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INFLUENCE OF OREGANO ESSENTIAL OIL ON THE INHIBITION OF SELECTED PATHOGENS IN "ALHEIRA" DURING STORAGE

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ABSTRACT

Background. Plant-derived essential oils (EOs) have shown remarkable antimicrobial potential against spoilage and pathogenic microorganisms in meat and meat products. The aim of this study was to investigate the influence of oregano EO on the inhibition of *Salmonella* Enteritidis, *Listeria monocytogenes* and *Staphylococcus aureus* in an internal mixture of "Alheira" during storage.
Material and methods. Different concentrations of oregano EO (4%, 1.5%, 0.5%, 0.195% and 0.0975%) were evaluated against the selected pathogens during 21 days of storage at 4°C. The pH and water activity values and lactic acid bacteria (LAB) counts were also evaluated. Finally, sensory assessment was performed.
Results. The antibacterial effect varied according to the oregano EO concentration used, and target pathogen. Oregano EO at 4% demonstrated the highest antimicrobial activity against all the pathogens tested. The lowest concentrations used (0.195% and 0.0975%) resulted in ~2–3 log reduction, but only for *L. monocytogenes* after 21 days of storage. Counts of LAB were ~10^9 CFU/ml for all samples and no differences in the pH and a_w values were detected between samples. However, at a concentration of 0.195%, Oregano EO had a negative impact on consumer acceptance of "Alheira".
Conclusion. These results could be interesting for the meat industry, as a starting point for other studies that have now to concentrate on strategies to "mask" unpleasant sensorial alterations caused by EOs in "Alheira" and helping the industry to ensure the microbiological safety of its products.

Keywords: foodborne pathogens, fermented meat sausage, oregano essential oil, food safety

INTRODUCTION

Fermented meat products are unique and important elements in the economy of certain regions. Their culinary heritage is related to the flavour and texture (organoleptic characteristics) of the product (Ojha et al., 2015). In Portugal, there are a wide variety of sausages, predominantly manufactured in Northern (mainly in Trás-os-Montes) and Southern regions of the country (mainly in Alentejo).

Production of traditional sausages relies on natural contamination by environmental microorganisms. This contamination occurs during slaughtering and increases during manufacturing (Albano et al., 2009).

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Dry fermented sausages are generally considered to be microbiologically safe products. Factors such as pH, temperature, water activity (a_w), nutrients and salt content constitute hurdles which play a critical role in suppressing the growth of microorganisms. Moreover, values of a_w close to 0.90 were proposed to increase microbiological stability, increasing the shelf life of these kind of products (reviewed by Kumar et al., 2017). This safety assurance relies on sufficient anti-pathogen effects of multiple antimicrobial factors according to the so-called "hurdle concept" (Heir et al., 2013). However, in cases of the initial contamination of the raw materials with high levels of pathogenic bacteria and/or insufficient control of the antimicrobial factors, the safety of these products can become compromised (Heir et al., 2013; Oliveira et al., 2018).

"Alheira" is a traditional fermented meat sausage typical of the North of Portugal and is made from a combination of pork meat, pork lard, poultry, wheat bread and olive oil mixed with salt, garlic and spices to achieve the desired taste (Albano et al., 2009). "Alheiras" are cooked before consumption either by frying, grilling or boiling, according to regional traditions or consumer preferences. In recent years, "Alheiras" have been characterized by their chemical and microbiological characteristics (Albano et al., 2009; Esteves et al., 2008; Ferreira et al., 2006). Several authors have observed that LAB constitute the predominant microbiota of "Alheiras", with particular incidence of *Lactobacillus* spp. and *Enterococcus* spp. (Albano et al., 2009; Esteves et al., 2008). A high number of *Enterococcus* spp. has been found in fermented meat products, since they usually grow during early fermentation and remain constant at a level of 4–6 log cfu/g until the end of the whole process (Giraffa, 2002). This high number could also mean a failure in hygiene and is therefore of great importance. Besides this, enterococci are intrinsically resistant to several antimicrobials and they can easily acquire resistance (Barbosa et al., 2017). However, enterococci are applied in food fermentation processes to improve the sensorial quality of foods (Foulquié et al., 2006). Therefore, the exclusion of virulence factors and/or antibiotic resistances is relevant for safety assessment and to ensure that there is no possibility of inter- species transfer of resistance between foods (Barbosa et al., 2017). Pathogenic organisms, such as *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus*, microorganisms that may influence the safe consumption of this food, have already been detected in "Alheiras" (Esteves et al., 2008; Ferreira et al., 2006). Regarding physicochemical parameters and composition, "Alheiras" have a pH ~5.1, 1.1% NaCl, a_w of ±0.95, 52.3% of moisture, 13% of lipids, 14% total protein and 13% of carbohydrates. Generally, these indicated that, according to the accepted limits for these parameters, pH, salt content and moisture *per se*, do not assure the microbiological safety of this product. Moreover, even this is a product that is consumed cooked, Felício et al. (2011) suggesting that internal temperatures may often not be sufficient to kill all of the pathogens originally present, or the thermostable toxins that could be produced, such as staphylococcal enterotoxins, the causative agent of staphylococcal food poisoning (Le Loir et al., 2003). This justifies the use of natural antimicrobial components such as essential oils.

Plant-derived essential oils (EOs) have shown remarkable antimicrobial potential against spoilage and pathogenic microorganisms in meat and meat products. Essential oils are among the most important active constituents of aromatic plants and spices (Krisch et al., 2010). Their use as flavouring agents in the food industry has also been increasing in order to avoid the use of traditional chemical additives (Ghabraie et al., 2016). Due to consumers' negative perceptions about chemical food additives, natural preservation methods and natural preservatives receive greater attention from the food industry (García-Diez et al., 2016). The differences in antimicrobial activity between each oil are usually associated with their chemical compositions, which change according to the seasons, geographical location and/or the method of EO extraction (García-Diez et al., 2016; Kokkini et al., 1997).

This study aimed to evaluate the influence of oregano essential oil in an internal mixture of "Alheira" on *Salmonella* spp., *L. monocytogenes* and *St. aureus* during 21 days of storage at 4°C.

MATERIAL AND METHODS

Composition of oregano essential oil by Head Space Solid Phase Micro Extration (HS-SPME)

The EO used in this study was Oregano (*Origanum vulgare* L.), kindly provided by Ventós Chemical (Barcelona, Spain).

The oregano EO was diluted in water (1:10000) and 10 ml of sample with 20 µl of internal standard (3-octanol 50 mg/L), and 0.5 g of sodium sulphate was added in a vial with a maximum volume of 20 ml. The SPME fiber (DVB/CAR/PDMS; Supelco; Bellefonte, PA, USA) was inserted into the headspace of the vial. Pre-incubation was performed at 40°C for 5 min at 500 rpm, and extraction was carried out for 10 min at 40°C, at 250 rpm. Volatile compounds were desorbed by inserting the fiber into the injection port of a Varian CP-450 gas chromatograph (Walnut Creek, CA, USA) operated in splitless mode for 2.5 min at 220°C. Helium was used as a carrier gas with a constant flow rate of 1 mL/min. The compounds were separated on a VF-WAXms column (Lake Foreste, CA, USA) measuring 15 × 0.15 mm and with a 15 µm film thickness. A Varian Saturn 240 mass spectrometry detector (Walnut Creek, CA, USA) was used and detection was carried out on the total ion current obtained. The mass range acquisition was 33–350 m/z. The compounds were quantified with ion extraction (m/z) and the retention time (rt) was determined in minutes.

Microorganisms and growth conditions

The strains used in this study were *L. monocytogenes* L7946, *L. monocytogenes* L7947 (McLauchlin et al., 1997), *L. monocytogenes* SCOTT A, *S.* Enteritidis, *S.* Enteritidis 417536, *S.* Enteritidis 545047, *St. aureus* 18N, *St. aureus* 2037 M1 (ESB culture collection (Escola Superior de Biotecnologia, UCP, Porto, Portugal) and *St. aureus* ATCC 292 13. All microorganisms were stored at –20 C in Tryptic Soy Broth (TSB, Pronadisa, Madrid, Spain) with 6 g/l of YE (Lab M, Bury, UK) containing 30% (v/v) glycerol (Sigma, Steinheim, Germany), and sub-cultured twice before being used in assays.

Each bacterial strain was grown on Tryptic soy agar (TSA, Pronadisa) with 6 g/l of YE (Lab M) at 37°C for 24 h.

Inhibitory effect of the oregano essential oil against selected pathogens in an internal mixture of "Alheira"

The "Alheiras" used in this study had rooster meat (40%), wheat bread (wheat flour, baking powder and salt), pork, cooking broth, olive oil, onion, parsley, spices and soy as their main ingredients. Before starting, the casing was removed and only the internal mixture of the "Alheira" was used. In order to ensure a homogeneous sample, an internal mixture of 12 different "Alheiras" was well mixed together in the same bag (~2.5 kg).

Individual strains of each target pathogen were sub-cultured twice (24 h at 37°C) in 10 ml Mueller-Hinton Broth (MHB, Biokar, France) using a 1% v/v inoculum. Each culture was centrifuged, and cocktails of individual species were prepared mixing individual the pellets in 10 ml of Ringer's solution (Biokar, Beauvais, France). An aliquot (2 ml) of each cocktail suspension (10^9 cfu/ml for each strain) was added to 200 g of internal mixture of "Alheira" in stomacher bags. This procedure was done in order to reach 10^7 CFU/g in each sample of "Alheira". Then, 8 ml of each concentration of EO (previously prepared in Mueller-Hinton Broth) was also added in order to reach the desired final concentration in the internal mixture of "Alheira": 4%, 1.5%, 0.5%, 0.195% and 0.0975% of EO. After assuring good mixing of the inoculum and EO with the mixture (manual massaging of the exterior of the bags), each 200 g was divided into 12 g portions and stored at 4°C for 21 days in stomacher bags. The samples were kept in a microaerobic atmosphere. The experimental conditions were: i) not inoculated internal mixture as control; ii) internal mixture inoculated with cocktail of *L. monocytogenes*; iii) internal mixture inoculated with cocktail of *L. monocytogenes* with 4%, 1.5%, 0.5%, 0.195% and 0.0975% of EO. The same was done for cocktails of *S.* Enteritidis and *St. aureus*.

After 4 hours and 3, 7, 15 and 21 days of storage, sample enumeration of the inoculated pathogens and LAB bacteria was performed. The pH and a_w values were also evaluated.

Two independent batches were analysed and, from each one, three samples were analysed for each time.

Microbiological analyses

Ten grams of internal mixture of "Alheira" was added to 90 ml of sterile Buffered Peptone Water (BPW, Biokar) and homogenized in the stomacher for 2 min. Appropriate decimal dilutions were prepared in sterile Ringer's solution for microbial enumeration: *L. monocytogenes* on Listeria Selective Agar Base (Prolabo, Leuven, Belgium) incubated at 37°C for 24 h; LAB on De Man, Rogosa and Sharpe agar (MRSa, Biokar), incubated for 48–72 h at 30°C, under microaerobic conditions; *S.* Enteritidis on Modified Semi-solid Rappaport-Vassiliadis Agar (MSRV, Biokar) and *St. aureus* on Baird-Parker Agar (BPA, Biokar), both incubated at 37°C for 48 h.

Chemical analyses

The pH value was determined directly with a Crison MicropH 2002 pH-meter (Crison, Barcelona, Spain). The a_w was measured with a calibrated electric hygrometer, Rotronic DT (Rotronic AG, Bassersdorf, Switzerland), according to the manufacturer's instructions.

Statistical analysis

An analysis of variance (two-way ANOVA) was carried out to assess the effects of the concentration of EO and time of storage on pathogens. For each time of storage, the comparison of the concentration of EOs was carried out by one-way analysis of variance (ANOVA). The Tukey-Kramer test was used to determine the significant differences ($p < 0.05$) among group means. Statistical analysis was done with SPSS 23.0 software for Windows, considering $p < 0.05$ as statistically significant.

Sensorial analysis

The sensory evaluation of "Alheira" made with 0.195% oregano EO was carried out by 57 consumers (73.7% female, 26.3% male; aged from 18 to 58 years old: 49.1% less than 30 years-old, 38.6% from 30 to 49 years-old and 12.3% over 50 years old). It was a condition of recruitment that subjects like "Alheira" and oregano.

Tests were performed in a controlled environment, at room temperature (20°C), under white fluorescent illumination (6500 K). Recruitment of consumers was done by e-mail or personal invitation among the university staff. Samples were composed of two small balls of internal mixture of "Alheira" of approximately 5 g each (cooked in an oven at 180°C for 15 min) which were placed in plastic dishes identified with three digit random numbers and presented simultaneously to the consumers. Spring water and unsalted biscuits were available to clean the mouth between tasting the samples. The consumer test was completed in one session, beginning with the control samples (without addition of oregano EO – 789) followed by samples with 0.195% of oregano EO (382).

Participants rated the samples for overall liking, on a 9-point hedonic scale from 1 (extremely dislike) to 9 (extremely like) (tasting and global appreciation) (Lawless and Heymann, 2010). Consumers also evaluated the adequacy of the aroma intensity, and flavour intensity of the EO applied using a 5-point JAR scale (1 – too weak, 3 – just about right, 5 – too strong; van Trijp et al., 2007). Just-about-right (JAR) scales are bipolar scales used to measure the level of an attribute relative to participants' ideal level, having a midpoint labelled just-about-right or just right. Consumers were also asked about their "willingness to consume" the products, using a scale from 0 to 10 (no and yes, respectively).

Statistical analysis

The comparison of the hedonic evaluation between "Alheira" with and without oregano EO (0.195%) was assessed by t-student test for independent samples (paired samples). Statistical analysis was carried out using Microsoft Excel 2013 for Windows 8, considering $p < 0.01$ as statistically significant. The frequencies of TW, JAR and TS ratings for the five sensory attributes evaluated were determined for each sample, and the resulting proportions calculated. A weighted penalty analysis (PA) was then conducted to relate attributed intensity ratings to OL for each sample and participant (Popper, 2014).

RESULTS AND DISCUSSION

The antimicrobial effect of oregano EO on some foodborne pathogens *in vitro* has already been described (Carvalho et al., 2018; Dussault et al., 2014; García-Diez et al., 2016); and research on its inhibitory effect *in vivo* on foodstuffs is increasing, mainly in traditional

meat products (García-Diez et al., 2016; Jayasena and Jo, 2013). In brief, EOs could: i) destroy the cell wall; ii) disrupt the phospholipid bilayer of the cytoplasmic membrane; iii) damage the membrane proteins leading to increased permeability of the cell membrane and loss of cellular constituents; iv) disrupt the proton motive force, electron flow and active transport; and v) coagulate the cell contents (Jayasena and Jo, 2013).

Thymol and carvacrol are two of the main compounds in oregano essential oil and in our study the major components of oregano essential oil were thymol (ion: 135 m/z; rt: 32.59 min), carvacrol (ion: 136; rt: 32.66 min), p-cymene (ion: 93; rt: 7.79 min), linalool (ion: 93; rt: 15.84 min), γ-terpinene (ion: 93; rt: 6.79 min), β-pinene (ion: 93; rt: 4.91 min), terpinen--4-ol (ion: 93; rt: 17.06 min), D-limonene (ion: 93; rt: 5.57 min), Eucalyptol (ion: 93; rt: 5.80 min) and borneol (ion: 95; rt: 19.76 min), which is in agreement with previous studies (reviewed by Burt, 2004; García-Díez et al., 2016; Khorshidian et al., 2018).

Counts of *S.* Enteritidis, *St. aureus* and *L. monocytogenes* during 21 days of storage at 4°C with and without oregano EO (4%, 1.5%, 0.5%, 0.195% and 0.0975%) are presented in Figures 1, 2 and 3, respectively. For all the experiments, there was no growth of *L. monocytogenes*, *Salmonella* spp., *St. aureus* or *E. coli* in the uninoculated control samples (data not shown). The antimicrobial effect was higher as the concentration of the oregano EO increased. Generally, the addition of EOs to the internal mixture of "Alheira" decreased the microbial counts of the pathogens tested during the storage time. For all the experiments, the concentration of EO used varied for each time ($p < 0.05$).

Regarding *S.* Enteritidis, the inhibitory effect of higher concentrations of oregano oil (4% and 1.5%) was observed at the beginning of the storage time, with a logarithmic reduction of ~3 log. In contrast, at lower concentrations (0.195% and 0.0975%), this effect took longer and had a smaller logarithmic reduction, 1.5 log and 1 log, respectively ($p < 0.05$; Fig. 1). Barbosa et al., (2009) also showed that 0.9% of oregano EO resulted in 1 log reduction for *S.* Enteritidis. García-Diez et al. (2016) showed no effect against *S.* Enteritidis with 0.005% of oregano EO in a fermented meat sausage.

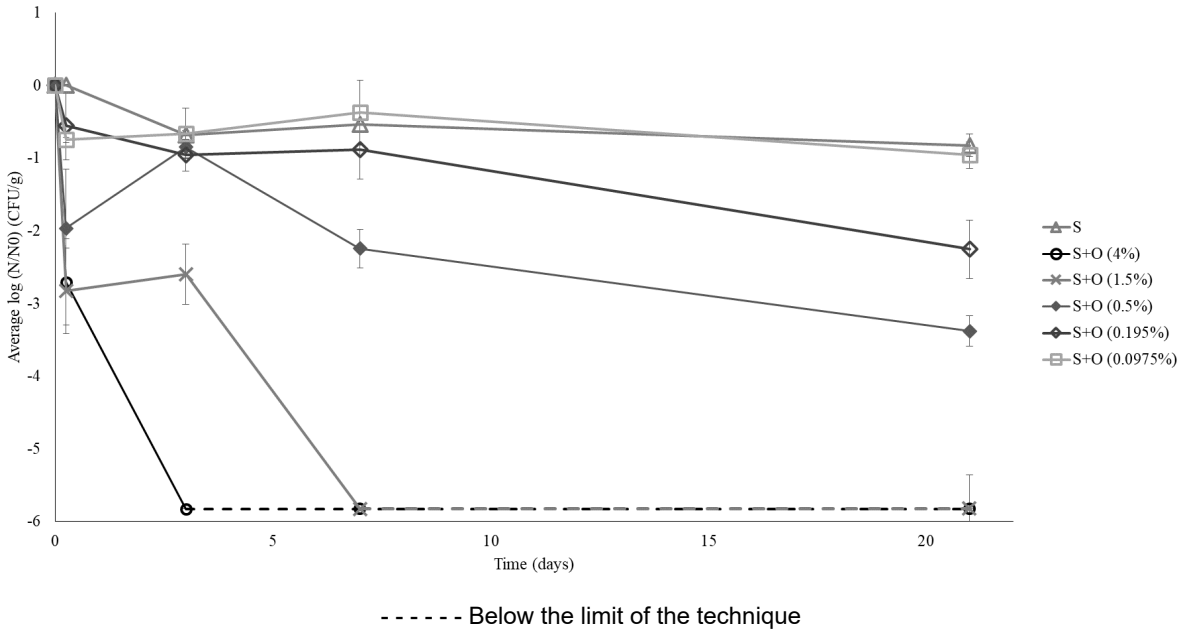


Fig. 1. The effect of different concentrations of oregano EO (4%, 1.5%, 0.5%, 0.195% and 0.0975%) on the survival of a cocktail of *S.* Enteritidis in an internal mixture of "Alheira" during 21 days at 4°C. Results are expressed as average of log (N/N0), CFU/g (means ±SD ($n = 3$))

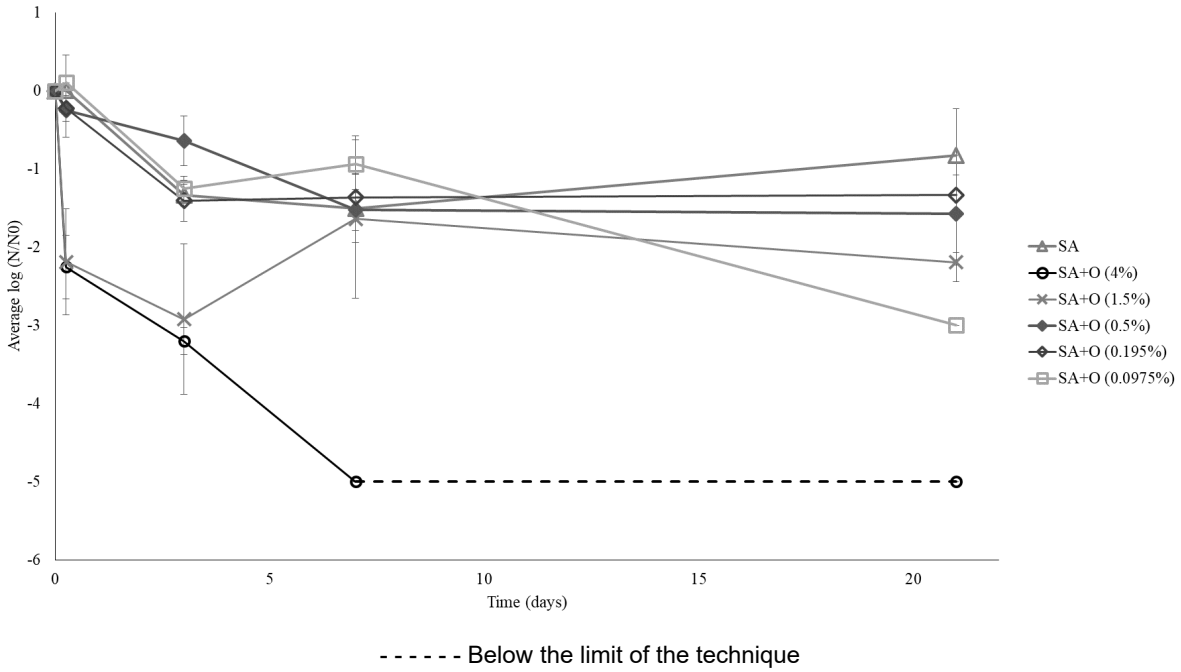


Fig. 2. The effect of different concentrations of oregano EO (4%, 1.5%, 0.5%, 0.195% and 0.0975%) on the survival of a cocktail of *St. aureus* in an internal mixture of "Alheira" during 21 days at 4°C. Results are expressed as average of log (N/N0), CFU/g (means ±SD (n = 3))

The activity of different concentrations of oregano EO against *St. aureus* is represented in Figure 2. The inhibitory effect of 4% of oregano essential oil was observed at the beginning of the storage time (2 log reduction). However, at lower concentrations, the reductions were only observed at the end of the storage and with a smaller logarithmic reduction ($p < 0.05$). Pesavento et al. (2015) observed a decrease of 1.5 log and 2.5 log for *St. aureus* in minced meat, with 0.5% and 2% of oregano EO, respectively, after 14 days of storage. Regarding the low concentrations, results obtained are in agreement with those obtained by García-Diez et al. (2016); i.e. there was no inhibition of *St. aureus* in "chouriço" with 0.05% and 0.005% EO. *Staphylococcus aureus* (considered one of the most osmotolerant foodborne pathogens) develops several mechanisms to survive under osmotic stress based mainly on modifications of the internal cell composition (Hennekinne et al., 2012). Changes on cell membrane composition, one of the main targets of EOs, could explain the lower antibacterial effect of EOs against *St. aureus* compared to that observed for *S*. Enteritidis and *L. monocytogenes*. For studies using *L. monocytogenes*, results are presented in Figure 3. A gradual decrease of *L. monocytogenes* over time was observed for all the concentrations used. At 4%, there was an immediate reduction after 4 h, and after 15 days, *L. monocytogenes* was not detectable. For the other concentrations used, the reduction of *L. monocytogenes* was continuous over time and a decrease between ~1 and 2 log was achieved. Different authors performed studies exposing *L. monocytogenes* to different concentrations of oregano EO and reductions in counts were also observed (Dussault et al., 2014; Pesavento et al., 2015). Survival of *L. monocytogenes* was clearly affected by the addition of oregano EO; for all the concentrations investigated, survivors decreased over the storage period.

For LAB (data not shown), pH values and a_w, there were no significant changes ($p > 0.05$) over time in the presence of EO (Table 1).

In short, 4% of oregano EO was the concentration with the greatest inhibitory activity. However, this is a very high concentration that could affect the aroma

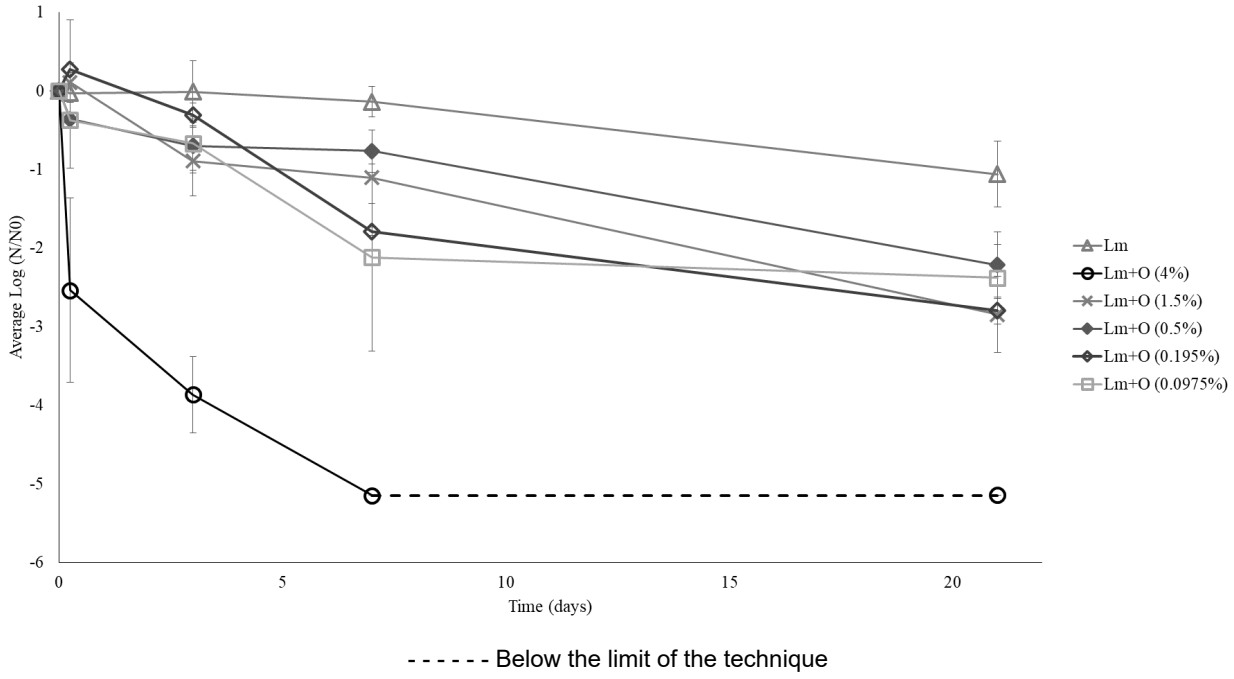


Fig. 3. The effect of different concentrations of oregano EO (4%, 1.5%, 0.5%, 0.195% and 0.0975%) on the survival of a cocktail of *L. monocytogenes* in an internal mixture of "Alheira" during 21 days at 4°C. Results are expressed as average of log (N/N0), CFU/g (means ±SD (n = 3))

and flavour, changing the characteristics of the selected product. Regarding lower concentrations (0.195% and 0.0975%), the highest antibacterial activity was observed for *L. monocytogenes*, reinforcing that Gram-positive bacteria are more sensitive than Gram-negative bacteria (Hyldgaard et al., 2012). One of the reasons could be related to the fact that Gram-positive bacteria only have a cell wall that allows hydrophobic molecules to readily penetrate into cells and act on both cell wall and cytoplasm. Gram negative bacteria have an outer membrane that could cause difficulties for the action of the EO, acting as a barrier against macromolecules and hydrophobic compounds. Also, and as stated above, thymol and carvacrol (hydrophobic compounds) were the major components of the oregano EO studied, which could explain the damage to the cytoplasmic membrane.

Moreover, the matrix used, i.e. internal mixture of "Alheira", could influence the EO efficiency, since factors present in complex food matrices can potentially decrease the efficacy of EOs, not allowing the oil to spread easily (Burt, 2004). It could also increase the concentration required to obtain sufficient antimicrobial activity (Hyldgaard et al., 2012), as our results have shown. Proteins present in "Alheira" (~14%) could interact with phenolic compounds present in oregano EO, while fats (~13%) surround the hydrophobic constituents of EO, restricting their availability to target sites of microorganisms (Burt, 2004). In addition, the physical structure of "Alheira" (a "soft" paste of meat, lard, spices and bread) could affect the spreading of oregano EO, limiting its availability to microbial cells (Gutierrez et al., 2008).

The sensory impact of EOs has been reported as one of the most negative aspects of their use (Chouliara et al., 2007). So, the concentration of 0.195% was selected to evaluate its sensory acceptance, since higher concentrations had previously been eliminated as not acceptable (data not shown). In the current work, most of the consumers (21%) consume "Alheira" once a month. Analysing the overall liking (OL), most of the consumers prefer "Alheira" without oregano EO to an internal mixture of "Alheira" with oregano EO, rejecting the null hypothesis ($p < 0.01$; $t = 8.01$; df = 56;

Table 1. pH and water activity in internal mixture of "alheira" inoculated with *S.* Enteritidis (S), *St. aureus* (SA) and *L. monocytogenes* (Lm) with and without different concentration of oregano EO (4%, 1.5%, 0.5%, 0.195% and 0.0975%) during 21 days of storage at 4ºC

Pathogens / Time	0.25 (4 h)		3		7		15		21	
	pH	a_w	pH	a_w	pH	a_w	pH	a_w	pH	a_w
C	5.09 ±0.015	0.981 ±0.004	5.00 ±0.006	0.980 ±0.006	4.96 ±0.055	0.986 ±0.015	4.87 ±0.021	0.987 ±0.004	4.95 ±0.021	0.985 ±0.005
C+O (4%)	5.06 ±0.040	0.985 ±0.005	5.12 ±0.065	0.979 ±0.016	4.98 ±0.085	0.989 ±0.004	4.88 ±0.070	0.986 ±0.004	4.78 ±0.54	0.990 ±0.005
C+O (1.5%)	5.05 ±0.050	0.990 ±0.005	4.84 ±0.062	0.978 ±0.012	4.67 ±0.080	0.987 ±0.005	4.44 ±0.15	0.984 ±0.006	4.40 ±0.051	0.991 ±0.016
C+O (0.5%)	5.03 ±0.047	0.991 ±0.016	4.80 ±0.070	0.980 ±0.005	4.60 ±0.015	0.981 ±0.010	4.50 ±0.057	0.985 ±0.002	4.33 ±0.051	0.984 ±0.006
C+O (0.195%)	5.07 ±0.027	0.986 ±0.010	5.01 ±0.029	0.987 ±0.004	4.91 ±0.010	0.976 ±0.004	4.99 ±0.24	0.978 ±0.012	4.97 ±0.040	0.985 ±0.002
C+O (0.0975%)	5.05 ±0.032	0.985 ±0.007	5.00 ±0.032	0.986 ±0.004	4.87 ±0.046	0.974 ±0.005	4.84 ±0.012	0.980 ±0.005	4.98 ±0.030	0.991 ±0.002
S	5.09 ±0.060	0.991 ±0.004	4.84 ±0.021	0.984 ±0.002	4.67 ±0.055	0.991 ±0.009	4.44 ±0.18	0.988 ±0.004	4.40 ±0.021	0.983 ±0.004
S+O (4%)	5.06 ±0.047	0.975 ±0.003	4.80 ±0.070	0.973 ±0.003	4.60 ±0.015	0.982 ±0.004	4.50 ±0.057	0.973 ±0.004	4.33 ±0.051	0.980 ±0.005
S+O (1.5%)	5.05 ±0.026	0.987 ±0.003	5.01 ±0.029	0.983 ±0.003	4.91 ±0.01	0.980 ±0.006	4.99 ±0.24	0.989 ±0.003	4.97 ±0.040	0.988 ±0.009
S+O (0.5%)	5.03 ±0.032	0.960 ±0.010	5.00 ±0.0058	0.976 ±0.010	4.87 ±0.046	0.979 ±0.016	4.84 ±0.012	0.982 ±0.002	4.98 ±0.03	0.980 ±0.002
S+O (0.195%)	5.07 ±0.017	0.987 ±0.009	5.01 ±0.021	0.980 ±0.005	4.89 ±0.01	0.978 ±0.012	4.87 ±0.029	0.990 ±0.005	4.94 ±0.032	0.979 ±0.006
S+O (0.0975%)	5.05 ±0.031	0.977 ±0.008	5.12 ±0.055	0.981 ±0.004	4.98 ±0.049	0.980 ±0.005	4.97 ±0.068	0.986 ±0.009	5.04 ±0.16	0.978 ±0.005
SA	5.07 ±0.056	0.991 ±0.002	4.68 ±0.057	0.985 ±0.005	4.72 ±0.13	0.987 ±0.004	4.54 ±0.029	0.985 ±0.011	4.51 ±0.14	0.989 ±0.014
SA+O (4%)	5.10 ±0.01	0.983 ±0.011	4.74 ±0.044	0.990 ±0.005	4.60 ±0.012	0.986 ±0.004	4.46 ±0.021	0.983 ±0.005	4.32 ±0.90	0.990 ±0.002
SA+O (1.5%)	5.09 ±0.017	0.986 ±0.008	5.03 ±0.067	0.991 ±0.016	4.90 ±0.035	0.984 ±0.006	4.82 ±0.021	0.985 ±0.004	4.89 ±0.02	0.990 ±0.002
SA+O (0.5%)	4.89 ±0.021	0.980 ±0.008	5.03 ±0.01	0.986 ±0.010	4.88 ±0.04	0.985 ±0.002	4.84 ±0.11	0.986 ±0.003	4.96 ±0.095	0.982 ±0.005
SA+O (0.195%)	5.05 ±0.021	0.986 ±0.015	4.99 ±0.0058	0.985 ±0.007	4.81 ±0.012	0.991 ±0.002	4.88 ±0.0058	0.990 ±0.003	5.13 ±0.16	0.987 ±0.004
SA+O (0.0975%)	5.08 ±0.035	0.989 ±0.004	5.10 ±0.01	0.991 ±0.003	5.04 ±0.015	0.990 ±0.001	4.69 ±0.015	0.984 ±0.007	4.64 ±0.36	0.989 ±0.003
Lm	5.04 ±0.012	0.987 ±0.005	4.78 ±0.076	0.982 ±0.008	4.72 ±0.031	0.985 ±0.006	4.59 ±0.093	0.984 ±0.006	4.50 ±0.071	0.985 ±0.003
Lm+O (4%)	5.12 ±0.098	0.981 ±0.010	4.69 ±0.032	0.983 ±0.003	4.58 ±0.01	0.980 ±0.010	4.47 ±0.031	0.979 ±0.005	4.35 ±0.07	0.980 ±0.003
Lm+O (1.5%)	5.07 ±0.025	0.976 ±0.004	5.31 ±0.34	0.980 ±0.003	4.81 ±0.01	0.978 ±0.005	4.84 ±0.015	0.980 ±0.011	5.00 ±0.045	0.976 ±0.003
Lm+O (0.5%)	4.97 ±0.036	0.974 ±0.005	4.92 ±0.012	0.974 ±0.010	4.85 ±0.021	0.978 ±0.006	4.82 ±0.01	0.979 ±0.002	5.01 ±0.021	0.984 ±0.001
Lm+O (0.195%)	5.05 ±0.015	0.975 ±0.012	4.54 ±0.035	0.976 ±0.004	4.54 ±0.032	0.980 ±0.011	4.41 ±0.042	0.978 ±0.006	4.39 ±0.012	0.977 ±0.004
Lm+O (0.0975%)	5.06 ±0.015	0.980 ±0.005	4.73 ±0.023	0.981 ±0.003	4.70 ±0.065	0.981 ±0.003	4.64 ±0.074	0.985 ±0.004	4.73 ±0.07	0.985 ±0.004

Results are expressed as mean ±standard deviation.

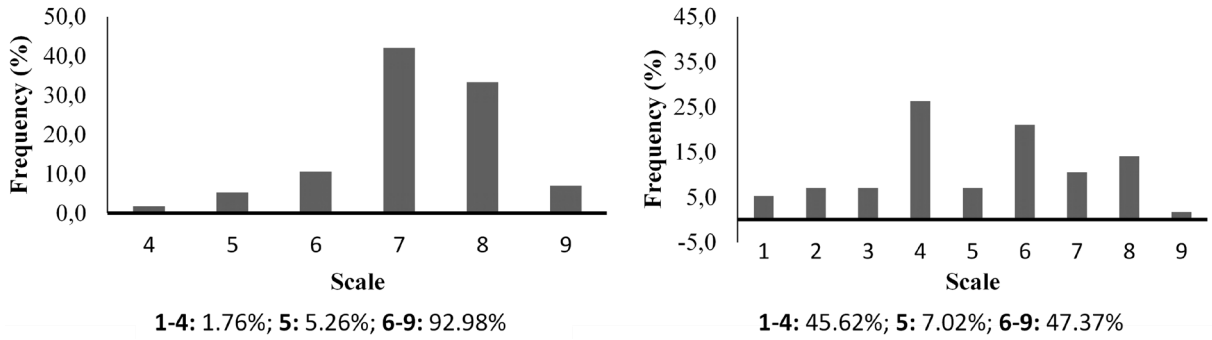
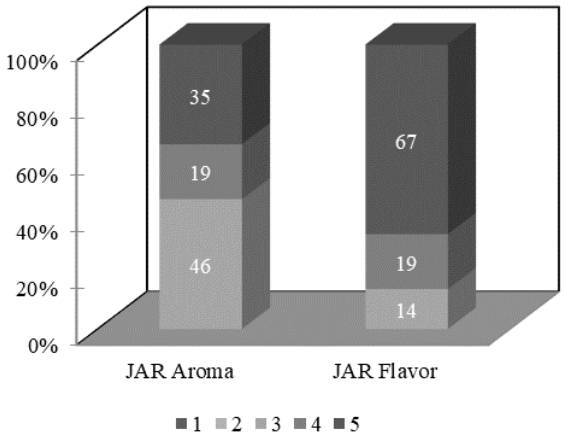


Fig. 4. Absolute frequency of overall liking in samples without oregano EO and with 0.195% oregano EO, respectively (1–9 = hedonic scale)

Fig. 4). Most of the consumers commented that samples with oregano EO had a very intense flavour and the after-taste was not pleasant. In line with the above-referred comments of the consumers, 54% and 86% of respondents rated the oregano aroma and flavour too strong, respectively (Fig. 5). Corresponding to a mean drop of 2.52 and to a probability $p < 0.01$, the overly strong flavour had an important impact on the low acceptance of this sample.

According to the results obtained in sensorial analysis, 0.195% of oregano EO has a negative effect on the consumers' acceptance of "Alheira". These results are in accordance with García-Diez et al. (2016), which states that the application of this type of concentration



Variable	Level	Frequencies	Percentage	Sum (OL 382)	Mean (OL 382)	Mean drops	Standardized difference	*p*-value
JAR Aroma	too little	0	0.0					
	JAR	26	45.6	146.0	5.62			
	too much	31	54.4	144.0	4.65	0.97	1.80	0.077
JAR Flavour	too little	0	0.0					
	JAR	8	14.0	58.0	7.25			
	too much	49	86.0	232.0	4.74	2.52	3.50	0.001

Fig. 5. Percentages for the JAR (just-about-right) levels of aroma and flavour (1 – too weak, 2 – weak, 3 – just about right, 4 – strong, 5 – too strong) of samples with oregano EO and penalties of JAR Aroma and JAR Flavour

in a fermented sausage gives it a strong taste. Other authors have also reported that the production of an off-flavour or a strong odour limits the use of EOs as food preservatives to increase the safety and shelf life of food products (Bajpai et al., 2012; Solorzano and Miranda-Novales, 2012). The EO concentration used was revealed to be inapplicable in practice, due to sensory reasons, once about 25% or less of consumers indicated, "will consume it".

CONCLUSION

The utilization of oregano EO in an internal mixture of "Alheira" seems to result in an interesting strategy to assure safety against *Salmonella* spp., *L. monocytogenes* and *St. aureus*, but with sensory limitations which do not allow its use in high concentrations that are more effective for pathogen inhibition. *In situ* assays of the antimicrobial effects on foodborne and spoilage bacteria have been described, although their application in foodstuffs is scarce, probably due to the differences in the antimicrobial effects on the food matrix and also their sensorial impact. Therefore, their utilization as antimicrobial agents in food must be assessed in the food product. In this work, it was concluded that high concentrations of oregano (4% and 1.5%), decreased counts of *L. monocytogenes*, *S.* Enteritidis and *St. aureus* present in internal mixtures of "Alheira". Regarding lower concentrations, the reduction was lower, although significant for *L. monocytogenes*.

This study maybe considered a starting point for other studies that have now to concentrate on ways to "mask" the unpleasant sensorial effects caused by EOs in "Alheira". Moreover, further studies could focus on the combination of lower concentrations of EOs with other technologies to achieve a balance between the microbial safety and sensory acceptability of "Alheira".

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