale delle Scienze 4, 90128 Palermo, Italy.

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the sensory, chemical, and microbiological characteristics of salted anchovies. Lee et al. (2015) reported that the quality of salted-fermented anchovies changes by varying the amount of salt during production at 15°C for 110 days. Sensory analysis has resulted in a product with a minimum amount of salt (15%) acceptable to consumers. Furthermore, the different concentration of salt during salted fish storage might affect many enzyme activities; thus, the chemical composition of the final products could change (Lee et al., 2015).

Previous microbial characterization of salted anchovies reported a variety of microbial populations, such as halotolerant and halophilic microorganisms (often belonging to the *Halobacteriaceae* family) (Casealio and Caramaschi, 1993; Hernandez-Herrero et al., 1999; Villar et al., 1985), lactic acid bacteria, *Enterobacteriaceae*, enterococci, and staphylococci (Aponte et al., 2010; Hernandez-Herrero et al., 1999). Halophilic microorganisms have been investigated not only in saline environments (Birbir et al., 2007; Grant et al., 1998; Oren, 2006), but also in food ecosystems (Aponte et al., 2010; Lee, 2013; Tapinkage et al., 2010; Yeannes et al., 2011), as they are considered to be the spoilage bacteria of meat and fish products due to their red/pink color and enzymatic activities (Eddy, 1958; Petter, 1931).

Previously, several species of *Halobacterium* sp. have been considered as potential spoilage microorganisms during ripening of salted fishes (Bronwn, 2008; Hall, 1997; Heredia et al., 2009; Troller and Christian, 1978). Recently, several studies of food ecosystems, especially those carried out on the conservation of fish products, tested the use of archaea as the starter during the ripening of salted anchovies. These fish products normally require a long time before reaching the consumer.

Many other studies investigated the ability of extremely halophilic archaea (EHA) strains to produce enzymes, in particular proteases, for food applications (Akolkar et al., 2010; Gimenez et al., 2000; Izotova et al., 1983; Kamekura et al., 1992; Ryu et al., 1994; Schmitt et al., 1990; Shi et al., 2006; Stepanov et al., 1992; Studdert et al., 2001; Vidyasagar et al., 2006). Most of the proteases produced by halophilic bacteria are extracellular serine proteases, which retain their enzymatic capacities even at high salt concentrations. Akolkar et al. (2010) reported that the inoculation of *Halobacterium* sp. SP1 (a red-pink color shape culture) into salted fishes shortened the ripening and improved the chemical composition (amino acids profile) and the flavor of the final products. Furthermore, Aponte et al. (2010) clearly showed that the use of red-pink color shape archaea halophilic strains as the starter might significantly improve both the safety and the sensory quality of salted anchovies (*Engraulis encrasicolus*), while no color alteration was recognized by panelists.

During the production of salted anchovies, several physico-chemical changes occur in fish tissues. First, the diffusion of salt occurs with the consequent elimination of water from fish tissues. Temperature, freshness of fish, and the relative fat content, as well as the thickness of muscle, might vary the rate of salt penetration (Clucas, 1982). The ripening is induced by enzymes that decompose proteins and fats (Voskresensky, 1965). The enzymatic pathways that occur are attributed to tissue and/or microbial enzymes (Hernandez-Herrero et al., 1999). The value of water activity of salted products might affect the growth of microbial population as well as the activities of many enzymes (Filsinger, 1987). Another main factor during salted anchovy production is the generation of histamine (Lehane et al., 2000), which, at high concentration, is detrimental to the quality and safety of the final product (EFSA, 2011). The European Commission has issued two Commission Regulations (EC No. 1441/2007; EC No. 1019/2013) establishing the histamine limit for ripened brined/salted fishery products at 400 mg/kg of fish. The histamine compound is mainly produced by histamine decarboxylase enzymes originating from many species of microorganisms and enzymes of animal origin. On the other hand, many bacteria strains used as starter strains for food production have been evaluated for their ability to degrade histamine compounds. Thus, the use of those starter cultures might be a useful tool to reduce the amount of histamine in foods (Bakke et al., 2005; Ienistea et al., 1971). Recently, Tapingkae et al. (2010) reported the degradation of histamine to a large extent by EHA that are the microbial group isolated from salted fish products.

To our knowledge, no studies have investigated the effect of the salt amount on the chemical, microbiological, and sensory quality of salted anchovies. Hence, the present research was aimed to

study (I) the effect of different concentrations of sea salt to produce salted anchovies and concomitantly (II) the effect of eight EHA strains isolated from different sea salts. Microbiological, chemical, and sensory parameters were monitored during the entire anchovy production in order to evaluate the final quality and safety of the resulting products.

Materials and methods

Collection of salt samples and microbiological analyses

A total of 14 samples (Table 1) of commercial salt from salt pans and mines were purchased from local markets or salt enterprises of Sicily (southern Italy). Salts were transferred into sterile Stomacher bags and kept at room temperature until analysis. The sampling was performed in triplicate on different days.

Salt samples were screened for the presence of EHA according to the protocol reported by Moschetti et al. (2006). To this purpose, each sample (10 g) was inoculated into a 500 ml conical flask containing 250 ml *Halobacterium* liquid medium (HLM) for the enrichment (Oren et al., 1999). Flasks were incubated for two weeks at 44°C under constant shaking (150 rpm) and lighting. Ten milliliters of each culture was transferred to a new flask with 250 ml HLM. After three subcultures, 100 μl were spread on *Halobacterium* medium agar (HMA) and incubated at 44°C for two weeks. Analyses were performed in triplicate. All media and the supplements were supplied from Oxoid (Milan, Italy).

Isolation and phenotypic characterization of EHA

Presumptive EHA (at least four colonies with the same color, morphology, edge, surface, and elevation) were collected from HMA plates. The isolates were purified by successive sub-culturing, and the purity of the isolates was checked microscopically.

Rod-shaped cell, Gram-negative, catalase (determined in presence of H_2O_2 5%, v/v), and oxidase (determined with tetramethyl phenylendiamine-HCl) positive isolates were stored in HLM containing 20% (v/v) glycerol at −80°C until further experimentations. EHA were initially subjected to a phenotypic characterization based on nitrate reduction (Hassanshahian, 2011), gas production from nitrate (Hassanshahian, 2011), anaerobic growth in presence of L-arginine (Oren and Litchfield, 1999), and production of acids from glucose (Rodriguez-Valera et al., 1983).

All tests were carried out in three independent replicates according to the protocol reported by Holt et al. (2005).

Table 1. Salt samples used in this study and origin of the EHA isolates

Samples	Company	Geographical origin	Source	nb of isolates
SS1	Saline Ettore e Infersa S.R.L.	Marsala (TP)	Salt pan	61
SS2	Sosalt S.P.A.	Trapani/Marsala (TP)	Mix salt pans	84
SS3	Sale e Mediterraneo S.R.L.S.	Trapani (TP)	Salt pan	25
SS4	Sikeli'a S.R.L.	Marsala/Trapani (TP)	Mix salt pans	65
SS5	Sosalt S.P.A.	Trapani/Marsala (TP)	Mix salt pans	0
SS6	Sale e Mediterraneo S.R.L.S.	Trapani (TP)	Salt pan	32
SS7	Sikeli'a S.R.L.	Marsala/Trapani (TP)	Mix salt pans	0
SS8	Italkali S.P.A.	Petralia (PA), Realmonte, Racalmuto (AG)	Mix salt mines	0
SS9	Sale di Raimondo	Trapani/Marsala (TP)	Mix salt pans	0
SS10	Iblea Sale di Borrometi G. & C. S.N.C.	Trapani/Marsala (TP)	Mix salt pans	0
SS11	Naso Carlo	Trapani	Mix salt pans	0
SS12	Sale Cucchiara S.R.L.	Trapani/Marsala (TP)	Mix salt pans	0
SS13	Roccella Ettore	Trapani/Marsala (TP)	Mix salt pans	0
SS14	Italkali S.P.A.	Petralia (PA), Realmonte, Racalmuto (AG)	Mix salt mines	0

Genotypic investigation of EHA at the strain and species level

Genomic DNA from EHA cultures was extracted with the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Before DNA extraction, cell cultures were washed twice in sterile 25% (w/v) salt water.

Strain differentiation was performed by polymorphic random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis in a 25 µL reaction mix using the single primers XD9, PRIMM 239, and XD4 as previously described by Moschetti et al. (2006).

RAPD amplicons were separated by electrophoresis on a 2% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France), stained with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA) and subsequently visualized by UV transillumination. The GeneRuler 100 bp Plus DNA Ladder (M-Medical S.r.l., Milan, Italy) was used as a molecular size marker. RAPD patterns were analyzed using the Gelcompare II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

One representative strain for each RAPD profile was identified at species level by 16S rRNA gene sequencing as described by Moschetti et al. (2006). The primers used to amplify the 16S rRNA gene were ARCH1 and ARCH2 (Moschetti et al., 2006). The 16S rRNA gene amplicons were visualized as reported above.

DNA sequencing reactions were performed by PrimmBiotech srl (Milan, Italy). The identities of the sequences were determined by BLASTN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov (Altschul et al., 1997) and those available at EzTaxon-e database at http://www.ezbiocloud.net/eztaxon (Chun et al., 2007).

Production of experimental anchovies

All strains belonging to the EHA group were independently inoculated into commercial salt (Sale Cucchiara S.R.L., Trapani/Marsala, Italy), according to the procedure described by Aponte et al. (2010). All salt samples used for the experimental trials were subjected to heat treatment for 2 h at 160°C. Subsequently, the salt was sterilized at 121°C for 20 min (Prasad et al., 1995). Furthermore, microbiological analysis and PCR amplification of the archaeobacterial 16S rRNA gene were performed to ascertain the absence of halophilic archaea in sterilized commercial salt to be used in the experimental trials (Moschetti et al., 2006; Prasad et al., 1995). The inoculated salts were used to produce experimental salted anchovies. Fresh anchovies were purchased from a local fish market located in Palermo (Italy) and immediately transferred to the laboratory. The experimental production of salted anchovies was carried out according to the protocol of Aponte et al. (2010). Each jar was filled with 2 kg of anchovies. The jars with 125 and 175 g of inoculated salt per each kg of anchovies represented the trials *a* and *b*, respectively. Per each EHA strain, both amounts of salt (*a* and *b*) were included in the experimental design. In addition, three different control productions (without EHA addition) were included for comparison: control 1-*a*, control 1-*b*, and control 2 were performed with 125, 175, and 500 g/kg of salt, respectively.

To maintain anchovies under constant pressure, a weight of 2 kg was placed above the jars for the first month, and after that, it was reduced to 1 kg until the end of ripening (150 days).

The ripening was carried out at 20°C for 150 days, and it was periodically monitored. Samples of salted anchovies (about 50 g) were collected before and immediately after the addition of sterile salt and at 3, 6, 12, 24, 48, 96, and 150 days of ripening. The experiment was conducted in triplicate (three jars per trial).

Monitoring of microbial populations of experimental anchovies

Samples of anchovies were suspended in Ringer's solution (Sigma-Aldrich, Milan, Italy) at a ratio of 1:10 (w/v), homogenized with a stomacher (BagMixer®400, Interscience, St Nom. France) for 4 min at the maximum speed, and subjected to decimal dilutions. The following microbial groups were

enumerated: the total aerobic mesophilic microorganisms on plate count agar (PCA) incubated at 30°C for 72 h; mesophilic lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe (MRS) agar incubated at 30°C for 48 h; *Enterobacteriaceae* on double-layer violet red bile glucose agar (VRBGA), incubated at 37°C for 24 h; staphylococci on Baird Parker (BP) and coagulase positive staphylococci (CPS) on BP added with RPF supplement, incubated at 37°C for 48 h (APHA, 2015). For the enumeration of halophilic microbial populations, samples of anchovies were suspended in a modified Ringer's solution (25% w/v NaCl) (Moschetti et al., 2006; Aponte et al., 2010), ratio 1:10 (w/v). Furthermore, they were counted on *Halobacterium* medium after incubation at 44°C for 15 days under constant light. All media and supplements were purchased from Oxoid. All analyses were performed in triplicate.

The presence of EHA strains inoculated into salt was monitored by phenotypic and genotypic analysis as reported above.

Histamine determination

The content of histamine in salted anchovies at 150 days of ripening was determined by acid extraction and derivatization of histamine. The analysis was performed by the modification of the methods reported by Erola et al. (1992) and Moret et al. (1996). Stock standard solutions containing histamine dihydrochloride (Sigma-Aldrich) and 1.7 diaminoheptane (as internal standard) were prepared by adding a weighed amount of histamine in ultrapure water (Easypure II, Thermo) at a concentration of 1000 mg/l. The standard solution was stored at 4°C until use. Homogenized anchovies (5 g) were added to 10 ml of HCl 0.1 M, containing 100 mg/l of the internal standard and homogenized with an Ultra-Turrax system (T 25 basic IKA labortechnik, Staufen, Germany). The mixture was centrifuged at 4000 rpm for 30 min at 4°C, and the supernatant was separated through 0.45 µm filters (Sartorius, Muggiò, Italy). The extraction was repeated for the solid residue. The two acid extracts were mixed and diluted up to 25 ml with HCl 0.1 M. An aliquot of 1 ml of the acid extract was mixed with 0.5 ml of saturated $NaHCO_3$ solution and 1.0 ml of dansyl chloride solution (5mg/ml in acetone) and kept in darkness for 1 h at 40°C. The residual dansyl chloride was removed by adding 300 µl of ammonia solution (30%), and each sample, shielded from light, reacted for 15 min at room temperature. Each sample was extracted twice with 1 ml of diethyl ether. The combined extracts were dried, and the residue was dissolved with 1 ml of acetonitrile and then injected into a high performance liquid chromatography (HPLC) system. The analyses and quantification of dansylated histamine were carried out by an Agilent 1200 HPLC system equipped with a G1329A high performance autosampler G1316A Thermostated Column Compartment and G1315D DAD detector (Diode Array Detector) injecting 20 µl of each sample. The peaks were integrated at 254 nm. The separation was carried out using an Agilent Eclipse XDB-C18 (4.6 × 150 mm, 5 µm) column. The mobile phases were ultrapure water (A) and acetonitrile (B) eluting under gradient condition with a flow rate of 1 ml/min. The gradient elution program was as follows: 0–12 min, 50–80% B, 12–25 min, 80–100% B, 25–30 min 100–50% B. An external calibration was obtained by analyzing six standard solutions at different concentrations derivatized as describe above for the samples. All analyses were performed in triplicate.

Volatile organic compounds

Volatile organic compounds (VOCs) were determined at 150 days of ripening of salted anchovies by solid-phase micro-extraction-gas chromatography-mass spectrometer (SPME-GC/MS) technique. Five grams of homogenized salted anchovies were transferred into 20 ml vials with pierceable polytetrafluoroethylene (PTFE)/silicone septa 27136 (Supelco, Bellefonte, Italy).

A SPME fiber of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; Supelco, Bellefonte, Italy) was used and conditioned at 250°C for 30 min in the GC/MS injector before each extraction. For the extraction, the SPME fiber was inserted into a sample vial through the septum and then exposed to the headspace gas for 30 min at 40°C. The fiber was retrieved and

injected into the GC/MS system. One hundred fifty μL of 1-heptanol solution (35mg/l 1-heptanol in 20% ethanol aqueous solution) was used as an internal standard.

The SPME fiber was directly inserted into a Finnegan Trace MS for GC/MS (Agilent 6890 Series GC system, Agilent 5973 Net Work Mass Selective Detector; Milan, Italy) equipped with a DB-WAX capillary column (Agilent Technologies; 30 m. 0.250 mm i.d. film thickness 0.25 μm, part no 122–7032). During the split less injection, the GC temperature was set at 40°C for the first 2 min, then increasing 4°C/min from 40°C to 60°C, followed by a break at 60°C for 2 min, and then from 60°C to 190°C, increasing 2°C/min, from 190°C to 230°C, increasing 5°C/min, and finally 230°C for 15 min. The GC injector was at 250°C, the Fid at 250°C, the transfer line at 230°C, with helium as carrier at a rate of 1 ml/min, and EM at 70 eV. Mass spectra were recorded by electronic impact at 70 eV using the ion source temperature of 200°C. All compounds of m/z 33–495 atomic mass units (amu) were detected with this scan mode. Individual peaks were identified by comparing their retention indices to those of control samples and by comparing their mass spectra with those within the NIST/EPA/NIH Mass Spectral Library database (Version 2.0d. build 2005). Volatile compounds were expressed as μg/kg. All solvents and reagents were purchased from WWR International (Milan, Italy). All analyses were performed in triplicate.

Sensory analysis

The evaluation of the sensory profiles of the ripened salted anchovies was performed following the descriptive method ISO 5496:2006.

Ten judges were trained in preliminary sessions using different samples of commercial salted anchovies in order to develop a common vocabulary for the description of the sensory attributes as well as to familiarize them with scales and procedures. Each attribute was extensively described and explained to avoid any doubt about the relevant meaning. The descriptors related to aspect (color uniformity and color intensity), texture (compactness, juicy, and gummy), odor (salt anchovies), flavor (ham taste, rancid, and putrid), and overall acceptability were included in the analyses. The anchovies were randomly evaluated by assigning a score between 1.00 (absence of descriptor sensation) and 9.00 (extremely intense) in individual booths under incandescent white light. Two panel replications were carried out per sample.

Statistical and explorative multivariate analyses

Data obtained from microbiological investigation and sensory evaluation were analyzed using a generalized linear model (GLM) that included the effects of samples; the Student “t” test was used for mean comparison. The analysis of variance (ANOVA) test was applied to identify significant differences among microbial counts. The post-hoc Tukey’s method was applied for pairwise comparison in case of microbial counts, histamine, and sensory scores. Statistical significance was attributed to p values of $p<0.05$.

In addition, an explorative multivariate approach was employed to investigate relationships among data obtained from the different experimentations.

A hierarchical cluster analysis (HCA) was carried out for grouping the experimental trials according to their similarity, measured by Euclidean distances; whereas cluster aggregation was based on the single linkage method (Todeschini, 1998). Furthermore, principal component analysis (PCAn) was employed to investigate relationships among samples. The input matrix used for HCA and PCAn consisted of the total area under growth/decline curves of the total aerobic mesophilic microorganisms, LAB, *Enterobacteriaceae*, CPS as well as histamine values, VOCs, and results of sensorial profiles (Bautista-Gallego et al., 2011; Blana et al., 2014; Rodríguez-Gómez et al., 2014). Areas were calculated by integration using the OriginPro 7.5 software (OriginLab Corporation, Northampton, MA, USA). The number of principal factors was selected according to the Kaiser criterion (Jolliffe, 1986); only factors with eigen-values higher than 1.00 were retained.

All data were preliminary evaluated using the Barlett’s Sphericity test (Dillon and Goldstein, 1984; Martorana et al., 2015) in order to check the statistically significant difference among samples within each dataset.

Statistical data processing and graphic construction were achieved using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and XLStat software version 2014.5.03. (Addinsoft, New York, NY, USA) for Excel.

Results and discussion

Isolation and characterization of EHA strains from salt samples

Three hundred fifteen colonies were isolated from the 14 salt samples. After purification and characterization based on colony appearance and microscopic inspection, a total of 267 rods were further phenotypically investigated. All cultures were considered presumptive EHA, because they were Gram-negative, oxidase and catalase positive, motile, as well as able to grow in 25% NaCl.

The reduction of nitrate, production of gas from nitrate and glucose, as well as the growth on arginine were negative for all strains.

All isolates were subjected to RAPD analysis (data not shown), revealing the presence of 8 strains (Figure 1). The DNA from the eight strains were subjected to specific PCR analyses that target a 16S rRNA fragment highly conserved in *Halobacteriaceae*. The results of sequencing (Figure 1) showed that all strains belonged to species *Hbt. salinarum* (identity of at least 98.66% in EzTaxon-e database) (Stackebrandt and Goebel, 2006). Within the *Halobacteriaceae* family, the genus *Halobacterium* is mostly studied, and the species *Hbt. salinarum* was the first halophilic archaeon described. *Hbt. salinarum* was isolated from salt rich environments as salted cowhide and cured codfish. In our investigation, *Hbt. salinarum* was the only species of extremely halophilic archaea detected in Sicilian sea salts.

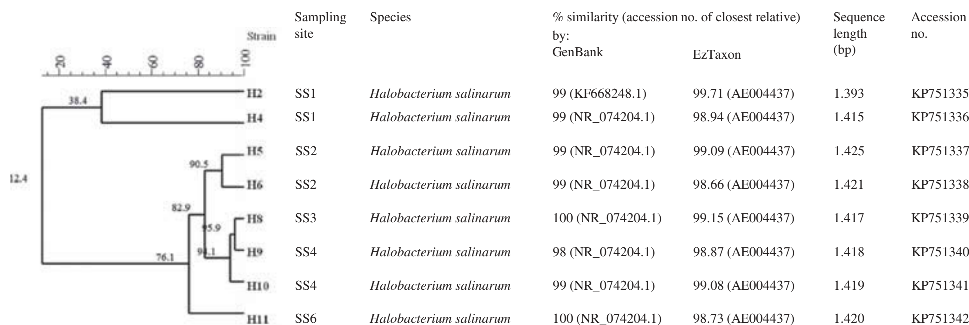
Evolution of microbiological parameters during production of experimental salted anchovies

The microbiological loads registered for the experimental salted anchovies are reported in Figure 2.

Salt samples inoculated with the selected strains showed a concentration of EHA population of about 4 Log CFU/g. Moreover, the PCR amplification of the archaeobacterial 16S rRNA gene confirmed that no salt sample contained DNA of archaeal origin.

Both experimental anchovy productions (*a* and *b*) obtained with inoculated salt showed concentration of EHA higher than that of controls. The experimental productions were not characterized for the presence of other microbial groups. During the entire period of monitoring, the EHA population was almost constant at values about 3.5 Log CFU/g in all inoculated trials. RAPD analysis showed that all inoculated strains dominated the EHA population during the entire ripening period of experimental productions.

At time zero, the total aerobic mesophilic microorganisms, LAB, *Enterobacteriaceae*, and staphylococci showed levels between 1.80–3.90 Log CFU/g in production *a*. Similar values (1.32–3.85 Log CFU/g) were recorded in samples of production *b*.



Sampling site	Species	% similarity (accession no. of closest relative) by: GenBank	EzTaxon	Sequence length (bp)	Accession no.
SS1	*Halobacterium salinarum*	99 (KF668248.1)	99.71 (AE004437)	1.393	KP751335
SS1	*Halobacterium salinarum*	99 (NR_074204.1)	98.94 (AE004437)	1.415	KP751336
SS2	*Halobacterium salinarum*	99 (NR_074204.1)	99.09 (AE004437)	1.425	KP751337
SS2	*Halobacterium salinarum*	99 (NR_074204.1)	98.66 (AE004437)	1.421	KP751338
SS3	*Halobacterium salinarum*	100 (NR_074204.1)	99.15 (AE004437)	1.417	KP751339
SS4	*Halobacterium salinarum*	98 (NR_074204.1)	98.87 (AE004437)	1.418	KP751340
SS4	*Halobacterium salinarum*	99 (NR_074204.1)	99.08 (AE004437)	1.419	KP751341
SS6	*Halobacterium salinarum*	100 (NR_074204.1)	98.73 (AE004437)	1.420	KP751342

Figure 1. Identification of EHA strains isolated from sea salts. The dendrogram obtained from combined RAPD-PCR patterns of EHA strains generated with three primers (XD9, PRIMM 239, and XD4). The upper line indicates the percentage of similarity.

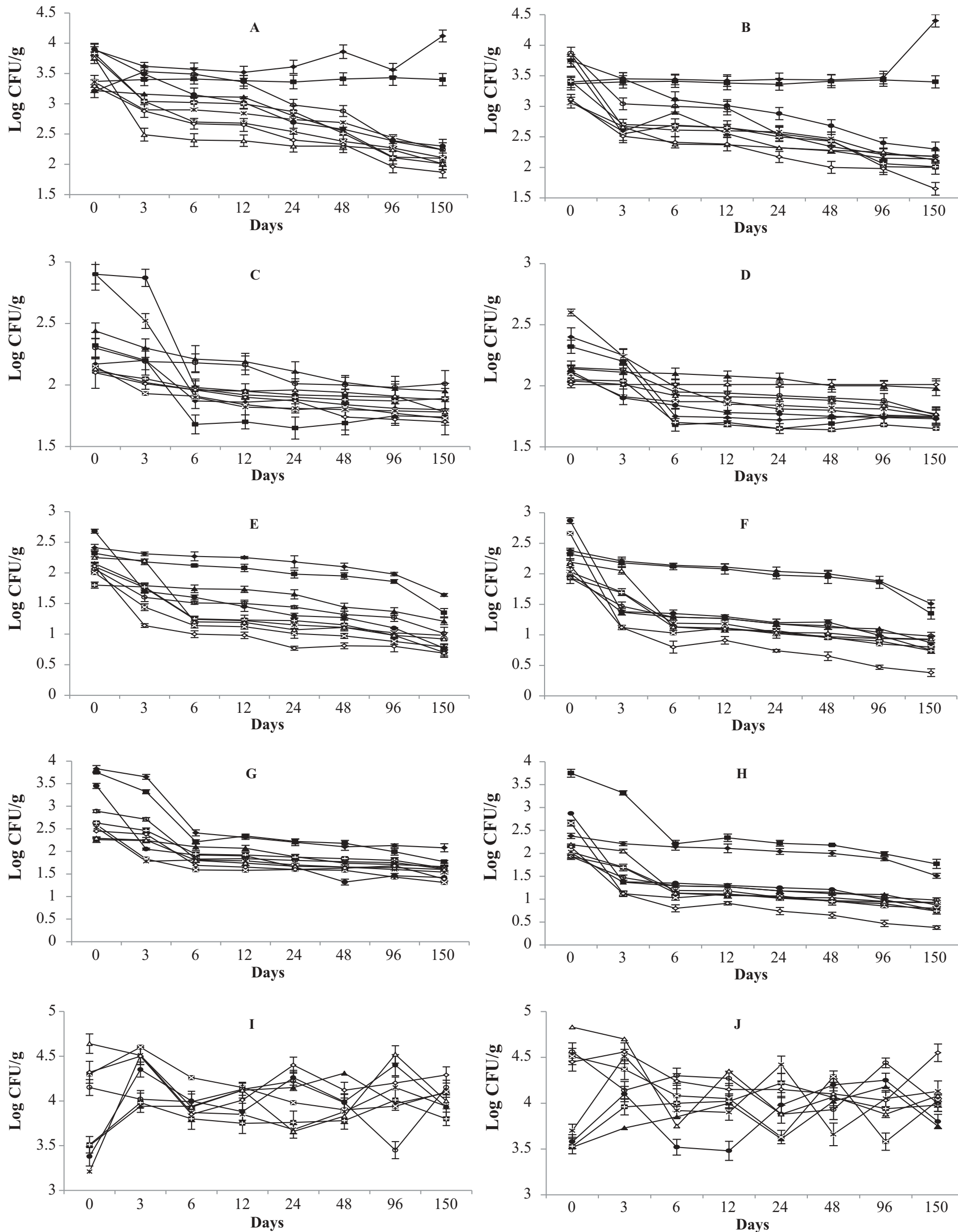


Figure 2. Microbiological concentrations (Log CFU/g) of samples during production of salted anchovies. **A**, PCA trial *a*; **B**, PCA trial *b*; **C**, MRS trial *a*; **D**, MRS trial *b*; **E**, VRBGA trial *a*; **F**, VRBGA trial *b***;** **G**, BP trial *a*; **H**, BP trial *b*; **I**, HMA trial *a*; **J**, HMA trial *b*. Symbols: ♦, Control 1; ■, Control 2;▲, H2;, H4;*, H5; ●, H6;, H8;○, H9;, H10; ◊, H11. Abbreviation: PCA, plate count agar for total aerobic mesophilic microorganisms; MRS, Man-Rogosa-Sharpe agar for mesophilic rod LAB; VRBGA, violet red bile glucose agar for Enterobacteriaceae; BP, baird parker for Staphylococcaceae; HMA, halobacterium medium agar for extremely halophilic bacteria. The italic letters, *a* and *b*, refer to the amounts of salt, 125 and 175 g/kg respectively, used to produce salted anchovies. Control 2, experimental production obtained by 500 g salt per 1 kg anchovies. H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum.

During ripening, the microbial counts of all experimental productions *b* (175 g/kg of salt) were slightly lower than *a* (125 g/kg of salt) for all media. Overall, the inoculum of EHA resulted in a decrease of microbial populations of *Enterobacteriaceae* and staphylococci. The microbial growth of *Enterobacteriaceae* and staphylococci decreased. HMA counts were almost constant during ripening, and the presence of LAB did not seem to be influenced by the inoculation of different strains of EHA used.

In detail, the total aerobic mesophilic microorganisms showed concentrations of about 3 Log CFU/g in samples produced with inoculated salt until 96 days of ripening. Subsequently, the microbial loads were lower than 2 Log CFU/g, except for controls, which showed values higher than 3.5 Log CFU/g. Furthermore, both productions *a* and *b* inoculated with the strain H11 showed the lowest count of all microbial groups until the end of ripening. On the contrary, control productions showed counts of *Enterobacteriaceae* and staphylococci significantly higher than the inoculated trials.

Data obtained in the present study comply with the trend reported in the literature by several authors (Aponte et al., 2010; Hernández-Herrero et al., 1999). The reduction of microbial loads detected for the total aerobic mesophilic microorganisms, *Enterobacteriaceae*, and *Staphylococcaceae* might be due to the high concentration of salt that determines a low water activity (Barros and Lenovich, 1992; Lupin et al., 1981). However, these determined favorable conditions for halophilic archaeal growth (Campello, 1985; Perez-Villarreal and Pozo, 1992). The population of the LAB was not influenced by the amount of salt or the presence of EHA inoculated in the different productions. No statistically significant differences were found in terms of microbial counts between the control 1-*b* and control 2.

Concentration of histamine

The concentrations of histamine in ripened anchovies are shown in Figure 3. The highest concentration (54.61 mg/kg) was found for control 1-*a*, which was also characterized by the highest counts of microbial populations (total aerobic mesophilic microorganisms, *Enterobacteriaceae*, staphylococci) and is statically different ($p<0.05$) from H11-*a* and H11-*b* productions. On the other hand, the lowest values of histamine (27.16 mg/kg and 33.61 mg/kg in H11-*a* and *b*, respectively) were obtained for salted anchovies inoculated with the strain H11, even though only the H11-b thesis showed statistically significant differences ($p <0.05$) with Control 1-*b*, H2-*a*, H4-*a*, H4-*b*, H6-*a*, H8-*a*, H9-*a*, H9-*b* and H10-*a*. The differences in terms of histamine contents might be related to EHA strains used as inoculum and the amount of salt used. EHA strains determined the inhibition of the microbial groups involved in histamine generation. Trial H11-b had very low counts in BP medium compared to the other trials, and *Staphylococcus* spp. has been identified as a histamine forming bacteria (Hernendez-Herrero et al., 1999). However, all samples showed histamine contents lower than that (400 mg/kg) indicated by Official Regulation (EC No. 1019/2013).

VOCs in salted anchovies

The results of VOCs emitted by salted anchovies sampled at day 150 are reported in Table 2. A total of 25 different compounds, including mainly hydrocarbons, alcohols, aldehydes and ketones were identified. About 90% of VOCs were represented by hydrocarbons, followed by alcohols (5%), and aldehydes (1%).

Among hydrocarbons, pentadecane and hexadecane were the most represented compounds. Pentadecane is one of the indicators of fish quality (Edirisinghe et al., 2007). This compound was found at high concentration for all productions *b* inoculated with halophilic strains and mainly for the trials inoculated with the strain H11.

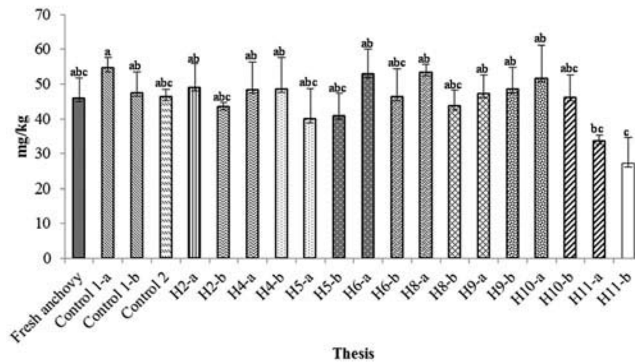


Figure 3. Values of histamine (mg/kg) determined in the experimental salted anchovies at the end of the ripening period (150 days). H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum. The italic letters *a* and *b*, associated to codes of samples refer to the amounts of salt, 125 and 175 g/kg respectively, used to produce salted anchovies; Control 2, experimental production obtained by 500 g salt per 1 kg anchovies. Different letters superscript on quantities of histamine indicate significant differences according to Tukey's test between experimental productions for $p \leq 0.05$.

Within the class of alcohols, 2-propyl-1-pentanol reached the highest concentration. This compound is generally associated with green banana flavor (Baranska et al., 2013). The 1-octen-3-ol, a compound associated with mushroom-like flavor (Triqui et al., 1995a), was mainly detected in controls 1-*a* and 1-*b*. Aldehydes were mainly represented by benzaldehyde, which is recognized as a final product of the amino acid catabolism (Sinz et al., 2012), as well as by (E)-2-heptenal, (E,E)-2,4-heptadienal, and (E,E)-3,5-octadien-2, reported to derive from the oxidation of linoleic (Grosch, 1987), α-linolenic, and eicosapentaenoic acids (Frankel, 1983; Kawai et al., 1996), respectively. In addition, the (E,E)-2,4-heptadienal was found responsible for fatty and rancid flavors (Triqui, 1995a). The concentrations of these compounds were significantly higher in the control productions.

Among ketones, 2,3-octanedione and 2-nonanone have been found to be associated with oxidation (Young et al., 1997) and autoxidation (Dehaut et al., 2014) of fatty compounds, respectively. The 2-nonanone and (E,E)-3,5-octadien-2-one were associated with the green and marine algae-like sensory descriptors in salted anchovies (Triqui et al., 1995a). Thus, many VOCs from oxidation and/or autoxidation of fatty compounds might be responsible for the characteristic flavor of salted anchovies (Czerner et al., 2011; Triqui et al., 1995a,b, 1999).

The effect of the salt amount on flavor of salted anchovies has been shown by Triqui et al. (1995b), who found a correlation between fatty oxidations, VOCs composition, and the amount of salt used to produce salted anchovies.

Sensory analysis

The sensory evaluation of the salted anchovies is shown in Table 3. The anchovies inoculated with EHA were similar to that of control production in terms of color uniformity, color intensity, compactness, gumminess, and ham flavor. The main differences were estimated in terms of juiciness. Overall, the inoculated anchovies were the most appreciated by the tasters, and the productions H2 and H11 with salt amount *b* showed the highest scores of overall enjoyment. In detail, the inoculated trials were characterized by higher scores of juicy descriptor, while the values of rancid and putrid were variable. The values of rancid and putrid scores might be due to proteolytic and lipolytic activities of different EHA strains used as starter. Both the hydrolytic activity on lipids and proteins, as well as the ability to decarboxylate amino acids is highly variable and depends on species, strains, and environmental conditions (Bardocz et al., 1999; De Lourdes

Table 2. Analysis of the volatile organic compounds (VOCs) of salted anchovies at the end of the ripening period (150 days)

Chemical compounds	Control 1-*a*	Control 1-*b*	Control 2	H2-*a*	H2-*b*	H4-*a*	H4-*b*	H5-*a*	H5-*b*
Alcohols									
1-Penten-ol	12.30 ± 1.20^{d}	40.43 ± 4.04^{a}	42.12 ± 0.87^{a}	20.50 ± 2.11^{bc}	4.31 ± 0.41^{e}	19.56 ± 1.69^{bc}	4.11 ± 0.86^{e}	17.15 ± 1.26^{cd}	3.13 ± 0.76^{e}
1-Penten-3-ol	13.09 ± 1.31^{c}	21.24 ± 2.10^{b}	24.21 ± 2.24^{a}	2.13 ± 0.21^{e}	7.35 ± 0.78^{d}	2.03 ± 0.22^{e}	7.02 ± 0.25^{d}	1.76 ± 0.62^{e}	5.86 ± 0.13^{d}
1-Butanol.2-methyl	8.72 ± 0.90 ^{c}	17.08 ± 1.7 ^{b}	20.23 ± 1.90^{a}	4.21 ± 0.43^{d}	4.46 ± 0.42^{d}	4.02 ± 0.44^{d}	4.26 ± 0.87^{d}	3.42 ± 0.79^{d}	3.57 ± 0.80^{d}
3-Buten-2-ol	11.93 ± 1.20^{a}	n.d.^{d}	8.84 ± 1.54^{b}	1.64 ± 0.15^{d}	5.01 ± 0.50^{c}	1.56 ± ± 0.11^{d}	4.78 ± 0.33^{c}	1.31 ± 0.14^{d}	4.21 ± 0.87^{c}
1-Octen-3-ol	37.04 ± 3.59 ^{c}	73.75 ± 7.39^{a}	65.01 ± 0.59^{b}	29.14 ± 2.92^{cde}	6.12 ± 0.62^{f}	27.80 ± 2.33^{de}	5.84 ± 0.39^{f}	25.84 ± 2.59^{de}	5.20 ± 0.07^{f}
2-Propyl-1-pentanol	114.65 ± 12.07^{bcd}	160.91 ± 15.98^{a}	134.82 ± 19.02^{ab}	104.74 ± 10.48^{bcde}	76.46 ± 7.89^{efg}	99.92 ± 9.44^{cdef}	72.94 ± 7,75^{fg}	92.56 ± 9.27^{cdefg}	67.94 ± 6.34^{g}
1-Octanol	4.17 ± 0.42^{b}	n.d.^{c}	0.25 ± 0.02^{c}	n.d. ^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}
(2E)-2,7-Octadien-1-ol	39.78 ± 4.01^{b}	65.9 ± 6.79^{a}	71.9 ± 2.42^{a}	25.58 ± 2.56^{cd}	5.37 ± 0.76^{e}	24.41 ± 2.89^{cd}	5.13 ± 0.55^{e}	24.22 ± 2.87^{cd}	5.20 ± 0.70^{e}
2-Phenylethanol	8.00 ± 0.85^{a}	n.d.^{c}	0.67 ± 0.05^{b}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}
Aldehydes									
Octanal	14.41 ± 1.45^{c}	24.94 ± 2.50^{b}	31.12 ± 1.27^{a}	3.95 ± 0.40^{d}	2.60 ± 0.21^{d}	3.76 ± 0.33^{d}	2.48 ± 0.25^{d}	3.20 ± 0.77^{d}	2.82 ± 0.73^{d}
2-Heptenal	n.d.^{b}	n.d.^{b}	n.d.^{b}	1.02 ± 0.10^{a}	1.09 ± 0.01^{a}	0.97 ± 0.04^{a}	1.04 ± 0.09^{a}	0.89 ± 0.09^{a}	1.20 ± 0.57^{a}
(E.E)-2,4-Heptadienal	16.01 ± 1.32^{c}	143.11 ± 12.44^{a}	121.51 ± 7.26^{b}	8.37 ± 0.89^{d}	6.69 ± 0.71^{d}	7.98 ± 0.81^{d}	6.39 ± 0.19^{d}	7.41 ± 0.29^{d}	5.73 ± 0.12^{d}
Benzaldehyde	49.72 ± 4.98^{bcd}	61.21 ± 6.22^{b}	84.27 ± 3.87^{a}	33.48 ± 3.29^{efghi}	46.20 ± 4.17^{cd}	31.94 ± 3.64^{efghi}	44.07 ± 0.45^{cde}	29.68 ± 2.51^{hi}	40.78 ± 4.42^{defgh}
Aromatic hydrocarbons									
Styrene	104.87 ± 10.53^{a}	63.93 ± 6.38^{b}	42.39 ± 5.25^{c}	28.67 ± 2.65^{d}	13.76 ± 1.82^{fg}	27.35 ± 2.28^{d}	13.13 ± 1.31^{g}	25.00 ± 2.05^{de}	12.57 ± 1.69^{g}
Carboxylic acid									
Acetic acid	6.29 ± 0.54^{bc}	9.99 ± 1.46^{a}	11.43 ± 1.24^{a}	5.73 ± 0.47^{bcd}	4.99 ± 0.40^{bcd}	5.47 ± 0.52^{bcd}	4.76 ± 0.23^{bcd}	5.17 ± 0.07^{bcd}	4.34 ± 0.88^{cd}
Alpha-hydrogen aldehydes									
3-(Methylthio)propanal	4.59 ± 0.39^{bc}	6.01 ± 0.65^{b}	8.98 ± 1.44^{a}	2.33 ± 0.22^{d}	3.01 ± 0.76^{cd}	2.22 ± 0.67^{d}	2.87 ± 0.30^{cd}	2.44 ± 0.21^{d}	2.27 ± 0.67^{d}
Hydrocarbons									
1,3,7-Octatriene	26.49 ± 2.77^{a}	n.d.^{e}	n.d.^{e}	5.02 ± 0.51^{d}	10.39 ± 1.84^{bc}	4.79 ± 0.43^{d}	9.91 ± 0.11^{bc}	4.88 ± 0.45^{d}	9.63 ± 0.51^{bc}
Tetradecane	59.39 ± 6.02^{a}	28.68 ± 2.91^{def}	12.75 ± 2.27^{g}	23.97 ± 2.42^{f}	40.02 ± 4.46^{bc}	22.86 ± 0.27^{f}	38.18 ± 3.36^{bcd}	2.74 ± 0.72^{h}	35.81 ± 3.13^{cde}
Pentadecane	3705.80 ± 365.55^{def}	5085.58 ± 412.87^{bc}	4354.80 ± 294.35^{cde}	3142.18 ± 308.23^{ef}	5718.76 ± 571.42^{ab}	2997.7 ± 254.32^{f}	5455.83 ± 590.11^{abc}	2947.12 ± 259.21^{f}	5185.65 ± 563.11^{bc}
Hexadecane	99.45 ± 10.01^{ab}	56.92 ± 5.72^{cd}	32.17 ± 1.01^{d}	108.94 ± 10.98^{ab}	95.87 ± 9.14^{b}	103.93 ± 10.84^{ab}	91.46 ± 9.59^{b}	97.63 ± 9.31^{ab}	86.04 ± 8.15^{b}
Ketones									
2,3-Octanedione	9.74 ± 0.98^{b}	11.08 ± 1.03^{b}	13.24 ± 0.88^{a}	3.15 ± 0.27^{c}	2.08 ± 0.26^{c}	3.00 ± 0.75^{c}	1.98 ± 0.25^{c}	2.74 ± 0.82^{c}	1.67 ± 0.61^{c}
(4E)-4-Hepten-2-one	33.75 ± 3.34^{a}	n.d.^{c}	n.d.^{c}	0.99 ± 0.02^{c}	5.00 ± 0.53^{b}	0.95 ± 0.05^{c}	4.77 ± 0.48^{b}	0.87 ± 0.09^{c}	4.31 ± 0.88^{b}
2-Nonanone	13.21 ± 1.32^{cdef}	24.36 ± 2.44^{a}	20.11 ± 1.32^{b}	9.12 ± 0.71^{fgh}	14.77 ± 1.02^{cd}	8.70 ± 0.42^{gh}	14.09 ± 1.41^{cde}	8.02 ± 0.37^{h}	13.93 ± 1.84^{cde}
(3E,5E)-3,5-Octadien-2-one	9.90 ± 1.07^{b}	25.88 ± 2.65^{a}	3.99 ± 2.45^{cd}	4.98 ± 0.51^{cd}	2.74 ± 0.28^{cd}	4.75 ± 0.25^{cd}	2.61 ± 0.24^{cd}	4.62 ± 0.03^{cd}	2.21 ± 0.22^{d}
Phenols									
Phenol	25.03 ± 2.51^{bc}	41.11 ± 4.19^{a}	38.43 ± 3.12^{a}	21.30 ± 2.17^{bc}	24.49 ± 2.89^{bc}	20.32 ± 0.68^{c}	23.37 ± 2.78^{bc}	20.46 ± 2.10^{bc}	21.51 ± 2.60^{bc}

Chemical compounds	H6-*a*	H6-*b*	H8-*a*	H8-*b*	H9-*a*	H9-*b*	H10-*a*	H10-*b*	H11-*a*	H11-*b*	Statistical significance
Alcohols											
1-Penten-ol	18.73 ± 1.42^{bc}	3.93 ± 0.84^{e}	19.44 ± 1.51^{bc}	4.08 ± 0.41^{e}	17.44 ± 1.29^{cd}	3.66 ± 0.81^{e}	18.12 ± 1.36^{bc}	3.8 ± 0.83^{e}	23.57 ± 2.80^{b}	4.95 ± 0.05^{e}	***
1-Penten-3-ol	1.95 ± 0.64^{e}	6.72 ± 0.22^{b}	2.02 ± 0.65^{e}	6.97 ± 0.71^{b}	1.81 ± 0.63^{e}	6.26 ± 0.17^{d}	1.89 ± 0.64^{e}	6.5 ± 0.20^{d}	2.45 ± 0.6 ^{e}	8.54 ± 0.40^{d}	***
1-Butanol.2-methyl	3.85 ± 0.83^{d}	4.08 ± 0.8 ^{d}	3.99 ± 0.84^{d}	4.23 ± 0.87^{d}	3.58 ± 0.81^{d}	3.80 ± 0.83^{d}	3.73 ± 0.8^{d}	3.95 ± 0.41^{d}	4.84 ± 0.03^{d}	5.15 ± 0.06^{d}	***
3-Buten-2-ol	1.50 ± 0.60^{d}	4.58 ± 0.91^{c}	1.55 ± 0.06^{d}	4.75 ± 0.02 ^{c}	1.39 ± 0.59^{d}	4.26 ± 0.87^{c}	1.45 ± 0.65^{d}	4.44 ± 0.89^{c}	1.88 ± 0.63^{d}	5.76 ± 0.12^{c}	***
1-Octen-3-ol	26.62 ± 2.21^{de}	5.59 ± 0.14^{f}	27.64 ± 2.31^{de}	5.80 ± 0.13^{f}	24.79 ± 2.20^{e}	5.21 ± 0.07^{f}	25.79 ± 2.12^{de}	5.42 ± 0.09^{f}	33.50 ± 3.80^{cd}	24.40 ± 2.89^{e}	***
2-Propyl-1-pentanol	95.66 ± 9.11^{cdefg}	69.83 ± 6.53^{fg}	99.32 ± 9.48^{cdef}	72.50 ± 7.70^{fg}	89.09 ± 8.55^{defg}	65.03 ± 6.05^{g}	92.70 ± 9.72^{cdefg}	67.67 ± 6.31^{g}	120.39 ± 12.84^{bc}	73.97 ± 7.84^{efg}	***

(*Continued*)

Table 2. (Continued).

Chemical compounds	H6-*a*	H6-*b*	H8-*a*	H8-*b*	H9-*a*	H9-*b*	H10-*a*	H10-*b*	H11-*a*	H11-*b*	Statistical significance
1-Octanol	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	4.82 ± 0.03^{a}	***
(2E)-2,7-Octadien-1-ol	23.37 ± 2.78^{cd}	4.91 ± 0.04^{e}	24.26 ± 2.87^{cd}	5.09 ± 0.51^{e}	21.76 ± 2.62^{cd}	4.57 ± 0.90^{e}	22.64 ± 2.71^{cd}	4.75 ± 0.02^{e}	29.41 ± 2.59^{c}	19.60 ± 1.51^{d}	***
2-Phenylethanol	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	n.d.^{c}	***
Aldehydes											
Octanal	3.60 ± 0.81^{d}	2.38 ± 0.68^{d}	3.74 ± 0.82^{d}	2.47 ± 0.41^{d}	3.36 ± 0.79^{d}	2.22 ± 0.67^{d}	3.49 ± 0.35^{d}	2.31 ± 0.68^{d}	4.54 ± 0.90^{d}	4.86 ± 0.48^{d}	***
2-Heptenal	0.93 ± 0.05^{a}	1.00 ± 0.06^{a}	0.97 ± 0.02^{a}	1.03 ± 0.03^{a}	0.87 ± 0.09^{a}	0.93 ± 0.09^{a}	0.90 ± 0.07^{a}	0.97 ± 0.01^{a}	1.17 ± 0.06^{a}	0.00 ± 0.00^{b}	**
(E,E)-2,4-Heptadienal	7.64 ± 0.31^{d}	6.11 ± 0.16^{d}	7.94 ± 0.34^{d}	6.35 ± 0.64^{d}	7.12 ± 0.26^{d}	5.69 ± 0.11^{d}	7.41 ± 0.29^{d}	5.93 ± 0.14^{d}	9.62 ± 0.51^{d}	7.74 ± 0.32^{d}	***
Benzaldehyde	30.58 ± 3.01^{ghi}	42.19 ± 4.66^{cdefg}	31.75 ± 3.62^{fghi}	43.81 ± 4.83^{cdef}	28.47 ± 2.39^{i}	39.29 ± 3.57^{defghi}	29.63 ± 2.51^{hi}	40.89 ± 4.53^{cdefgh}	38.48 ± 3.39^{defghi}	53.16 ± 5.76^{bc}	***
Aromatic hydrocarbons											
Styrene	26.19 ± 2.16^{de}	12.57 ± 0.52^{g}	27.19 ± 2.25^{d}	13.05 ± 1.75^{g}	24.39 ± 2.88^{def}	11.71 ± 1.62^{g}	25.38 ± 2.08^{de}	12.18 ± 1.66^{g}	32.96 ± 3.74^{cd}	15.90 ± 1.14^{efg}	***
Carboxylic acid											
Acetic acid	5.24 ± 0.07^{bcd}	4.56 ± 0.90^{bcd}	5.44 ± 0.09^{bcd}	4.73 ± 0.02^{bcd}	4.88 ± 0.04^{bcd}	4.24 ± 0.87^{d}	5.08 ± 0.55^{bcd}	4.42 ± 0.89^{cd}	6.59 ± 0.20^{b}	5.75 ± 0.41^{bcd}	***
Alpha-hydrogen aldehydes											
3-(Methylthio) propanal	2.13 ± 0.66^{d}	2.75 ± 0.72^{cd}	2.21 ± 0.67^{d}	2.85 ± 0.73^{cd}	1.98 ± 0.64^{d}	2.56 ± 0.70^{cd}	2.06 ± 0.65^{d}	2.66 ± 0.71^{cd}	2.68 ± 0.71^{cd}	3.48 ± 0.79^{cd}	***
Hydrocarbons											
1,3,7-Octatriene	4.58 ± 0.90^{d}	9.49 ± 0.51^{bc}	4.76 ± 0.02^{d}	9.85 ± 0.99^{bc}	4.27 ± 0.88^{d}	8.84 ± 0.09^{c}	4.44 ± 0.89^{d}	9.19 ± 0.56^{bc}	5.77 ± 0.12^{d}	11.94 ± 1.64^{b}	***
Tetradecane	21.89 ± 2.63^{fg}	36.55 ± 3.20^{bcde}	22.73 ± 2.72^{f}	37.95 ± 3.34^{bcd}	20.38 ± 2.48^{fg}	34.04 ± 3.85^{cde}	21.21 ± 2.57^{fg}	35.42 ± 3.09^{cde}	27.55 ± 2.80^{ef}	45.92 ± 4.14^{b}	***
Pentadecane	2869.85 ± 241.40^{f}	5223.13 ± 567.58^{bc}	2979.65 ± 252.20^{f}	5422.96 ± 587.67^{abc}	2672.66 ± 222.21^{f}	4864.23 ± 441.68^{bcd}	2781.01 ± 278.55^{f}	5061.43 ± 506.59bc	3611.70 ± 361.62^{def}	6603.14 ± 660.76^{a}	***
Hexadecane	99.50 ± 9.50^{ab}	87.56 ± 8.30^{b}	103.31 ± 10.33^{ab}	90.91 ± 9.54^{b}	92.66 ± 9.71^{b}	81.54 ± 8.60^{bc}	96.42 ± 9.19^{b}	84.85 ± 8.03^{bc}	125.22 ± 12.07^{a}	106.33 ± 10.18^{ab}	***
Ketones											
2,3-Octanedione	2.87 ± 0.73^{c}	1.90 ± 0.64^{c}	2.98 ± 0.74^{c}	1.97 ± 0.25^{c}	2.68 ± 0.71^{c}	1.77 ± 0.62^{c}	2.78 ± 0.72^{c}	1.84 ± 0.63^{c}	3.62 ± 0.81^{c}	2.40 ± 0.69^{c}	***
(4E)-4-Hepten-2-one	0.91 ± 0.46^{c}	4.56 ± 0.90^{b}	0.94 ± 0.09^{c}	4.74 ± 0.47^{b}	0.84 ± 0.03^{c}	4.25 ± 0.87^{b}	0.88 ± 0.03^{c}	4.42 ± 0.89^{b}	1.14 ± 0.56^{c}	5.75 ± 0.13^{b}	***
2-Nonanone	8.33 ± 0.38^{h}	13.49 ± 1.79^{cde}	8.65 ± 0.87^{gh}	14.01 ± 1.85^{cde}	7.76 ± 0.32^{h}	12.57 ± 1.70^{defg}	8.07 ± 0.36^{h}	13.07 ± 1.75^{cdef}	10.48 ± 1.59^{efgh}	16.99 ± 1.24^{bc}	***
(3E,5E)-3.5-Octadien-2-one	4.55 ± 0.90^{cd}	2.50 ± 0.70^{d}	4.72 ± 0.42^{cd}	2.60 ± 0.71^{cd}	4.23 ± 0.87cd	2.33 ± 0.68^{d}	4.41 ± 0.89^{d}	2.42 ± 0.69^{cd}	5.72 ± 0.12^{c}	3.15 ± 0.77^{cd}	***
Phenols											
Phenol	19.45 ± 1.49^{c}	22.37 ± 2.68^{bc}	20.20 ± 2.43^{c}	23.23 ± 2.77^{bc}	18.12 ± 1.36^{c}	20.83 ± 2.53^{bc}	18.85 ± 1.43^{c}	21.68 ± 2.61^{bc}	24.48 ± 2.89^{bc}	28.08 ± 2.35^{b}	***

Abbreviations: n.d., not detected.
Results indicate mean values of three measurements and are expressed as relative peak areas (peak area of each compound/total area) × 100.
The italic letters, *a* and *b*, refer to the amounts of salt, 125 and 175 g/kg respectively, used to produce salted anchovies; Control 2, experimental production obtained by 500 g salt per 1 kg anchovies.
H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum.
Data within a line followed by the same letters are not significantly different according to Tukey's test (p value: ***, $p \le 0.001$; **, $p \le 0.01$)
Results indicate mean values of three measurement and are expressed as relative peak areas (peak area of each compound/total area) × 100.
The italic letters, *a* and *b*, refer to the amounts of salt, 125 and 175 g/kg respectively, used to produce salted anchovies; Control 2, experimental production obtained by 500 g salt per 1 kg anchovies.
H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum.
Data within a line followed by the same letters are not significantly different according to Tukey's test (p value: ***, $p \le 0.001$; **, $p \le 0.01$)

Table 3. Sensory scores of experimental salted anchovies at the end of the ripening period (150 days).

Descriptors	Control 1-*a*	Control 1-*b*	Control 2	H2-*a*	H2-*b*	H4-*a*	H4-*b*	H5-*a*	H5-*b*	H6-*a*	H6-*b*	H8-*a*	H8-*b*	H9-*a*	H9-*b*	H10-*a*	H10-*b*	H11-*a*	H11-*b*
Aspect:																			
Color uniformity	4.85 ±0.15^{bcde}	4.36 ±0.18^{fgh}	5.12 ±0.03^{b}	4.80 ±0.08^{bcde}	5.07 ±0.03^{bc}	5.87 ±0.10^{a}	3.91 ±0.14^{i}	4.88 ±0.04^{bcd}	5.55 ±0.14^{a}	4.46 ±0.09^{efg}	4.65 ±0.13^{def}	4.15 ±0.03^{ghi}	4.08 ±0.15^{hi}	4.51 ±0.09^{efg}	4.61 ±0.16^{def}	3.94 ±0.12^{i}	4.72 ±0.13^{cdef}	5.60 ±0.14^{a}	5.64 ±0.10^{a}
Color intensity	5.33 ±0.09^{f}	5.83 ±0.05^{cde}	5.93 ±0.14^{bcde}	5.70 ±0.12^{de}	5.92 ±0.05^{cde}	4.88 ±0.15^{g}	5.93 ±0.16^{bcde}	5.34 ±0.10^{f}	6.08 ±0.06^{bc}	6.25 ±0.01^{b}	6.66 ±0.12^{a}	5.69 ±0.15^{e}	5.63 ±0.10^{ef}	6.07 ±0.04^{bc}	5.88 ±0.08^{cde}	5.90 ±0.11^{cde}	5.63 ±0.12^{ef}	6.02 ±0.12^{bcd}	6.79 ±0.08^{a}
Texture:																			
Compactness	6.20 ±0.04^{a}	5.79 ±0.03^{bcde}	5.82 ±0.03^{bcd}	6.09 ±0.02^{a}	5.41 ±0.09^{fg}	6.11 ±0.10^{a}	5.82 ±0.06^{bcd}	6.13 ±0.10^{a}	5.54 ±0.09^{efg}	6.09 ±0.10^{a}	5.51 ±0.05^{fg}	6.10 ±0.06^{a}	5.57 ±0.08^{def}	6.09 ±0.10^{a}	5.52 ±0.04^{fg}	5.92 ±0.09^{abc}	5.67 ±0.13^{cdef}	6.05 ±0.09^{ab}	5.28 ±0.17^{g}
Juicy	3.36 ±0.07^{k}	3.80 ±0.05^{j}	4.00 ±0.11^{j}	4.92 ±0.21^{fgh}	5.41 ±0.04^{bc}	4.66 ±0.11^{hi}	4.77 ±0.09^{h}	4.40 ±0.05^{i}	4.87 ±0.21^{fgh}	4.86 ±0.04^{gh}	5.25 ±0.03^{bcd}	4.71 ±0.07^{h}	5.07 ±0.02^{defg}	4.83 ±0.07^{gh}	5.22 ±0.09^{bcde}	4.94 ±0.03^{efgh}	5.15 ±0.02^{cdef}	5.44 ±0.09^{b}	5.94 ±0.02^{a}
Gummy	4.32 ±0.08^{a}	3.66 ±0.16^{def}	3.52 ±0.13^{f}	3.97 ±0.05^{bc}	3.52 ±0.03^{f}	4.03 ±0.03^{bc}	3.80 ±0.06^{cde}	4.09 ±0.07^{ab}	3.57 ±0.06^{ef}	3.98 ±0.09^{bc}	3.59 ±0.05^{ef}	4.02 ±0.08^{bc}	3.62 ±0.03^{def}	3.99 ±0.03^{bc}	3.59 ±0.06^{ef}	3.86 ±0.08^{bcd}	3.70 ±0.09^{def}	3.85 ±0.11^{bcd}	3.47 ±0.08^{f}
Odor:																			
salt anchovies	4.71 ±0.05^{d}	5.25 ±0.21^{ab}	5.01 ±0.09^{bcd}	5.19 ±0.09^{ab}	4.97 ±0.04^{bcd}	5.11 ±0.09^{abc}	5.09 ±0.15^{abc}	5.03 ±0.09^{bcd}	5.06 ±0.11^{abc}	5.17 ±0.01^{abc}	5.00 ±0.02^{bcd}	5.13 ±0.01^{abc}	5.03 ±0.13^{bcd}	5.16 ±0.07^{abc}	5.00 ±0.09^{bcd}	5.13 ±0.05^{abc}	5.04 ±0.06^{bcd}	5.35 ±0.17^{a}	4.87 ±0.04^{cd}
Flavor:																			
Ham	3.27 ±0.06^{a}	2.46 ±0.11^{g}	2.09 ±0.04^{h}	2.99 ±0.06^{bcd}	2.76 ±0.02^{ef}	3.04 ±0.08^{abc}	2.85 ±0.09^{cdef}	3.09 ±0.09^{ab}	2.66 ±0.07^{fg}	3.00 ±0.07^{bcd}	2.78 ±0.05^{def}	3.03 ±0.09^{abc}	2.76 ±0.04^{ef}	3.01 ±0.09^{bc}	2.78 ±0.04^{def}	2.92 ±0.04^{bcde}	2.85 ±0.05^{cdef}	2.90 ±0.12^{bcde}	2.86 ±0.01^{cdef}
Rancid	3.70 ±0.19^{b}	3.75 ±0.10^{ab}	3.68 ±0.05^{b}	3.21 ±0.06^{cdef}	3.89 ±0.06^{ab}	3.29 ±0.09^{cd}	3.23 ±0.17^{cdef}	3.37 ±0.06^{c}	3.18 ±0.04^{cdef}	3.23 ±0.04^{cdef}	3.97 ±0.02^{ab}	3.27 ±0.04^{cde}	3.07 ±0.04^{def}	3.23 ±0.03^{cdef}	2.99 ±0.06^{f}	3.17 ±0.01^{cdef}	3.04 ±0.05^{ef}	1.04 ±0.03^{g}	0.60 ±0.03^{h}
Putrid	3.76 ±0.05^{ab}	3.63 ±0.08^{b}	3.24 ±0.08^{cd}	3.14 ±0.02^{cde}	3.87 ±0.07^{ab}	3.24 ±0.11^{cd}	3.18 ±0.11^{cde}	3.35 ±0.08^{c}	3.12 ±0.13^{cde}	3.16 ±0.09^{cde}	3.94 ±0.04^{a}	3.22 ±0.17^{cd}	3.03 ±0.06^{de}	3.17 ±0.04^{cde}	2.96 ±0.05^{e}	3.11 ±0.01^{cde}	3.00 ±0.09^{de}	1.93 ±0.02^{f}	0.61 ±0.05^{g}
Overall enjoyment	3.70 ±0.09^{j}	5.42 ±0.11^{ef}	5.12 ±0.10^{fgh}	5.04 ±0.02^{gh}	6.31 ±0.21^{ab}	4.82 ±0.07^{hi}	5.41 ±0.05^{ef}	4.60 ±0.05^{i}	6.01 ±0.10^{bc}	4.99 ±0.06^{gh}	6.08 ±0.10^{bc}	4.86 ±0.09^{hi}	5.95 ±0.09^{c}	4.97 ±0.07^{h}	6.06 ±0.07^{bc}	5.32 ±0.07^{efg}	5.78 ±0.04^{cd}	5.49 ±0.04^{de}	6.60 ±0.27^{a}

The italic letters, *a* and *b*, refer to the amounts of salt, 125 and 175 g/kg respectively, used to produce salted anchovies; Control 2, experimental production obtained by 500 g salt per 1 kg anchovies.

H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum.

Different letters superscript on sensory scores indicate significant differences according to Tukey's test between experimental productions for $p \leq 0.05$

Moreno et al., 2009; Kameura et al., 1986). However, both productions carried out with the H11 strain showed the lowest scores of rancid and putrid and were significantly lower than the score associated with the ham descriptor.

Multivariate statistical analyses

HCA classified the productions in accordance to their mutual dissimilarity and relationship. This analysis classified trials using 19 variables selected on the basis of results from microbial monitoring, histamine, VOCs profiles, and sensorial analyses (Figure 4). Except for controls, all experimental productions were clearly separated into two mega-clusters. Furthermore, within each mega-cluster, two groups at low level of dissimilarity (dissimilarity by Euclidean distance <20%) were found on the basis of the amount of salt used. Among trials, the production carried out with strain H11 and with a lower salt amount (*a*) was separated from all other experimental productions.

The results from microbial counts and histamine and VOC concentrations, as well as from sensory analysis were also subjected to PCAn (Figure 5), which mainly confirmed the distribution of trials obtained by HCA. In detail, a total of three factors (accounting for 51.57, 20.13, and 10.71% of the total variability) showing an eigen-value higher than 1 were found. These results indicated that the initial 19 variables (used as data matrix for PCAn) might be expressed as linear combination of three factors explaining up to 82.41% of the total variability.

The components of the PCAn were correlated to variables as represented by a bi-plot graph that explained 71.70% of the total variability as function of factors 1 and 2. In detail, Figure 5 mainly highlights the discrimination of experimental productions along Factor 1 on the basis of microbial counts and VOCs profiles. In addition, a visible separation resulted between inoculated productions and controls. With regard to factor 2, overall enjoyment, histamine, and hydrocarbons mainly affected the distribution of samples, grouping them according to the amounts of salt used. The production obtained with strain H11-*b* were slightly separated from all others trials.

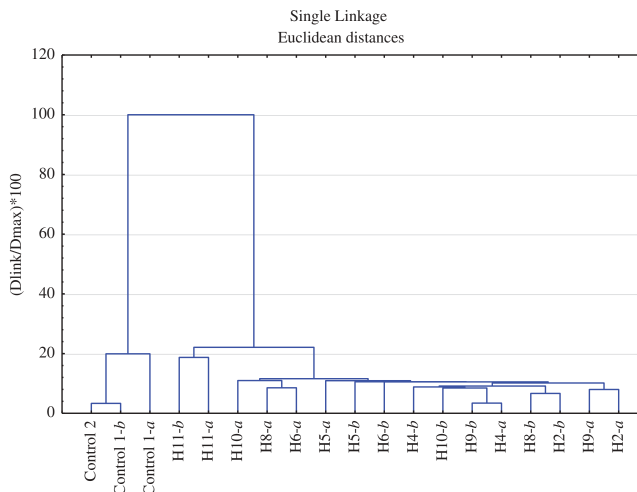


Figure 4. Dendrogram of salted anchovy samples resulting from HCA analysis based on values of microbiological loads, histamine and VOC concentrations, as well as sensory scores. H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum. The italic letters *a* and *b*, associated to codes of samples refer to the amounts of salt, 125 and 175 g/kg respectively, used to produce salted anchovies; Control 2, experimental production obtained by 500 g salt per 1 kg anchovies.

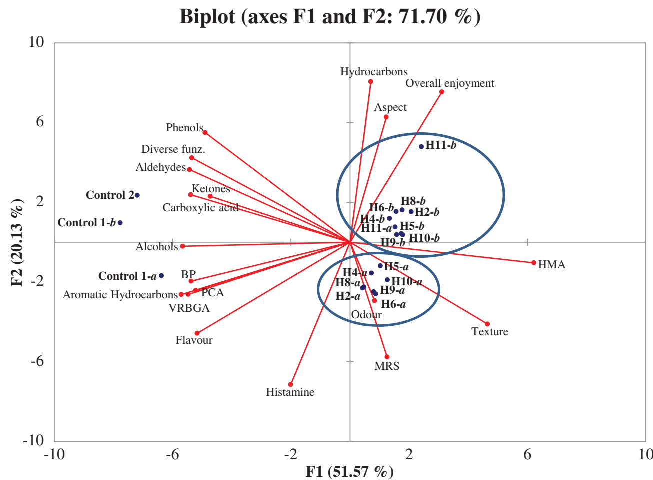


Figure 5. PCAn analysis based on microbiological loads, histamine and VOCs concentrations, as well as sensory scores of the experimental salted anchovies. Bi-plot graphs show relationships among factors, variables, and samples. H2, H4, H5, H6, H8, H9, H10, H11 refer to codes of *Halobacterium salinarum* strains used as inoculum. The italic letters *a* and *b*, associated to codes of samples refer to the amounts of salt, 125 and 175 g/kg, respectively, used to produce salted anchovies; Control 2, experimental production obtained by 500 g salt per 1 kg anchovies.

Conclusion

The inoculum of strain *Hbt. salinarum* H11 in concomitance with a low amount of salt inhibited the growth of undesired microbial populations. In particular, salted anchovies produced with strain H11 showed the lowest histamine content in respect to the other experimental productions and also gained the best sensory evaluation. Furthermore, results obtained by using 175 g of salt per kg of anchovies compared to the amount used in traditional protocols (500 g/kg) did not alter the quality of the final product. Even though the application of strain *Hbt. salinarum* H11 at an industrial level needs to be further investigated, based on these findings, it can be concluded that both inoculum of EHA strains and a salt amount might affect quality of salted anchovies.

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