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Effect of the lemon essential oils on the safety and sensory quality of salted sardines (*Sardina pilchardus* Walbaum 1792)

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ABSTRACT

The main aim of this research was to investigate the biopreservative effects of lemon essential oil (EO) micro-emulsions on salted sardines. The experimental design included two experimental trials, SR1 and SR2 carried out with 25 ml of lemon EO micro-emulsion at 0.3 and 1.0% (v/v), respectively, and a control trial performed without EO addition. Chemical analyses on salted sardines inoculated with the EOs clearly showed a substantial persistence of several volatile organic compounds (VOCs) belonging to groups of monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons derived from EOs during the entire period of ripening. In particular, the molecules mostly represented were limonene, p-cymene and β-pinene. Immediately after the addition of EOs, the concentrations of all microbial groups decreased. The presence of Enterobacteriaceae, staphylococci and rod lactic acid bacteria (LAB) observed in the trials SR1 and SR2 was significantly lower than that registered for the control trial during the entire period of monitoring. Furthermore, the addition of EOs determined a lower accumulation of histamine in sardines compared to those of the control trial. The highest scores of sensory evaluation were registered for flavour and overall acceptability of the experimental trials in presence of EOs. On the basis of the increasing interest toward novel food preservatives, we conclude that the use of EOs to produce salted fishes represents a valid strategy to improve safety and sensory characteristics of salted sardines. This work has also economic implications, since the flavour improvement due to the addition of lemon EOs might increase the consumption of sardines by regular and new consumers.

1. Introduction

Essential oils (EOs) are lipophilic substances known since antiquity for their flavouring properties (Guenther, 1948). These compounds, as first reported by De la Croix in 1881, show also bactericidal properties (Boyle, 1955). Nowadays, the application of EOs as natural antimicrobial products is receiving great attention by "green consumers", mainly because they represent an alternative to common food preservatives of chemical synthesis (Burt, 2004; Settanni et al., 2012). Furthermore, the emergence of pathogens resistant to classical preservatives has determined an urgent necessity for alternative antimicrobial agents. Several plant compounds are commonly used as natural agents for food preservation (Nychas, Tassou, & Skandamis, 2003). EOs exert different biological properties (Bakkali, Averbeck, Averbeck, & Idaomar, 2008) and enjoy the "generally recognized as safe" (GRAS) status conferred by the US Foods and Drugs Administration (FDA).

Many factors affect the chemical composition of EOs and their antimicrobial properties (Burt, 2004; Settanni et al., 2014). The biological activity of EOs is due to the occurrence of synergistic and/or antagonistic effects amongst its various components. For food applications, the effectiveness of a specific EO as natural antimicrobial additive is studied considering the EO itself as a whole

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ingredient, rather than a mixture of components (Militello et al., 2011). Recent reports (Randazzo et al., 2016; Settanni et al., 2012, 2014) demonstrated that EOs extracted from *Citrus limon* (cv. Femminello) cultivated in Sicily (south Italy) are highly effective against food spoilage and/or pathogenic microorganisms both *in vitro* and *in vivo* using edible film formulations.

One of the major limit for EO applications is due to the interference with the natural taste and flavour of foods when they are added in high concentrations. In fact, a major problem to the *in situ* efficacy of a given EO derives from its interaction with the food components and/or other additives (Settanni et al., 2012). Thus, higher concentrations of EOs could be needed in food (*in vivo*) to achieve the effect registered *in vitro* (Burt, 2004). The high inhibitory activity of EOs at low concentrations determines a lower amount of EOs to be added to the food matrices, limiting the unwanted changes of the sensory features (Settanni et al., 2012).

The biological activity of EOs especially from aromatic plants have been studied *in vitro*, using various food models (Burt, 2004; Holley & Patel, 2005), as well as commercial food products, such as seafood (Goulas & Kontominas, 2007; Harpaz, Glatman, Drabkin, & Gelman, 2003; Mejlhom & Dalgaard, 2002) and meat (Ismaiel & Pierson, 1990; Skandamis, Tsigarida, & Nychas, 2002). However, limited data are available on the application of EOs, in particularly from citrus fruits, for the extension of shelf-life of fish products.

Lemon juice and lemon fruit are extensively used as flavouring ingredients in a wide variety of foods. These ingredients are commonly added to fishes consumed raw and after cooking, especially in the Mediterranean basin (Goulas & Kontominas, 2007; Özogul, Polat, & Özogul, 2004). Thus, lemon aroma is well accepted for fishery products and the addition of lemon EOs could be positively applied also on salted fishes.

Food salting process represents an effective hurdle to the microbial growth in fishery products, in particular, anchovies and sardines (Aponte, Blaiotta, Francesca, & Moschetti, 2010). However, when salt fails to penetrate fish tissues potential pathogenic and spoilage microorganisms can easily grow. One of the main issues related to the microbial development on fish tissues is represented by the increase of histamine content into final product (EC No. 1019/2013; FDA, 1998).

Italian fishery sector is one of the most important and competitive industry of the Mediterranean area (Crescimanno, Galati, & Bal, 2014; Crescimanno, Galati, Siggia, & Farruggia, 2013). Anchovies and sardines constitute 18% (31,842 tons in 2014) and 14.6% (25,729 tons) of the total Italian seafood production, respectively (INEA, 2015). Although sardines are more abundant than anchovies in the Mediterranean Sea, they have a lower commercial value. The annual average price registered in 2014 for anchovies ranged between 1.51 and 2.45 Euros per kilogram, while sardines were sold at 1.02–1.58 Euros per kilogram (ISMEA, 2016). The total revenue reached 52.4 million Euros for anchovies and 18.5 for sardines, representing 6.4 and 2.3%, respectively, of the revenue of the entire sector (INEA, 2015). For these reasons, a high fishing pressure is exerted on anchovies (García & Palomera, 1996). The implementation of innovative processes for the transformation and preservation of sardines might represents an important economic opportunity for several manufacturing companies located along the Mediterranean basin.

The main scope of the present research was to improve the safety and the sensory quality of salted sardines obtained by the addition of lemon EOs. The specific aims of the work were to characterize the chemical composition of EOs obtained from *Citrus limon* cultivar Femminello and to evaluate the effect of EO micro-emulsion addition on the chemical, microbiological and sensory parameters of salted sardines during the entire period of ripening.

2. Materials and methods

2.1. Lemon fruits, extraction and chemical characterization of EOs

The EOs were obtained from the peels of lemon fruits collected from mature trees of Femminello Siracusano 2Kr cultivated in the "Azienda Sperimentale Palazzelli C.R.A. - Centro di ricerca per l'agrumicoltura e le colture mediterranee Contrada Palazzelli Scordia" (Sicily, Italy, N 37° 17′ 32.424″, E 14° 50′ 58.628″). The peels were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus (Comandè, Palermo, Italy) and the EOs were collected in hexane and dried over anhydrous sodium sulphate as reported by Randazzo et al. (2016). A Gas Chromatography device with Mass Spectrometer detector (single quadrupole) (GC-MS) was used to qualitative assessment of essential oil volatile compound constituent.

2.2. Micro-emulsion of EOs

Due to the limited solubility of EOs in water and to improve their wetting properties, the micro-emulsions of EOs (final volume 25 ml) were set up. The EOs were mixed with surfactant solutions of soybean lecithin and sucrose octanoate ester (SOE) (5:5 mass ratio) prepared in water at a final concentration of 40% (w/w) (Zhang, Critzer, Davidson, & Zhong, 2014). Two EO micro-emulsions (0.3% and 1% v/v) were prepared and stored at room temperature until their use.

2.3. Salted sardine manufacturing and sample collection

Fresh sardines were purchased from a local market located in Palermo (Italy) and immediately transported with a portable fridge (4–6 °C) to the laboratory of Agricultural Microbiology (Department of Agricultural and Forest Science, University of Palermo). Sardines were manually beheaded, gutted and pre-salted. Subsequently, they were transferred in glass jars (known as "arbanelle") of 3 l volume capacity and divided into three aliquots of 1.5 kg representing two experimental trials, SR1 and SR2 carried out with 25 ml of lemon EO micro-emulsion at 0.3 and 1.0% (v/v), respectively, and the control trial performed without EO addition. The EO micro-emulsion was applied on sardine surface by a sprayer (Zhang et al., 2014). The sardines were disposed "head-tail" in layers (Aponte et al., 2010) and spaced out with salt, provided by "Salt Cucchiara srl" company (Marsala, Italy). The sardines were kept under constant pressure with 2 kg weight put on the top of the jars for the first month, reduced to 1 kg until the end of ripening (Aponte et al., 2010).

The ripening of sardines was carried out at 20 °C for 150 d. Samples of sardines (about 50 g) were collected before and immediately after the addition of salt and EOs, and at 3, 6, 12, 24, 48, 96 and 150 d of ripening. The experiment was performed in triplicate (three jars per trial). The entire sardine production was performed twice following the same experimental design as reported above. Two independent productions were carried out at 3 d interval in the same week.

2.4. Microbiological analysis

Sardines (25 g) were suspended at the ratio 1:10 (w/v) in Ringer's solution (Sigma-Aldrich, Milan, Italy) and homogenized in a stomacher (BagMixer®400, Interscience, St Nom. France) for 4 min at the maximum speed. Different microbial groups were enumerated as follows: total aerobic mesophilic microorganisms on plate count agar (PCA) incubated aerobically at 30 °C for 72 h; mesophilic rod lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe

(MRS) agar, incubated anaerobically at 30 °C for 48 h; Enterobacteriaceae on violet red bile glucose agar (VRBGA), incubated aerobically at 37 °C for 24 h; staphylococci on Baird Parker (BP) and coagulase positive staphylococci (CPS) on BP added with RPF supplement, incubated aerobically at 37 °C for 48 h. Analyses were performed in triplicate. Samples were also analysed for the presence of halophilic populations in accordance to the protocol described by Moschetti et al. (2006). To this purpose, each sample (10 g) was transferred into 500 ml conical flask containing 250 ml *Halobacterium* liquid medium (HLM) for the enrichment as described by Oren and Litchfield (1999). Flasks were incubated for 2 weeks at 44 °C under constant conditions of shaking (150 rpm) and lighting. After incubation, 10 ml of each broth culture was transferred into a new flask with 250 ml HLM. After three subcultures, 100 μl were spread on *Halobacterium* agar medium (HAMe) and incubated at 44 °C for 15 d under constant conditions of lighting. All media and the supplements were supplied from Oxoid (Thermofisher, Basingstoke UK).

2.5. Histamine determination

Sardine samples were analysed for histamine content by acid extraction and derivatization applying the methods of Eerola, Hinkkanen, Lindfors, and Hirvi (1992) and Moret, and Conte (1996) with some modifications. To this purpose, the sardines analysed were those sampled before salting and at 75 and 150 days of ripening. Stock standard solutions containing histamine dihydrochloride (Sigma-Aldrich) and 1.7 diaminoheptane (as internal standard) were prepared by adding weighed amount of each compound in ultrapure water (Easypure II, Thermo) at concentration of 1 g/l. The standard solution was stored at 4 °C until use. Aliquots of 5 g of minced sardine samples were added with 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 using 0.45 μm filters (Sartorius, Muggiò, Italy). The solid residue was newly extracted as described above. 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 (5 mg/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%, v/v) and each sample was kept protected from light for 15 min at room temperature. The sample was extracted twice with 1 ml of diethyl ether. The combined extracts were dried and the residue dissolved with 1 ml of acetonitrile and then injected (20 μl) in Agilent 1200 high performance liquid chromatography (HPLC) system equipped with a G1329A high performance autosampler G1316A Thermostated Column Compartment and G1315D DAD detector (Diode Array Detector) for quantification of dansylated histamine. The peaks were integrated at 254 nm. Separation was carried out using an Agilent Eclipse XBD-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 analysing six standard solutions at different concentrations derivatized as described above. All the measurements were performed in triplicate to ensure stability and reproducibility of the method.

2.6. Volatile organic compounds

The VOCs were determined on 5 g of salted sardines, chopped and placed into 250 ml glass vials sealed with silicon septum. After 12 h of equilibration at 25 °C, the SPME (DVB/CAR/PDMS, 50 μm, Supelco) fiber was inserted in the silicon septum by a manual holder system. After 10 min at 25 °C, the SPME fiber was recovered and inserted into the injector port of the gas chromatograph, allowing for 1 min desorption at 250 °C.

A GC instrument (Agilent 6890), equipped with a mass selective detector (Agilent 5975 c), was used for the chromatographic analyses. A fused silica capillary column Carbowax (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness; Supelco) served as the stationary phase. The injector was used in splitless mode at 250 °C. Experimental chromatographic conditions were as follows: Helium carrier gas at 1 ml/min and an oven temperature program with a 5 min isotherm at 40 °C followed by a linear temperature increase of 4 °C min up to 200 °C, where it was held for 2 min. The MS scan conditions were: source temperature 230 °C, interface temperature 280 °C, E energy 70eV, and mass scan range 33–350 amu. A commercial library (NIST05) was used interactively with the MS data for compound identification. The relative proportions of the essential oil constituents were expressed as percentages obtained by GC-MS peak area normalisation, with all relative response factors being taken as one. Three replicates of each sample were analysed.

2.7. Sensory analysis

The sensory characteristics of salted sardines were evaluated according to ISO 5496:2006 at 75, 120 and 150 days of ripening.

The descriptive panel consisted of twelve judges (6 females and 6 males, 25–40 years old) which regularly perform sensory analyses of food products, including salted sardines. Judges were trained in preliminary sessions to gain consensus on the sensory descriptors and the use of the evaluation scale. Preliminary sessions were carried out using commercial salted sardines without the addition of lemon EOs. The same judges were also trained with lemon EOs as previously reported. Each attribute was extensively described and explained to avoid any doubt about the relevant meaning. The parameters related to texture (compactness, juicy and gummy), odour (salt sardines) and flavours (ham taste, rancid and putrid) were assessed into a 10-point quality scale [from 0.00 (absence of the descriptor) to 10.00 (extremely intense)]. Furthermore, the sensory-derived effects of adding EOs to fish were evaluated by an acceptance test (Ekhtiarzadeh et al., 2012), and the overall acceptability of samples were evaluated by panellists using a 10-point scale. The evaluations were carried out by ten trained judges in individual booths under incandescent light. Samples were three-digit coded and the order of serving was determined by random permutation. Two panel replications were carried out for each sample.

2.8. Statistical and explorative multivariate analysis

Results from physico-chemical and microbiological investigation, as well as VOCs and sensory data were analysed using a generalized linear model (GLM) based on ANOVA model that included the effects of treatment (addition of EOmicro-emulsions) and time of ripening, as well as the interaction between EO concentrations and time of ripening. The post-hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to p values of <0.05.

In order to graphically represent the values of concentration of EOs and VOC, a heat map clustered analysis (HMCA), based on double hierarchical dendrogram with heat map plot, was employed to represent the individual content values contained in the data matrix as colours. The relative values of VOC concentration were

depicted by colour intensity from yellow (lowest concentration) to red (highest concentration). Heat map analysis of the volatile levels was performed using the autoscaled data.

Graphic construction were achieved by using XLStat software version 7.5.2 (Addinsoft, New York, USA) for excel.

Furthermore, the explorative multivariate analyses [principal component analysis (PCAn)] was applied to investigate sensory differences among experimentations as reported by Martorana et al. (2015, 2016). All data were preliminary evaluated by using the Barlett's sphericity test (Dillon & Goldstein, 1984; Mazzei, Francesca, Moschetti, & Piccolo, 2010) in order to check the statistically significant difference among samples within each data set. The number of principal factors was selected according to the Kaiser criterion (Jolliffe, 1986). Only the factors with eigen-values higher than 1.00 were retained. Statistical data processing and graphic construction were achieved by using software as reported above.

3. Results

3.1. Determination of chemical composition of lemon EOs

A total of 37 volatile compounds were identified and quantified by GC/MS analysis (Table 1). Three phytochemical groups including the monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons were recognized. The most quantitatively relevant monoterpene hydrocarbons were D-limonene, β-pinene, γ-terpinene and p-cymene. The α-terpineol, α-citral, β-citral, neryl acetate and 4-terpineol accounted for the highest concentration within group of oxygenated monoterpenes; the sesquiterpene hydrocarbons were also detected but at low concentration.

3.2. Microbiological counts

The untreated sardines showed concentrations of TMC, coccus and rod LAB, Enterobacteriaceae and staphylococci of 4.20, 4.33, 3.90, 3.10 and 2.24 Log CFU/g, respectively; halophilic bacteria were not detected. The viable counts of the microbial groups investigated during the ripening of salted sardines are reported in Fig. 1. Immediately after the addition of EOs and salt (day 0), the concentrations of all microbial groups decreased. In details, the addition of EOs caused the reduction of TMC, Enterobacteriaceae and staphylococci of about one order of magnitude reaching the levels of 3.40, 2.15 and 1.65 Log CFU/g, respectively.

During the entire period of monitoring, the levels of Enterobacteriaceae, staphylococci and rod LAB registered for the trials SR1 and SR2 were significantly lower than those of the control trial. The highest differences between the experimental and control trials were, on average, 1.5 Log cycles and were found for rod LAB. During the ripening period, significant differences were also found for staphylococci which reached the highest concentration in SR2 samples between 10 and 120 d. The lowest levels of Enterobacteriaceae were found for the trial SR2 between 15 and 60 d.

Furthermore, some differences between the trials SR1 and SR2 were registered for the levels of members of Enterobacteriaceae family and staphylococci between 15 and 90 d; in particular, these two populations were less concentrated in trial SR2 (<1.5 Log CFU/g).

At the end of ripening (day 150), Enterobacteriaceae and staphylococci disappeared from both experimental trials and they were detectable only in the sardines of the control trial at 1.2 Log CFU/g. The halophilic bacteria appeared from day 30 (1.0 Log CFU/g) onwards until the end of ripening (3.5 Log CFU/g) both in experimental and control trials.

Table 1
Chemical composition of lemon EOs.

Chemical compounds[a]	Experimental lemon EO
Monoterpene hydrocarbons	88.69
α-Thujene	0.27
α-Pinene	1.53
Camphene	0.07
Sabinene	1.23
β-Pinene	12.74
β-Myrcene	1.13
4-Carene	n.d.
p-Cymene	7.23
D-Limonene	59.48
Ocimene	0.15
γ-Terpinene	3.78
2-Carene	0.19
Limonene epoxide	0.45
Limonene oxide. trans-	0.48
Oxygenated monoterpenes	10.45
Octanal	0.19
1-Octanol	0.05
Linalol	0.36
Nonanal	0.22
Fenchol	0.05
cis-β Terpineol	0.05
β-Terpinol	n.d.
Citronellal	0.05
Borneol	0.07
4-Terpineol	1.08
α-Terpineol	2.01
Decanal	0.10
trans-Carveol	0.10
cis-Geraniol	0.27
β-Citral	1.42
Carvone	0.07
Trans-Geraniol	0.30
α-Citral	1.98
1.2-Cyclohexanediol. 1-methyl-4-(1-methylethenyl)	0.18
Geranyl acetate	0.81
Neryl acetate	1.12
Sesquiterpene hydrocarbons	0.86
α-Bergamotene	0.30
β-Farnesene	n.d.
α-Caryophyllene	n.d.
β-Bisabolene	0.24
Caryophyllene	n.d.
Spathulenol	0.26
Caryophyllene oxide	0.06

Abbreviations: n.d., not detectable.
[a] Data are means percentage of three replicate of all samples analysed against the total peak area.

3.3. Histamine determination

Data from determination of histamine content in samples collected during the sardine ripening are depicted by Fig. 2. The highest concentrations of histamine were found in the untreated sardines (71.09 μg/g) and in the control trials (between 61.06 and 48.23 μg/g) during the entire period of ripening. In particular, both trials treated with lemon EO showed the most rapid decrease of histamine concentration from day 75 onwards. Although trial SR2 showed the lowest values of histamine, no significant differences were found between the two experimental trials during ripening of salted sardines.

3.4. Monitoring of VOC concentration

The profiles of the VOCs emitted from the sardines were monitored during ripening (Table 2). Limonene and p-cymene were the compounds detected at the highest levels in both experimental trials. In particular, these two VOCs were quite constant during the entire period of ripening. β-pinene was also detected in both trials

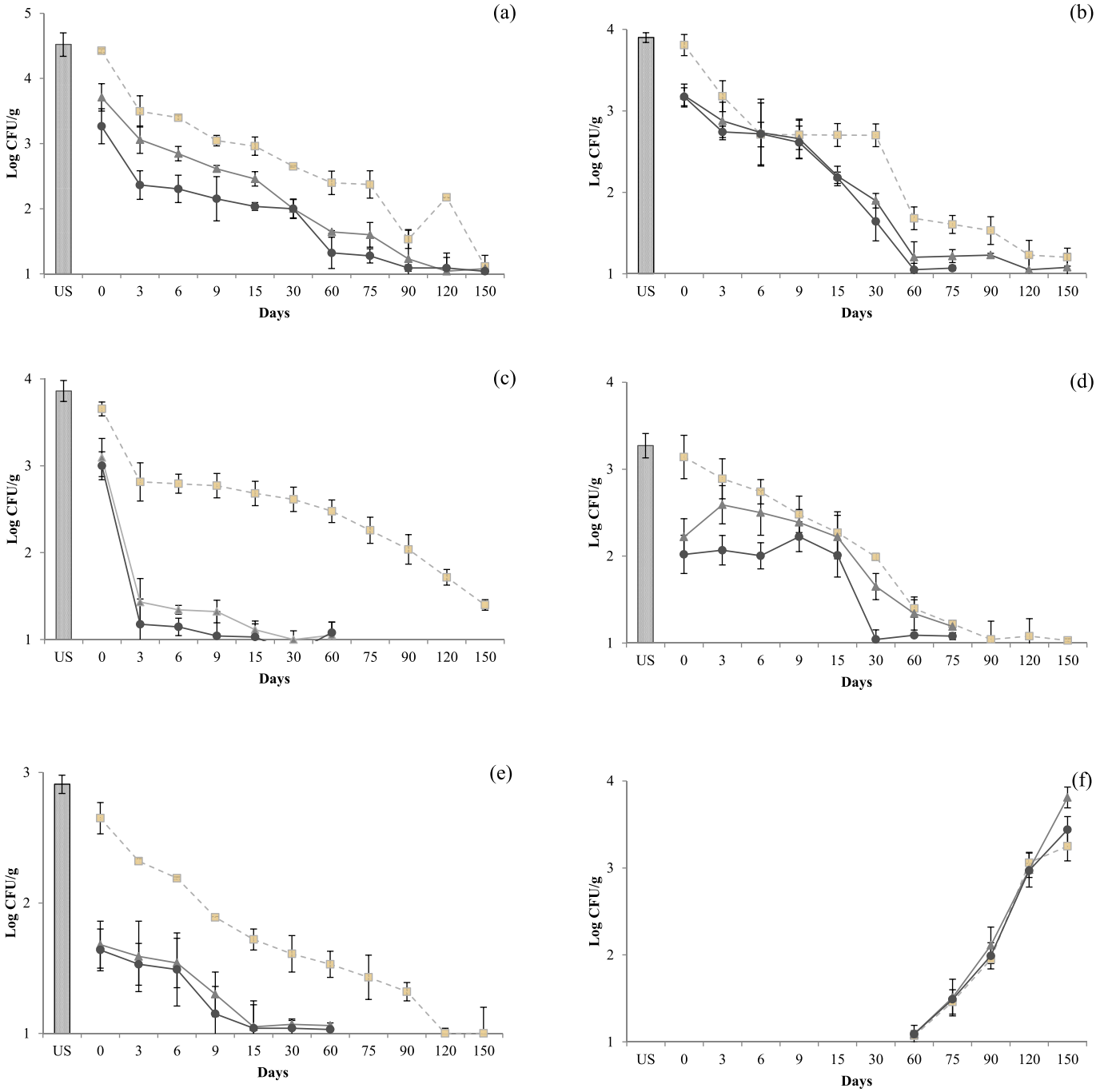

Fig. 1. Microbiological concentrations (log CFU/g) during the ripening period [from days 0 (just after the addition of EOs and salt) until day 150 (end of ripening)] of salted sardines. Results indicate mean values ± standard deviation of three measurements. Letters between brackets per each graphs indicate: (a), PCA, total aerobic mesophilic microorganisms; (b), M17, coccus lactic acid bacteria; (c), MRS, rod lactic acid bacteria; (d), VRBGA, Enterobacteriaceae; (e), BP, Staphylococcaceae; (f), HMAm, extremely halophilic archaea bacteria. Abbreviation: US, untreated sardines (before the addition of EOs and salt). Symbols: ■, control; ▲, SR1; ●, SR2.

and during the entire period of sardine manufacturing, but at lower levels than limonene and p-cymene. No VOCs derived from EOs were detected in salted sardine samples of control trial.

3.5. Sensory analysis

The experimental salted sardines were evaluated by expert panellists in sensory analysis of salted fishes (Table S1). Samples inoculated with the micro-emulsions of lemon EO differed significantly ($p < 0.05$) from those of the control trials in terms of VOC emission. The main differences among the trials were registered for salted sardines and ham flavour. The sardines of the experimental trial SR2 showed the right overall acceptability. Both experimental trials showed the lowest values of rancid and putrid descriptors.

3.6. Statistical and explorative multivariate analysis

The analysis of variance for the three experimental productions of salted sardines showed significant effect among experimental treatment [addition of lemon EOs at two (0.3 and 1%)

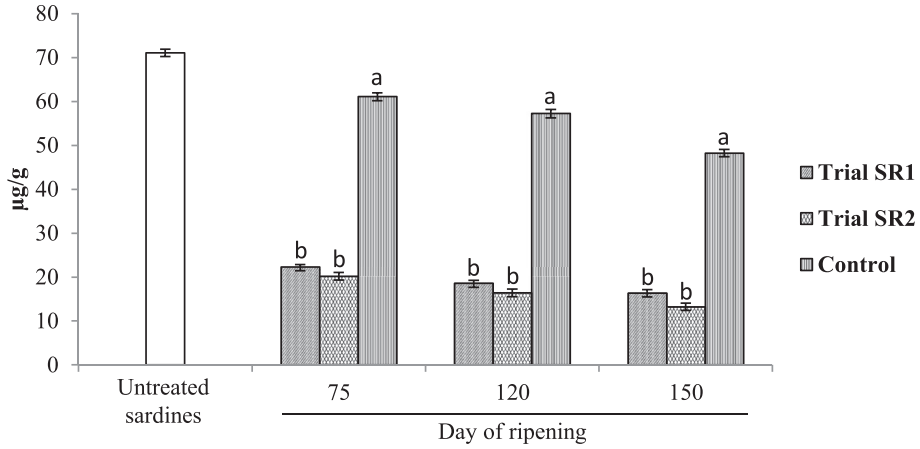

Fig. 2. Values of histamine (µg/g) determined in the experimental productions of salted sardines during manufacturing and ripening period. Results indicate mean values of three measurements. Different letters indicate significant differences (for $p < 0.05$) among experimental productions (SR1, SR2 and control) within the same day of sampling.

Table 2
Concentration of VOCs derived from EOs during the ripening of salted sardine.

VOCs	Trial SR1					Trila SR2					Control[a]
	Day 3	Day 15	Day 75	Day 120	Day 150	Day 3	Day 15	Day 75	Day 120	Day 150	
α-Pinene	1.37	1.21	1.18	1.25	1.32	0.70	0.59	0.54	0.62	0.65	n.d.
β-Pinene	7.70	6.71	6.50	6.02	5.43	7.31	7.24	7.49	6.98	6.20	n.d.
Sabinene	1.02	0.89	0.80	0.73	0.67	2.01	1.67	1.82	1.57	1.36	n.d.
β-Myrcene	0.98	0.84	0.80	0.72	0.67	0.97	1.17	1.42	0.98	0.97	n.d.
Limonene	73.07	71.28	74.35	71.82	70.87	74.02	76.08	77.11	74.20	73.44	n.d.
γ-Terpinene	1.08	1.18	1.22	1.04	1.41	2.01	1.93	1.74	1.87	1.85	n.d.
p-Cymene	14.08	16.99	14.11	17.22	18.76	12.12	10.51	9.75	13.41	14.77	n.d.
5-hepten-2-one-6-methyl	0.70	0.90	1.04	1.20	0.87	0.86	0.81	0.13	0.37	0.76	n.d.

Results indicate mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant and common peaks to all samples) × l00.

[a] All VOCs presented in the table were not detected (n.d.) in the salted sardines of control trial during the entire period of monitoring.

concentrations], time of ripening and dependent variables corresponding to microbiological data, histamine concentration, VOC composition and sensory scores.

The Barlett's sphericity test was applied to all data matrix inputs and differences statistically ($p < 0.001$) significant were found among trials.

To better investigate the effects of the addition of EOs to sardines, the results obtained from VOC analysis were also subjected to HMCA (Fig. 3). In details, the heat map plot graphically depicted the relative percentages of many VOCs (in particular limonene, β-pinene and p-cymene) into experimental salted sardines until 150 d of ripening. Furthermore, the clustering dendrogram of heat map plot grouped all samples into mega-clusters characterized by different relative abundances of many VOCs. Samples from the trials SR1 and SR2 resulted significantly separated during the entire period of monitoring as effect of the two concentrations of EOs added to salted sardines.

The multivariate statistical analysis was concluded by using data of sensory analysis of salted sardines sampled on day 75, 120 and 150 of ripening as illustrated by biplot graphs in Fig. 4 (a, b, c). Both trials SR1 and SR2 were closely associated with salted sardines odour and taste, and overall enjoyment descriptors that positively characterized the final products. On the contrary, the control trial showed the closest correlation with descriptors (putrid, rancid, gummy) that negatively affect the salted sardines.

4. Discussion

The scope of the present work was to apply a new technology based on the addition of lemon EO micro-emulsions for the manufacture of salted sardines. According to the scientific literature, up to now there are no studies on the effect of EO addition on the microbiological, chemical and sensory characteristics of salted fish, as well as during manufacturing of salted sardines.

The results of the chemical analysis on salted sardines inoculated with EO micro-emulsions clearly showed a substantial persistence, at high concentration, of several VOCs derived from EOs during the entire period of ripening. D-limonene (4-isopropenyl-1-methylcyclohexene), a natural monoterpene with a lemon-like odour, is a major constituent of several citrus derived EOs such as orange, mandarin, lemon, lime, and grapefruit (Randazzo et al., 2016; Settanni et al., 2012). Owing to its pleasant citrus fragrance, it is widely used in cosmetics, foods, and consumer's products. D-limonene is listed in the code of federal regulation as GRAS for use as a flavouring agent and in food preservation (Sun, 2007). Furthermore, several biochemical activities such as antimicrobial (Chikhoune, Hazzit, Kerbouche, Baaliouamer, & Aissat, 2013; Settanni et al., 2012; van Vuuren & Viljoen, 2007), antioxidant (Roberto, Micucci, Sebastian, Graciela, & Anesini, 2010), chemo-preventive (Crowell, Lin, Vedejs, & Gould, 1992; Wattenberg & Coccia, 1991), anticarcinogen (Crowell & Gould, 1994), as well as antidiabetic proprieties (Murali, Karthikeyan, & Saravanan, 2012) have been reported for D-limonene. The p-cymene, a biological precursor of carvacrol, is a hydrophobic chemical that might cause swelling of the cytoplasmic membrane of microorganisms (Ultee, Bennink, & Moezelaar, 2002). The p-cymene is not an effective antibacterial when used alone (Dorman & Deans, 2000; Juven, Kanner, Schved, & Weisslowicz, 1994; Ultee, Kets,

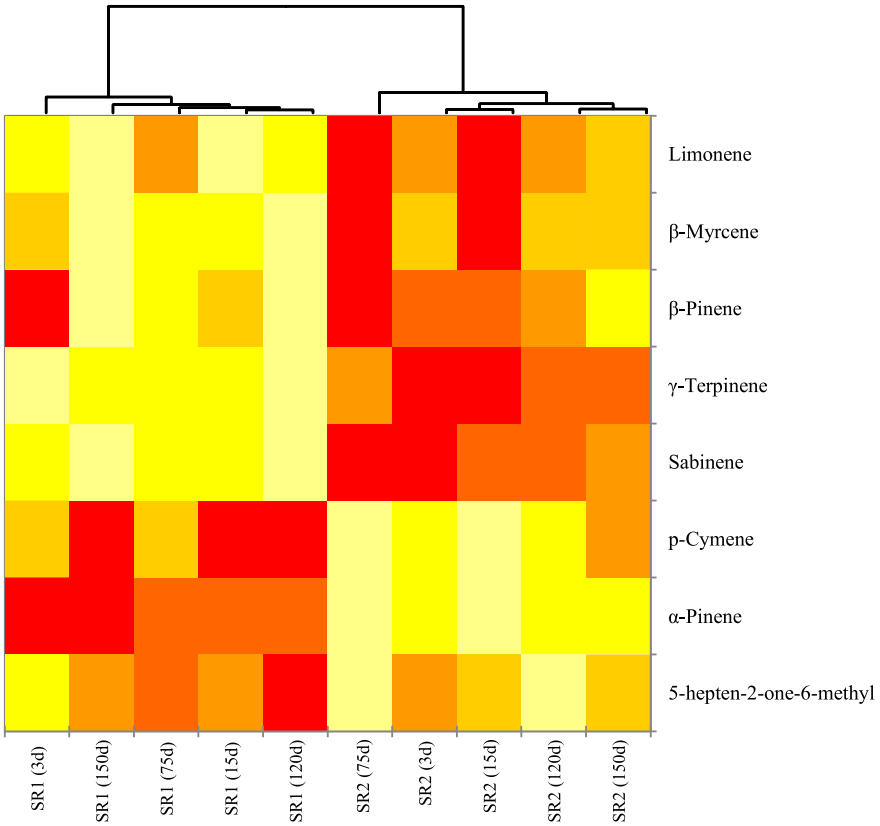

Fig. 3. The clustering dendrogram of heat map plot depict the distribution of relative percentage of VOCs (y-axis) derived from EOs among salted sardine samples (x-axis) collected at 3, 15, 75, 120 and 150 days of ripening. The relative percentages for VOCs are depicted by colour intensity from yellow (lowest concentration) to red (highest concentration). Codes (SR1 and SR2) correspond to experimental trials. The numbers associated to each code correspond to day of ripening per each experimental trials. The VOCs derived from EOs were not detected in salted sardine samples of control trial, thus results from control samples have been not reported (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Alberda, Hoekstra, & Smid, 2000), but when combined with other compounds derived from EOs, a potential synergism is observed against pathogenic microorganisms (Ultee et al., 2000).

The antimicrobial activity of the EOs in foods is important to inhibit proliferation as well as to reduce the populations of pathogenic or spoilage bacteria. In our study, the addition of EOs to salted sardines determined low concentrations of spoilage and pathogenic microorganisms during the entire period of sardine ripening.

Microbial pathogens associated to foods mainly originate from the raw materials and/or cross-contamination during food processing (Nguyen-the & Carlin, 1994; Beuchat, 1996; Seymour & Appleton, 2001). The incidence of foodborne outbreaks caused by contaminated foods has increased in recent years (WHO, 2002; EFSA, 2015; Mukherjee et al., 2006).

Within microbial populations of salted fishes, the Enterobacteriaceae family includes several species and strains recognized as pathogens and opportunistic pathogens for humans. They are commonly isolated from gut microbiota of humans (van Schaik, 2016) and other animals (Schierack, Walk, Reiter, Weyrauch, & Wieler, 2007). Thus, the risk associated to Enterobacteriaceae contamination should be reduced during food manufacturing. In meat and fish foods, the decarbossilation activity of Enterobacteriaceae strains might result in biogenic ammine production such as putrescine, cadaverine and histamine.

So far, several species of halophilic bacteria have been considered as potential spoilage microorganisms during ripening of salted fishes. Recently, Lee (2013) reported the use of halophilic bacteria for industrial food application. The inoculum of halophilic bacteria as starter culture into salted fishes might shorten the ripening period of salted anchovies and might improve the safety and sensory quality of the final products due to decrease of the levels of Enterobacteriaceae and staphylococci. Aponte et al. (2010) and Akolkar, Durai, and Desai (2010) clearly showed that the use of archaea halophilic strains (red-pink colour shape) as starters improve significantly the safety and the sensory quality of salted anchovies without colour alteration of processed fishes. Thus, the presence of halophilic bacteria during the ripening of salted fishes, including pink-colored shape strains, does not affect negatively the quality of the product. Furthermore, the pink spoilage of ripened fishes depends on the strains.

Although, the decrease of the microbial concentrations is commonly reported during the ripening period of salted fishes (Aponte et al., 2010), as well as for the majority of salted foods (Francesca et al., 2016), the presence of many microbial populations during ripening of salted fishes has been clearly associated to an increase of histamine content in the final products (EC No. 1441/2007; EC No. 1019/2013; FDA, 1998).

In this work, the addition of EOs at two different concentrations (0.3 and 1.0%) determined lower accumulation of histamine in sardine than control trial until the end of ripening of salted sardines. Several studies showed that the histamine is mainly produced by histamine decarboxylase enzymes originated from many species of microorganisms, as well as from enzymes of animal origin. On the other hand, Tapingkae, Tanasupawat, Parkin, Benjakul, and Visessanguan (2010) reported the histamine-degrading (either oxidases or dehydrogenases) activities by extremely halophilic archaea bacteria that are commonly

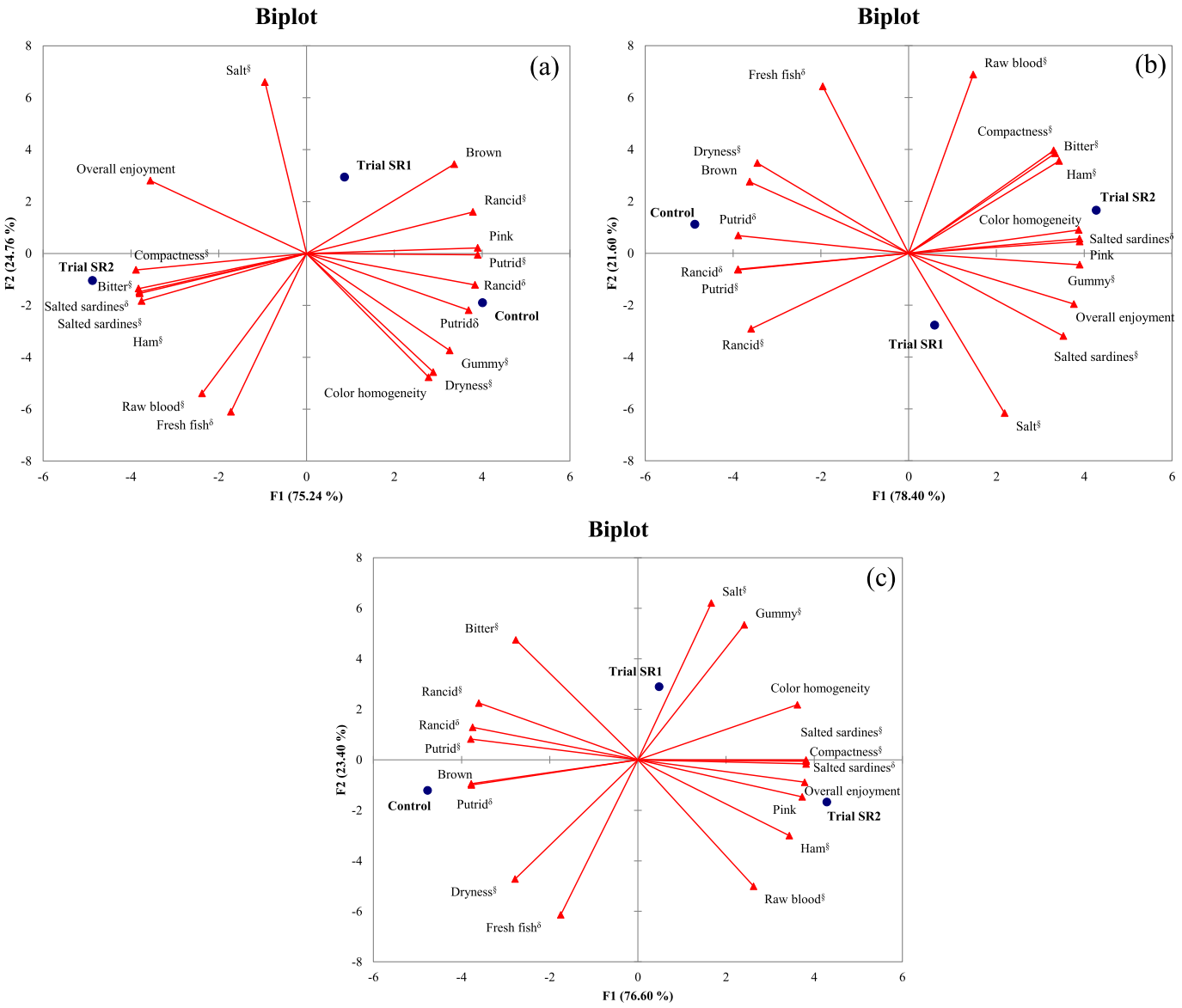

Fig. 4. PCA for sensory data of salted sardines at day 75 (Fig. a), day 120 (Fig. b) and day 150 (Fig. c) of ripening. Biplot graphs show relationships among factors, variables and treatments. Codes (SR1 and SR2) correspond to experimental trials. Symbols: δ, refers to odour descriptors; §, refers to taste descriptors.

associated to salted food products. However, several studies in the scientific literature have highlighted the presence of histamine in sardines preserved in ice (Erkan & Özden, 2008) and marinades (Kilinc & Cakli, 2005), but no studies are available on the variation of the content of histamine in sardines subjected to salting process.

Fishery foods present a high risk of histamine intoxication and many regulations have been worldwide legislated to limit this risk (EC No. 1441/2007; EC No. 1019/2013; FDA, 1998). The histamine limits for ripened brined and/or salted fishery products have been established at 400 and 500 ppm in Europe and USA, respectively. In contrast to several data on fish preservation obtained by salting process (Bellagha, Sahli, Farhat, Kechaou, & Glenza, 2007; Boudhrioua, Djendoubi, Bellagha, & Kechaou, 2009), as well as on the use of EOs as food bio-preservatives (Can, 2011; Goulas & Kontominas, 2007; Mahmoud, Yamazaki, Miyashita, Shin, & Suzuki, 2006), no data have been reported on the use of EOs to inhibit microbial growth and to limit the histamine formation during the ripening of salted fishes.

With regards to sensory analysis, the salting process leads to obtain a tender fishery product with a specific, pleasant aroma and taste (Aponte et al., 2010; Hernández-Herrero, Roig-Sagués, López-Sabater, Rodríguez-Jerez, & Mora-Ventura, 1999), firstly due to the diffusion of salt into fish tissues and, subsequently, to the enzymatic pathways that decompose proteins and fats during the ripening stage (Hernández-Herrero, Roig-Sagués, López-Sabater, Rodríguez-Jerez, & Mora-Ventura, 2002; Voskresensky, 1965). The enzymes that determine the autolysis of tissues may have a fish tissue and/or a microbial origin. In the present work, the sensory-derived effects of lemon EO addition to salted fishes were positively registered by panellists. Furthermore, no off-odours were recognized into the experimental salted sardines after 75, 120 and 150 d of ripening.

A great number of EO components have been registered by the European Commission for use as flavouring in foodstuffs. Among these chemicals, limonene, p-cymene, eugenol, menthol and many others have been clearly referred as no dangerous for the consumers' health.

In conclusion, our study provided insights of the changes of several microbial populations that characterize salted sardines

produced by the addition of micro-emulsions of lemon EOs, as well as chemical and sensory characteristics of the final products. It can be assumed that in terms of microbiological data (higher inhibition of microbial concentrations of alterative and pathogen microorganisms during ripening), chemical characteristics (higher reduction of histamine content) and sensory scores (higher overall acceptability) of sardines, the SR2 treatment showed the best results.

On the basis of the increasing interest toward novel food preservatives, in particular to novel natural antimicrobial agents, we conclude that the use of EOs to produce salted fishes might represent a valid strategy to improve safety and sensory characteristics of salted sardines. It can be also speculated on the economic implications of our findings. This treatment might have a positive impact on the fishery industries and sardine market. In particular, the flavour improvement pursued by this study might offer new and significant opportunities to increase the consumption of sardines by regular and new consumers.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2016.10.046.

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