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Influence of salt of different origin on the microbiological characteristics, histamine generation and volatile profile of salted anchovies (*Engraulis encrasicolus* L.)

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ABSTRACT

The effect of six salts of different geographical areas on the quality of salted anchovies was evaluated. The crude salts were chemically characterized by determination of inorganic and volatile organic compounds (VOCs). Salted anchovies, corresponding to six experimental trials, were subjected to microbiological, chemical (including histamine content) and sensory analysis during the entire period of ripening (150 days). The salts were characterized by marked differences in terms of major cations and trace element amounts. Among the 27 VOCs detected, octadecane was the most abundant compound and the main differences of the salts were registered for alkanes and alcohols. During maturation, significant microbiological differences between the salts were found for the levels of total aerobic mesophilic microorganisms, lactic acid bacteria, *Staphylococcaceae* and *Enterobacteriaceae* counts. All salted anchovies contained histamine below the thresholds allowed by current regulations, but statistical differences were registered for the concentrations of the different trials. Consistent differences were also revealed for their sensory profiles, in particular concerning odour and taste and overall acceptability. Several differences were also detected for dryness, brown colour, putrid odour, rancid and raw blood taste sensory attributes. Especially the differences in the composition (chemical and VOC's) of the raw salts used for the production of salted anchovies has a significant effect on the sensory characteristics of the final product.

1. Introduction

Salting is an ancient technology applied to preserve fishes (Hall, 1992). Once in contact with fish tissues, NaCl induces several physico-chemical changes (Hernandez-Herrero, Roig-Sagues, Lopez-Sabater, Rodriguez-Jerez, & Mora-Ventura, 2002; Roldan, Barassi, & Trucco, 1985; Shenderyuk & Bykowski, 1990). Furthermore, in this condition, the selection of a microbial community occurs and their enzymatic activities affect the lipid and protein component of fish tissues (Czerner, Tomás, & Yeannes, 2011; Hernández-Herrero, Roig-Sagués, López-Sabater, Rodríguez-Jerez, & Mora-Ventura, 1999a) during the ripening process (duration 3–6 months). This process determines changes in color, juiciness, texture, odor and flavor (Coppes, Pavlisko, & Vecchi, 2002; Triqui & Reineccius, 1995a). Marine salts used for salting of anchovies represent the natural habitat of several halophilic archaea (Moschetti et al., 2006). These agents inhabiting extreme environments might be of particular importance for the ripening of salted anchovies (Lee, 2013). Many studies investigated the ability of extremely halophilic archaea (EHA) strains to produce enzymes, in particular proteases, for food applications (Akolkar, Durai, & Desai, 2010; Giménez, Studdert, Sánchez, & De Castro, 2000; Izotova et al., 1983; Kamekura, Seno, Holmes, & Dyall-Smith, 1992; Ryu, Kim, & Dordick, 1994; Shi et al., 2006; Stepanov et al., 1992; Studdert, Seitz, Gil, Sanchez, & de Castro, 2001; Vidyasagar, Prakash, & Sreeramulu, 2006). Most of the proteases produced by EHA are extracellular serine proteases which retain their enzymatic

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capacity at high salt concentrations. With these regards, Akolkar et al. (2010) reported that the inoculum of *Halobacterium* sp. SP1 (a red-pink colour culture) into salted fishes shortened the period of ripening and improved the chemical composition (amino acids profile) and the flavour of the final products. Furthermore, Aponte, Blaiotta, Francesca, and Moschetti (2010) and Alfonzo et al. (2017b) clearly showed that the use of red-pink archaea halophilic strains applied as starters might improve significantly the safety and the sensory quality of salted anchovies (*Engraulis encrasicolus*).

Traditional production protocols of salted anchovies include the use of sea salt (Aponte et al., 2010). Sea salt is processed from seawater left in salt pans. The final product is obtained by crystallisation due to the combined effects of wind and sunlight (Gianguzza, Pellizzetti, & Sammartano, 2002). Before crystallization, seawater circulates along a series of successive ponds increasing the level of salinity due to continuous water evaporation. Contact with the surrounding environment is a potential source of volatile compounds affecting sea salt composition (Silva, Coimbra, Barros, Marriott, & Rocha, 2015). Recent studies have shown that the geographical area of origin influences the chemical composition of sea salts, especially the volatile organic fraction (Silva et al., 2015). Thus, some chemicals can be used as markers to identify univocally each salt type.

Several salt pans are present in Italy with Cervia, Marsala and Pula representing the most important area of sea salt production (Maffeis, 2013). In Europe, the sites of Andiran in France and Santa Pola in Spain are also important. Up to date, the safety and sensory characteristics of salted anchovies have been only associated to the quality of fresh fish and the technological processes applied for production (Lee, 2013), but the role of salt has not been investigated yet. For these reasons, this study, was performed to examine the influence of salt from different geographical origins on the physico-chemical, microbiological and sensory characteristics of salted anchovies.

2. Materials and methods

2.1. Collection, microbiological and chemical analysis of salt samples

Five samples of sea salt and one of mine salt were collected from different European sites (Table 1). Mine salt was included for comparison. All samples were stored at room temperature in a glass vacuum dessicator before analysis of EHA, heterotrophic marine bacteria, lactic acid bacteria (LAB), *Enterobacteriaceae* and *Staphylococcaceae*.

EHA were investigated after enrichment in *Halobacterium* liquid medium (HLM) (Oren & Litchfield, 1999). The enrichement procedure was conducted as reported by Moschetti et al. (2006) inoculating 10 g of each salt sample in 250 of HLM into 500 ml volume conical flasks incubated under constant shaking (150 rpm) and lighting for two weeks at 44 °C. Ten milliliters of each culture were subcultured in the same conditions three times. Finally, 100 μl from each broth were spread on *Halobacterium* medium agar (HMA) and incubated at 44 °C for two weeks.

The other microbial groups were investigated after decimal serial dilutions in Ringer's solution (Sigma-Aldrich, Milan, Italy): heterotrophic marine bacteria on Difco™ Marine Agar 2216 (Becton Dickinson, Milan, Italy) incubated at 25 °C for 72 h; mesophilic LAB rods and cocci on de Man-Rogosa-Sharpe (MRS) agar and M17, respectively, incubated in anaerobiosis at 30 °C for 48 h; members of *Enterobacteriaceae* and *Staphylococcaceae* families were investigated as reported by Alfonzo et al. (2017a). Analyses were performed in triplicate. All media and the supplements were supplied from Oxoid (Milan, Italy).

The six salts were also analyzed for the composition of cations and anions. Thirty-five grams of each sample were mixed in highly purified water (18 milliohm cm-1 water Form a mill-QRG Millipore ultra-pure water System) and brought to 1 L. The cations Na^{+}, K^{+}, Ca^{+2} and Mg^{+2} were measured with an atomic absorption spectrometer Varian AA240 Fast (Kawashima & Nishiyama, 1989). The major anions Cl^{-} and SO_4^{-2} were determined by ion chromatography (Dionex ICS-1100 in chromatograph with Dionex IonPac AS9-HC column) (Atkinson & Bingman, 1997). The remaining elements, Ba, Be, Cr, Cu, Mn, Co, Ni, Cd, Pb, Zn were measured by Inductively Coupled Plasma emission spectroscopy (ICP) (Atkinson & Bingman, 1997).

2.2. Production of experimental anchovies

Fresh anchovies (40 kg) were purchased from a fish market located in Palermo (Italy) and transferred refrigerated (by a portable fridge) to the Laboratory of Fermented Food Preparation of University of Palermo. The anchovies were gutted and put in 3.5 kg jar containing 2.0 kg of anchovies and 1.0 kg of each salt for six experimental trials (CER, FRA, SAR, SPA, MAR and MIN). The production of salted anchovies was performed as described by Aponte et al., 2010. The ripening of salted anchovies was carried out at 20 °C for 150 d. Samples of salted anchovies (about 50 g) were collected before (untreated anchovies, UA) and immediately after the addition of salt (0 day) and at 3, 6, 12, 24, 48, 96 and 150 d of ripening.

All productions of salted anchovies were carried out in triplicate (three jars per trial).

2.3. Monitoring of microbial populations

Samples of anchovies were suspended 1:10 (w/v) in Ringer's solution (Sigma-Aldrich, Milan, Italy), homogenized by a stomacher (BagMixer®400, Interscience, St Nom. France) for 4 min at the maximum speed and subjected to the serial decimal dilutions. Different microbial groups were enumerated: total aerobic mesophilic microorganisms on Plate Count Agar (PCA), incubated at 30 °C for 72 h; mesophilic cocci lactic acid bacteria (LAB) on M17 agar, incubated anaerobically at 30 °C for 48 h; mesophilic rod LAB on de Man-Rogosa-Sharpe (MRS) agar, incubated anaerobically at 30 °C for 48 h; *Enterobacteriaceae* on double-layer Violet Red Bile Glucose Agar (VRBGA), incubated at 37 °C for 24 h; total staphylococci on Baird Parker (BP) and coagulase positive staphylococci (CPS) on BP added with Rabbit Plasma Fibrinogen (RPF) supplement, incubated aerobically at 37 °C for 48 h (APHA, 2015). For the enumeration of halophilic microbial populations, samples of anchovies were suspended 1:10 (w/v) in a 25% (w/v) NaCl solution (Alfonzo et al., 2017b; Aponte et al., 2010; Moschetti et al., 2006). The microbial suspensions were inoculated into Halobacterium medium (HMA) and the count was performed after incubation at 44 °C for 15 d under constant lighting. All media and the supplements were purchased from Oxoid (Thermofisher, Basingstoke, UK). All microbiological analyses were performed in triplicate.

Table 1
Salt samples used in this study.

Samples	Company	Geographical origin	Source
CER	Salina di Cervia	Cervia (RA, Italy)	Salt pan
FRA	Danival	Andiran (France)	Salt pan
MAR	Sale Cucchiara S.R.L.	Marsala (TP, Italy)	Salt pan
SAR	Su Sali Sanpaolo2	Pula (CA, Italy)	Salt pan
SPA	Natural park of Santa Pola	Santa Pola, Alicante (Spain)	Salt pan
MIN	Italkali S.P.A.	Petralia (PA, Italy)	Salt mine